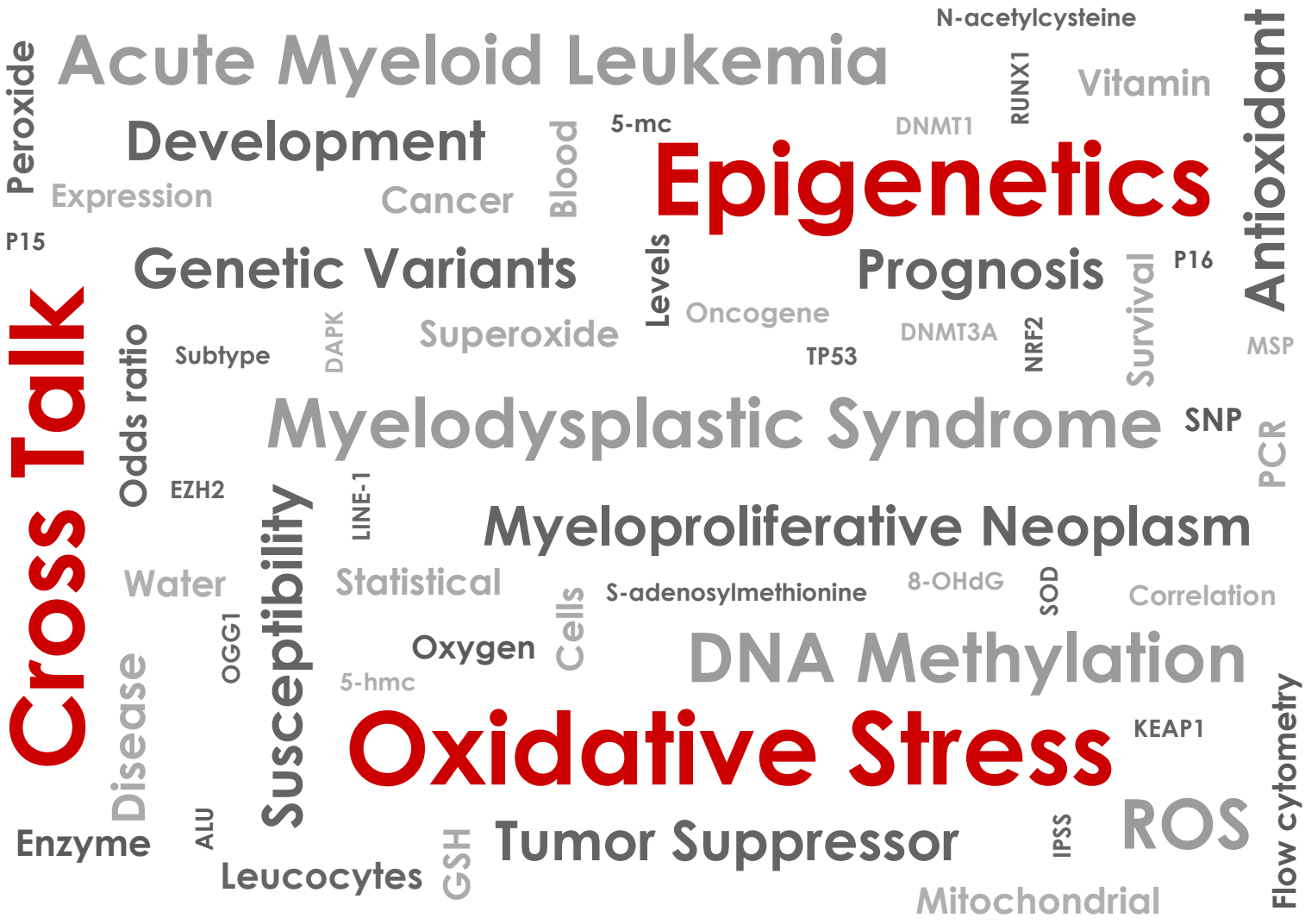


Ana Cristina Pereira Gonçalves

Oxidative stress *versus* epigenetic

– Role in susceptibility, development, and progression of myeloid neoplasms –

Tese de doutoramento do Programa de doutoramento em Ciências da Saúde, ramo de Ciências Biomédicas, orientada pela Senhora Professora Ana Bela Sarmento Ribeiro, pelo Senhor Professor José Manuel Nascimento Costa e pela Senhora Investigadora Luisa Mota Vieira, e apresentada à Faculdade de Medicina da Universidade de Coimbra

Setembro 2015

· U C ·

Universidade de Coimbra

Ana Cristina Pereira Gonçalves

Oxidative stress *versus* epigenetic

– Role in susceptibility, development, and progression of myeloid neoplasms –

Tese de doutoramento do Programa de doutoramento em Ciências da Saúde, ramo de Ciências Biomédicas, orientada pela Senhora Professora Ana Bela Sarmento Ribeiro, pelo Senhor Professor José Manuel Nascimento Costa e pela Senhora Investigadora Luisa Mota Vieira, e apresentada à Faculdade de Medicina da Universidade de Coimbra

Setembro 2015

UNIVERSIDADE DE COIMBRA

Cover:

Cross talk between oxidative stress and epigenetic

by Raquel Alves, 2015

Institutions and Funding

The research work presented in this thesis involved the following institutions and was supported by:

Publications

The following original articles and abstracts have been published in peer-scientific journals, within the scope of the present PhD thesis:

Original articles

Gonçalves, A.C., Cortesão, E., Oliveiros, B., Alves, V., Espadana, A.I., Rito, L., Magalhães, E., Lobão, M.J., Pereira, A., Nascimento Costa, J.M., Mota-Vieira, L., Sarmento-Ribeiro, A.B. *Oxidative stress and mitochondrial dysfunction play a role in myelodysplastic syndrome development, diagnosis, and prognosis: A pilot study.* (2015) *Free Radic Res,* **49,** 1081-94.

Gonçalves, A.C., Cortesão, E., Oliveiros, B., Alves, V., Espadana, A.I., Rito, L., Magalhães, E., Pereira, S., Pereira, A., Nascimento Costa, J.M., Mota-Vieira, L., Sarmento-Ribeiro, A.B. (2015) Oxidative stress levels are correlated with P15 and P16 gene promoter methylation in myelodysplastic syndrome patients. *Clin Exp Med*, [Epub ahead of print, 2015 May 17].

Gonçalves, A.C., Alves, R., Baldeiras, I., Cortesão, E., Carda, J.P., Oliveiros, B., Brás, M., Pereira, A., Nascimento Costa, J.M., Mota Vieira, L. & Sarmento Ribeiro, A. B. Localized DNA hypermethylation and global DNA hypomethylation are correlated with oxidative stress levels in myeloid neoplasms. (Under review, Sept 2015)

Gonçalves, A.C., Alves, R., Baldeiras, I., Cortesão, E., Carda, J.P., Branco, C.C., Oliveiros, B., Loureiro, L., Pereira, A., Nascimento Costa, J.M., Sarmento Ribeiro, A. B. & Mota Vieira, L. Genetic variants involved in oxidative stress, base excision repair, DNA methylation, and folate metabolism pathways influence myeloid neoplasms susceptibility and prognosis. (Under review, Sept 2015)

Gonçalves, A.C., Alves, R., Ribeiro, I.P., Pires, A., Jorge, J., Laranjo, L., Botelho, M.F., Nascimento Costa, J.M., Mota-Vieira, L., Carreira I.M. & Sarmento-Ribeiro A.B. Acute and chronic exposure to hydrogen peroxide modulates the methylome of normal and malignant hematological cells. (In preparation, Sept 2015)

Abstracts

Gonçalves, A.C., Cortesão, E., Espadana, A.I., Magalhães, E., Moucho, C., Rito, L., Pereira, A., Sousa, I., Teixeira, A., Mota-Vieira, L., Nascimento Costa, J.M.,

Sarmento-Ribeiro, A.B. Oxidative stress can influence gene methylation status in MDS patients. (2012) *Haematologica*, **97(S1),** 135.

Gonçalves, A.C., Cortesão, E., Espadana, A.I., Magalhães, E., Moucho, C., Rito, L., Pereira, A., Sousa, I., Teixeira, A., Mota-Vieira, L., Nascimento Costa, J.M., Sarmento-Ribeiro, A.B. MNSOD and GPX1 polymomorfisms – a possible role in MDS susceptibility. *Acta Med Port,* **1(2011/2012),** 7.

Gonçalves, A.C., Alves, R., Cortesão, E., Baldeiras, I., Carda, J.P., Barbosa-Ribeiro, A., Borges, A., Pereira, A., Ribeiro, L., Mota-Vieira, L., Nascimento Costa, J.M., Sarmento-Ribeiro, A.B. The interplay between oxidative stress and epigenetic profile in myeloid neoplasias. (2014) *Haematologica*, **99 (S1),** 87.

Gonçalves AC, Alves R, Cortesão E, Freitas-Tavares P, Carda JP, Espadana AI, Magalhães E, Moucho C, Rito L, Loureiro L, Pereira A, Oliveiros B, Ribeiro L, Mota-Vieira L, Nascimento-Costa JM, Sarmento-Ribeiro AB. Predisposition to myeloid neoplasias - The role of oxidative stress genes polymorphisms. (2014) *Haematologica*, **99 (S1),** 659.

Gonçalves, A.C., Alves, R., Pires, A., Jorge, J., Baldeiras, I., Cortesão, E., Carda, J.P., Rito, L., Silva, N., Pereira, A., Oliveiros, B., Ribeiro, L., Mota-Vieira, L., Nascimento Costa, J.M., Sarmento-Ribeiro, A.B. The interplay between oxidative stress and dna methylation – role in development, diagnosis and prognosis of myeloid neoplasias. (2015) *Rev Port Pneumol*, **21(Esp Cong 1),** 15.

Gonçalves, A.C., Alves, R., Pires, A., Jorge, J., Mota-Vieira, L., Nascimento-Costa, J.M., Sarmento-Ribeiro, A.B. Chronic exposure to oxidative stress inducers influence methylation status in normal and neoplastic hematological cells. (2015) *Rev Port Pneumol*, **21(Esp Cong 1),** 18.

Gonçalves, A.C., Alves, R., Pires, A., Jorge, J., Mota-Vieira, L., Nascimento-Costa, J.M., Sarmento-Ribeiro, A.B. Exposure to ROS induces DNA damage and influences DNA methylation in normal and malignant hematological cell lines. (2015) *Haematologica*, **100(S1),** 366.

"…each human cell is made up of 95 trillion water molecules, 60 billion proteins, 2 trillion fat molecules, 5 trillion sugars and amino acids, 60 billion RNA molecules and six feet of DNA… "

S. Naylon & J. Chen

in "Unraveling human complexity and disease with systems biology and personalized medicine"

Acknowledgments

Embora, pela sua finalidade académica, uma dissertação seja um trabalho individual há contributos que não podem deixar de ser realçados. Por essa razão desejo expressar os meus sinceros agradecimentos:

Aos meus orientadores Professora Doutora Ana Bela Sarmento Ribeiro, Professor Doutor José Manuel Nascimento Costa e Doutora Luisa Mota Vieira pela orientação desta tese, assistência na definição deste projecto, apoio, disponibilidade, dedicação e empenho diários ao longo do seu desenvolvimento, pelas críticas e conselhos e, em especial, pelo apoio incondicional, pela partilha de conhecimento e experiência científica, pelos ensinamentos, pelo tempo que generosamente me dedicaram, pela amizade e confiança.

A todos os que durante o período de realização deste trabalho foram passando pelo Laboratório de Oncobiologia e Hematologia (LOH) da Faculdade de Medicina da Universidade de Coimbra pelo companheirismo e bom ambiente que sempre proporcionaram. Agradeço especialmente à Mestre Raquel Alves, pela partilha dos bons e maus momentos de que é feita Ciência, mas sobretudo pela amizade e pelo apoio que sempre me demonstrou. À Professora Doutora Marília Dourado pelo apoio e amizade.

À CIMAGO, Centro de Investigação em Meio Ambiente, Genética e Oncobiologia, em especial ao Professor Doutor Carlos Freire de Oliveira e à Professora Doutora Isabel Marques Carreira, antigo Presidente e Coordenadora deste Centro, respectivamente, pelo apoio financeiro que, em parte, permitiu a realização deste trabalho.

À Doutora Bárbara Paiva, Laboratório de Bioestatística e Informática Médica da Faculdade de Medicina da Universidade de Coimbra pela disponibilidade demonstrados na análise estatística dos resultados obtidos, mas também pela sua amizade.

À Doutora Letícia Ribeiro, Directora do Serviço de Hematologia Clínica do Centro Hospitalar e Universitário de Coimbra, à Drª Adriana Teixeira, à Drª Isabel de Sousa, à Doutora Emília Cortesão e à Drª Catarina Geraldes pela colaboração, disponibilidade, ensinamentos, e em especial pela amizade permanentemente prestada, um muito obrigada. Aos restantes elementos deste Serviço que também disponibilizaram algum do seu tempo para que este trabalho se realizasse.

À Doutora Amélia Pereira, Directora do Serviço de Medicina do Hospital Distrital de da Figueira da Foz, EPE e minha mãe, o meu mais sincero obrigada pela apoio e colaboração prestada a este trabalho, assim como a disponibilidade e os ensinamentos pessoais e científicos. À Drª Fátima Guedes e às enfermeiras do Hospital de Dia de Oncologia deste hospital pela disponibilidade e amizade que sempre demonstraram.

À Professora Doutora Catarina Resende de Oliveira, Bioquímica da Faculdade de Medicina de Coimbra, a todos os docentes, investigadores e funcionários, em especial à Dª Rosa Cardoso, que de uma ou outra forma permitiram que este trabalho se realizasse, um obrigada por tudo.

À Professora Doutora Filomena Botelho, Biofísica da Faculdade de Medicina de Coimbra, à Professora Doutora Margarida Abrantes e à Doutora Mafalda Laranjo pela amizade e apoio que sempre

demonstraram, mas também a todos os outros elementos desta Unidade.

À Professora Doutora Isabel Marques Carreira, Laboratório de Citogenética e Genomica (LCG) da Faculdade de Medicina de Coimbra, à Doutora Joana Melo, Mestre Ilda Ribeiro e aos restantes elementos deste LCG pelo apoio e disponibilidade prestadas.

Ao Professor Doutor Santos Rosa, Laboratório de Imunologia da Faculdade de Medicina de Coimbra, e em especial à Drª Vera Alves um muito obrigada pela disponibilidade e empenho demonstrados e que permitiram a realização de parte deste trabalho, pela partilha de conhecimentos e experiências, e pela amizade incondicional que sempre demonstrou.

À Professora Doutora Raquel Seiça, Fisiologia da Faculdade de Medicina de Coimbra, à Doutora Paula Moreira, ao Doutor Paulo Matafome e ao Sr. Simões pela amizade e apoio que sempre demonstraram.

Aos amigos e colegas de doutoramento Doutora Amélia Pereira, Doutora Emília Cortesão, Drª Catarina Geraldes e Mestre Fernando Mendes pela amizade e companheirismo.

A todos os que de algum modo contribuíram para a presente dissertação e que não foram especificamente designados, mas que, nem por isso foram esquecidos.

Um especial agradecimento a todos os meus amigos e familiares (que apesar de não serem mencionados sabem que são importantes para mim) pelo incentivo e apoio ao longo da realização deste trabalho e por pacientemente terem aceite as minhas incontáveis ausências...

Finalmente, quero agradecer aos meus pais, ao meu irmão João, à Isabel, ao Tó, à Inês e à Joana pelo amor, pela inspiração, pela dedicação, pelo estímulo e pela sensatez com que sempre me ajudaram a concretizar mais esta jornada da minha vida.

Table of contents

Abbreviations

$\Delta\psi_{mit}$ - Mitochondrial membrane potential
ΔNp73 - Oncogenic isoforms of p73 protein
γ-H2AX - Gamma histone variant H2AX
+8 - Trisomy 8
5,10 methylene THF - 5,10-Methylenetetrahydrofolate
5-caC - 5-carboxylcytosine
5-fC - 5-formylcytosine
5-hmC - 5-hydroxymethylcytosine
5-mC - 5-methylcytosine
5-methyl-THF - 5-methyltetrahydrofolate
8-OHdG - 8-hydroxy-2'-deoxyguanosine
α-KG - α-ketoglutarate

A

ABL1 - ABL proto-oncogene 1, non-receptor tyrosine kinase
$ABTS^+$ - 2,2' azino di [3 etilbenzotiazolin sulfonate radical cation
AE - Acute exposure
AKT - Protein kinase B
ALK - Anaplastic lymphoma receptor tyrosine kinase
ALL - Acute lymphoblastic leukemia
AML - Acute myeloid leukemia
ANC - Absolute neutrophil count
APC - Allophycocyanin
APC - Adenomatosis polyposis coli
APL - Acute promyelocytic leukemia
ARE - Antioxidant response elements
ARMS-PCR - Amplification refractory mutation system-polymerase chain
ASXL1 - Additional sex combs like transcriptional regulator 1
ASXL1 - Additional sex combs-like 1 protein
ATCC - American Type Culture Collection
ATM - ATM serine/threonine kinase
ATP - Adenosine triphosphate
AUC - Area under the curve

B

BACH1 - BTB and CNC homolog 1
BCL2 - B-cell CLL/lymphoma 2
BCL2L10 - BCL2-like 10
BCOR - BCL6 corepressor
BCR - Breakpoint cluster
BER - Base excision repair

BM - Bone marrow
BMI1 - BMI1 proto-oncogene, polycomb ring finger
BRAC1 - Breast cancer 1
BRAF - B-Raf proto-oncogene, serine/threonine kinase
BRCA1 - Breast cancer 1, early onset
BRCA2 - Breast cancer 2, early onset

C

CADM1 - Cell adhesion molecule 1
CALR - Calreticulin
CASP8 - Caspase 8, apoptosis-related cysteine peptidase
CASR - Calcium-sensing receptor
CAT - Catalase
CBFB - Core-binding factor, beta subunit
CBL - Casitas B-lineage lymphoma proto-oncogene
CBP - CREB-binding protein
CBS - Cystathionine-beta-synthase
CD - Cluster of Differentiation
CD27 - CD27 molecule
CD44 - CD44 molecule
CDH1 - Cadherin 1, type 1
CDH1 - Cadherin 1, type 1
CDH13 - Cadherin 13
CDK6 - Cyclin-dependent kinase 6
CDKN1B - Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN2A (P16) - Cyclin-dependent kinase inhibitor 2A
CDKN2B (P15) - Cyclin-dependent kinase inhibitor 2B
CEBPA - CCAAT/enhancer binding protein (C/EBP), alpha
CELF2 - CUGBP, Elav-like family member 2
CHFR - Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase
CHIP - Clonal hematopoiesis of indeterminate potential
CI - Confidence interval
c-KIT - Cellular homolog of the transforming gene of a feline retrovirus
CLPs - Lymphoid progenitor cells
CML - Chronic myeloid leukemia
CMPs - Multipotent myeloid cells
CNC - Cap 'n' collar
CO_2 - Carbon dioxide
COBRA - Combined bisulfite restriction analysis
CREM - cAMP responsive element modulator
CSFs - Colony-stimulating factors
c-SRC - SRC proto-oncogene, non-receptor tyrosine kinase

CTL - Control
CTNNB1 - Catenin (cadherin-associated protein), beta 1
CYBA - Cytochrome b-245, alpha polypeptide

D

DAPK - Death-associated protein kinase
DAPK1 - Death-associated protein kinase 1
$DCFH_2$-DA - 2',7'-dichlorodihydrofluorescein diacetate
DEK - DEK proto-oncogene
del - Deletion
del(20q) - Long arm deletion of chromosome 20
del(5q) - Long arm deletion of chromosome 5
del(7q) - Long arm deletion of chromosome 7
DFO - Deferoxamine
DHE - Dihydroethidium
DHF - Dihydrofolate
DHFR - Dihydrofolate reductase
DNA - Deoxyribonucleic acid
DNMT - Methyltransferase
DNMT1 - DNA (cytosine-5-)-methyltransferase 1
DNMT1B - DNA (cytosine-5-)-methyltransferase 1 beta
DNMT1O - DNA (cytosine-5-)-methyltransferase 1 oocyte
DNMT1P - DNA (cytosine-5-)-methyltransferase 1 pachytene
DNMT2 - DNA (cytosine-5-)-methyltransferase 2
DNMT3A - DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B - DNA (cytosine-5-)-methyltransferase 3 beta
DNMT3L - DNA (cytosine-5-)-methyltransferase 3 like
DSBs - Double-Strand DNA Breaks
DSMZ - German Collection of Microorganisms
dTMP - Deoxythymidine monophosphate
dUMP - Deoxyuridine monophosphate

E

ECACC - European Collection of Cell Cultures
EDTA - Ethylenediaminetetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
EM - Expectation maximization
EP300 - E1A binding protein p300
EPO - Erythropoietin
EPOR - Erythropoietin receptor
EPs - Unilineage-committed progenitors erythrocytes

ERK - Extracellular signal-regulated kinases
ERO1 - Endoplasmic reticulum oxidoreductin
ESR1 - Estrogen receptor 1
ET - Essential thrombocythemia
ETV6 - Ets variant 6
EVI1 - Ecotropic viral integration site 1 [alias *MECOM,* MDS1 and EVI1 complex locus]
EZH2 - Enhancer of zeste 2 polycomb repressive complex 2 subunit

F

FACS - Fluorescence-activated cell sorting
FapyG - 2,6-diamino-4-hydroxy-5-formamidopyrimidine
FDA - US Food and Drug Administration
FGFR1 - Fibroblast growth factor receptor 1
FHIT - Fragile histidine triad gene
FISH - Fluorescence in situ hybridization
FLT3 - Fms-related tyrosine kinase 3
F-SNP - Functional Single Nucleotide Polymorphism

G

GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GATA1 - GATA binding protein 1 (globin transcription factor 1)
GCLC - Glutamate-cysteine ligase, catalytic subunit
GCLM - Glutamate-cysteine ligase, modifier subunit
GCSFR - Granulocyte colony-stimulating factor receptor
GMPs - Granulocytes and macrophages progenitor cells
GNAS - Guanine nucleotide-binding proteins
GP - Genotypic profile
GPs - Unilineage-committed progenitors granulocytes
GPX - Glutathione peroxidase
GPX1 - Glutathione peroxidase 1
GR - Glutathione reductase
GS - Total glutathione
GSF3R - granulocyte colony stimulating factor 3 receptor
GSH - Reduced glutathione
GSR - Glutathione reductase
GSSG - Oxidized glutathione
GSTP1 - Glutathione S-transferase pi
GSTT1 - Glutathione S-transferases theta 1
GUSB - glucuronidase beta

H

H_20 - Water
H2A - H2A histone family
H2B - H2B histone family
H_2O_2 - Hydrogen peroxide
H3 - H3 histone family
H4 - H4 histone family
HAT - Histone acetyltransferase
Hb - Hemoglobin
HDAC - Histone deacetylase
HDAC1 - Histone deacetylase 1
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGNC - HUGO Gene Nomenclature Committee
HIC1 - Hypermethylated in cancer 1
HO-1 - Heme oxygenase-1
HPLC - High performance liquid chromatography
HR - Hazard ratio
HRR - Homologous recombination repair
HSCs - Hematopoietic stem cells
HuGE - Human Genome Epidemiology
HUGO - Human Genome Organisation
HWE - Hardy-Weinberg equilibrium

I

IDH1 - Isocitrate dehydrogenase 1
IDH2 - Isocitrate dehydrogenase 2
IFNs - Interferons
IKZF1 - IKAROS family zinc finger 1
IL-3 - Interleukin 3
Ils - Interleukins
Int-1 - Intermediate risk 1IPSS
Int-2 - Intermediate risk 2 IPSS
IPSS - International Prognosis Score System
IR - Ionizing radiation
ITD - Internal tandem duplications

J

JAK2 - Janus kinase 2
JC-1 - 5,5',6,6'-tethrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide

JC-1 M/A - JC-1 monomer/aggregate ratio
JNK - c-Jun N-terminal kinase

K

KEAP1 - Kelch-like ECH-associated protein 1
KIT - v-kit Hardy–Zuckerman 4 feline sarcoma viral
KLK3 - Kallikrein-related peptidase 3
KLLN - Killin, p53-regulated DNA replication inhibitor
KRAS - Kirsten rat sarcoma viral oncogene homolog

L

LINE 1 - Long interspersed nucleotide elements 1
LN - Lymphoid neoplasm
Low - Low risk IPSS
LR - Likelihood ratio
LSC - Leukemia stem cells

M

M - Methylated
M/m - Major/minor allele
MAF - Minor allele frequency
MAPK - Mitogen-activated protein kinase
MAT - Methionine adenosyltransferase
MBD1 - Methyl-CpG-binding domain protein 1
MBD2 - Methyl-CpG-binding domain protein 2
MBD4 - Methyl-CpG-binding domain protein4
MBP - Methyl binding proteins
MDA - adducts of malondialdehyde
MDS - Myelodysplastic syndrome
MDS/MPN - Myelodysplastic syndrome/myeloproliferative neoplasms
MECP2 - Methyl CpG binding protein 2
MEPs - Megakaryocytes and erythroid progenitor cells
MGMT - O-6-methylguanine-DNA methyltransferase
MIF - Mean intensity of fluorescence
miRNAs - microRNAs
MKL1 - Megakaryoblastic leukemia (translocation) 1
MKs - Unilineage-committed progenitors megakaryocytes
MLH1 - MutL homolog1
MLH3 - MutL homolog 3

MLL - Myeloid/lymphoid or mixed-lineage leukemia [alias *KMT2A*, lysine (K)-specific methyltransferase 2A]
MLLT3 - Myeloid/lymphoid or mixed-lineage leukemia translocated to 3
MMC - Mitomycin C
MMR - DNA mismatch repair pathway
MMR - Mismatch repair
MN - Myeloid neoplasm
MnSOD - Manganese superoxide dismutase
MO - Mercury orange dye
MOX - DNA adenine methylase
MPL - Myeloproliferative leukemia virus
MPN - Myeloproliferative neoplasms
MPO - Myeloperoxidase
MPPs - Multipotent progenitors
MPs - Unilineage-committed progenitors monocytes
MS-MLPA - Methylation-specific multiplex ligation-dependent probe amplification
MSH2 - mutS homolog 2
MSP - Methylation specific PCR
mtDNA - Mitochondrial DNA
MTHFR - Methylenetetrahydrofolate reductase
MTRR - 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
MYH11 - Myosin (heavy chain 11, smooth muscle)

N

N - Normal
NAC - *N*-acetylcysteine
NADP+ - Nicotinamide adenine dinucleotide phosphate
NADPH - Reduced nicotinamide adenine dinucleotide phosphate
nc - Not calculated
ncSNPs - Non coding SNPs
ND - Not determined
Neg - Negative
NEIL1 - Nei endonuclease VIII-like 1 (E. coli)
NER - Nucleotide excision repair
NF1 - Neurofibromin 1
NFE2L2 - Nuclear factor, erythroid 2-like 2 gene
NHEJ - Non-homologous end joining
NHL - Non-Hodgkin lymphoma
NK - Natural killer cells
NLR - Negative likelihood ratio
NO - Nitric oxide

No - Sample number
NOX - NADPH oxidase
NPM - Nucleophosmin
NPM1 - Nucleophosmin 1
NPV - Negative predictive value
NQO1 - NAD(P)H dehydrogenase quinone 1
NFE2L2 - Nuclear factor erythroid 2-like 2
NRAS - Neuroblastoma RAS viral (v-ras) oncogene homolog
NRF1 - Nuclear factor erythroid 2-like 1
NRF2 - Nuclear factor erythroid 2 like 2
NRF3 - Nuclear factor erythroid 2-like 3
NTBI - Non-transferrin-bound iron
NTC - no template control
NUP214 - Nucleoporin 214 kDa

O

$^{\bullet}OH$ - Hydroxyl radical
$^{1}O_2$ - Singlet oxygen
$O_2^{\bullet-}$ - Superoxide anion
OCT-1 -POU class 2 homeobox 1
OGG1 - 8-oxoguanine DNA glycosylase
OR - Odds ratio
Ox - Oxidized

P

P15 - alias *CDKN2B* (*cyclin-dependent kinase inhibitor 2B*)
P16 - alias *CDKN2A* (*cyclin-dependent kinase inhibitor 2A*)
p22phox - Cytochrome b-245 alpha subunit
p40phox - Neutrophil cytosolic factor 4
p45 NF-E2 - 45 kDa subunit of Nuclear factor erythroid-derived 2
p47phox - Neutrophil cytosolic factor 1
p67phox - Neutrophil cytosolic factor 2
P73 - Tumor protein p73
PAH - Phenylalanine hydroxylase
PARK2 - Parkin RBR E3 ubiquitin protein ligase
PB - Peripheral blood
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
PCR-RFLP - rRestriction fragment length polymorphism-polymerase chain reaction
PDGFR - Platelet-derivated growth factor receptor

PDGFRA - Platelet-derivated growth factor receptor alpha
PDGFRB - Platelet-derivated growth factor receptor beta
PDI - Protein disulfide isomerase
PerCP-Cy5.5 - Peridinin chlorophyll Cyanine 5.5
PHF6 - PHD finger protein 6
PI3K - Phosphoinositide 3-kinase
PKA - Protein kinase A
PKC - Protein kinase C
PLR - Positive likelihood ratio
PML - Promyelocytic leukemia
Pos - Positive
PPV - Positive predictive value
Pro-Bs - Unilineage-committed progenitors for B cells
Pro-NKs - Unilineage-committed progenitors NK cells
Prot Coding - Protein coding
Pro-Ts - Uunilineage-committed progenitors T cells
PRPF40B - PRP40 homolog, pre-mRNA processing factor B
PTEN - Phosphatase and tensin homolog
PTLs - Platelets
PTPN11 - Protein tyrosine phosphatase, non-receptor type 11
PV - Polycythemia vera

Q

qPCR - Quantitative PCR

R

RA - Refractory anemia
RAC - RAC GTPase
RAC1 - Ras-related C3 botulinum toxin substrate 1
RAD21 - Double-strand-break repair protein rad21
RAEB - Refractory anemia with excess of blasts
RAEB-1 - Refractory anemia with excess blasts type 1
RAEB-2 - Refractory anemia with excess blasts type 2
RARβ - Retinoic acid receptor beta
RARα - Retinoic acid receptor alpha
RARS - Refractory anemia with ring sideroblasts
RAS - Rat sarcoma
RASSF1 - RAS association (RalGDS/AF-6) domain family member 1
RB - retinoblastoma protein
RBM15 - RNA binding motif protein 15
RCMD - Refractory cytopenia with multilineage dysplasia

RCUD - Refractory cytopenia with unilineage dysplasia
Red - Reduced
Ref - Reference
RING-1 - Ring finger protein 1
R-IPSS - Revised IPSS
RNA - Ribonucleic acid
ROC - Receiver operating characteristic
ROS - Reactive oxygen species
RPMI 1640 - Roswell Park Memorial Institute 1640 medium
RPN1 - Ribophorin 1
RT - Room temperature
RUNX1 - Runt-related transcription factor 1
RUNX1T1 - Runt-related transcription factor 1 translocated to 1 (cyclin D-related)
RUNX3 - Runt-related transcription factor 3

S

SAH - S-adenosylhomocysteine
SAM - S-adenosylmethionine
SCF - Stem cell factor
SDF-1 - stromal cell-derived factor 1
SEM - Standard error of mean
SEN - Sensitivity
SF1 - Splicing factor 1
SF3A1 - Splicing factor 3a, subunit 1
SF3B1 - Splicing factor 3b, subunit 1
SH2B3 - SH2B adaptor protein 3
SHMT – Serine hydroxymethyltransferase
SHP1 – alias *PTPN6* (*protein tyrosine phosphatase, non-receptor type 6*)
SINEs - Short interspersed nuclear elements
SIRT1 - Silent information regulator 1
SLC19A1 - Solute carrier family 19, member 1
SMC1 - Structural maintenance of chromosomes protein 1
SMC1A - Structural maintenance of chromosomes protein 1 alpha
SMC2 - Structural maintenance of chromosomes protein 2
SMC3 - Structural maintenance of chromosomes protein 3
SNP - Single nucleotide polymorphism
SOCS1 - Suppressor of cytokine signaling 1
SOD - Superoxide dismutase
SOD1 - Copper-zinc superoxide dismutase
SOD1 - Superoxide dismutase 1
SOD2 - Manganese superoxide dismutase
SOD2 - Superoxide dismutase 2

SOD3 - Extracellular superoxide dismutase
SPE - Specificity
Splicing Reg - Splicing regulation
SRSF2 - Serine/arginine-rich splicing factor 2
SSBR - SSBS repair
SSBs - Single stranded DNA breaks
SSC - Side scatter
STAG2 - Cohesin subunit SA-2
STAT - Signal transducer and activator of transcription
STAT5 - Signal transducer and activator of transcription 5
SWI/SNF - SWItch/Sucrose Non-Fermentable pathway

T

TAp73 - Tumor suppressor isoforms of p73 protein
TAS - Total antioxidant status
TBA - Thiobarbituric acid
TDG - Thymine DNA glycosylase
TEL - Telomere elongation
TET - Ten-eleven translocation
TET2 - Tet methylcytosine dioxygenase 2
TIF2 - TNF-related apoptosis-inducing ligand
TIMP3 - Tissue inhibitor of metalloproteinase 3
TNKs - T and natural killer progenitor cells
TNF-α - Tumor necrosis factor alpha
TNFRSF1A - Tumor necrosis factor receptor superfamily, member 1A
TP53 - Tumor Protein P53
TP73 - Tumor protein p73
TPO - Thrombopoietin
TPOR - Thrombopoietin receptor (c-MPL)
TRAIL - TNF-related apoptosis-inducing ligand
Trans Reg - Transcriptional regulation
TSC2 - Tuberous sclerosis 2
TSG - Tumor suppressor genes
TYMS - Thymidylate synthase

U

U - Unmethylated
U2AF1 - U2 small nuclear RNA auxiliary factor 1
U2AF35 - U2AF small subunit
U2AF65 - U2AF large subunit
und - Undefined

UTR - Untranslated region
UV - Ultraviolet

V

vit A - Vitamin A
vit E - Vitamin E
VHL - von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase

W

WBC - White blood cells
WHO - World Health Organization
WPSS - WHO-based prognostic scoring system
WT1 - Wilms tumor 1

X

XRCC1 - X-ray repair complementing defective repair in Chinese hamster cells 1
XRCC3 - X-ray repair complementing defective repair in Chinese hamster cells 3

Z

ZRSR2 - Zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2

Abstract & Resumo

Abstract

Myeloid neoplasms (MN) are a group of heterogeneous diseases that includes myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), and acute myeloid leukemia (AML). These diseases can simultaneously harbor changes in reactive oxygen species (ROS) levels and in DNA methylation pattern. The oxidative stress (OS) results from the imbalance between ROS production, mainly in mitochondria, and their elimination by antioxidants. Moreover, MN recurrently show abnormal hypermethylation of tumor suppressor genes (TSG), such as *P15* and *P16* genes, and in less extent global hypomethylation of repetitive sequences such as long interspersed nucleotide elements 1 (LINE-1).

The present study aimed to analyze the involvement of OS and DNA methylation in the development and progression of MN, in order to evaluate their role as diagnostic and prognostic markers, as well as to identify susceptibility variants in genes involved in these mechanisms.

Frist, we conducted a proof of concept study to investigated the involvement of OS and mitochondrial dysfunction in MDS pathogenesis, as well as to assessed their diagnostic and prognostic value, and the relation of OS parameters (intracellular levels of peroxides, superoxide, and reduced glutathione – GSH) with methylation status of *P15* and *P16* gene promoters. To that end we used two technique: flow cytometry and methylation specific-PCR (MSP). We observed that bone marrow cells from MDS patients had higher peroxide levels and lower GSH content than control cells. Moreover, OS levels were dependent of MDS subtype and risk group. GSH showed to be an accurate MDS diagnostic marker, while ROS, GSH, and superoxide/peroxides ratio were good survival markers. MDS patients had higher *P15* and *P16* methylation frequencies than controls. Moreover, patients with methylated *P15, P16,* and *P15* or *P16* had higher OS levels than patients without methylation.

To confirm these results, we expanded the evaluation of OS (several enzymatic and non-enzymatic antioxidant defenses; oxidative damage) and DNA methylation

parameters [localized DNA methylation – methylation status of *P15*, *P16*, *TP53*, *MGMT*, *DAPK,* and *KEAP1* genes; global DNA methylation – levels of 5-methylcytosine (5-mC), 5-hydroxymetylcytosine (5-hmC), and LINE-1 methylation], as well as the studied pathologies (MDS and MPN). These biological parameters were evaluated in peripheral blood samples by colorimetry and fluorimetry assays, as well as by MSP and combined bisulfite restriction analysis (COBRA). MDS patients had lower levels of GSH and TAS (total antioxidant status), as well as higher levels of peroxide, peroxide/GSH, and peroxide/TAS than controls. Moreover, MDS and MPN patients had higher 5-mC levels and lower 5-hmC/5-mC ratio, as well as increased methylation of at least one methylated gene (*P15*, *P16*, *DAPK,* or *KEAP1*). *TP53* and *MGMT* genes were unmethylated in these patients. Peroxide levels and peroxide/GSH ratio were higher in patients with methylated genes than in those without methylation. LINE-1 methylation and 5-mC levels were correlated with peroxide levels and peroxide/GSH ratio.

Next, we assessed the association of variants of genes involved in OS, folate metabolism, DNA repair, and DNA methylation with the susceptibility and prognosis of MDS and AML. To that end, 16 SNPs (one *per* gene: *CAT*, *CYBA*, *DNMT1*, *DNMT3A*, *DNMT3B*, *GPX1*, *KEAP1*, *MPO*, *MTRR*, *NEIL1*, *NFE2F2*, *OGG1*, *SLC19A1*, *SOD1*, *SOD2*, and *XRCC1)* were genotyped by PCR techniques. We also analyzed OS (ROS/TAS), DNA damage (8-OHdG), and DNA methylation (5-mC) in a sub-cohort of MDS patients and controls. Results showed that five genes (*GPX1*, *NEIL1*, *NFE2L2*, *OGG1*, and *SOD2*) were associated with MDS, two (*DNMT3B* and *SLC19A1*) with AML, and two (*CYBA* and *DNMT1*) with both diseases. OS levels were correlated with *CYBA*, *GPX1*, and *SOD2* genotypes, DNA damage with *NEIL1* and *OGG1*, and 5-mC levels with *DNMT1*, *DNMT3A*, *DNMT3B*, and *MTRR*. Furthermore, *DNMT3A*, *MTRR*, *NEIL1*, and *OGG1* variants modulated AML transformation in MDS patients. Additionally, *DNMT3A, OGG1, GPX1*, and *KEAP1* variants influenced MDS and AML survival.

Finally, we investigated if acute and chronic exposure to hydrogen peroxide (H_2O_2) affects the methylome of normal and malignant hematological cells. In this investigation, we used four acute myeloid leukemia cell lines and a

normal B lymphocyte cell line, and analyzed the copy number and methylation status of several TSG, LINE-1 methylation, levels of 5-mC, 5-hmC, 8-hydroxy-2'-deoxyguanosine (8-OHdG), ROS, and GSH, as well as the gene expression of *DNMT1*, *DNMT3A*, *DNMT3B*, *MECP2*, *MBD1*, *HDAC1*, *EZH2*, *EP300*, and *TET2*. These analyses were performed by methylation-specific multiplex ligation dependent probe amplification (MS-MLPA), COBRA, colorimetry and fluorimetry assays, as well as real time PCR. Acute and chronic exposure to H_2O_2 increased TSG methylation (in cells with increased ROS/GSH ratio) and decreased LINE-1 methylation (in cells with increased GSH levels). TSG hypermethylation was cell line-dependent and accompanied by upregulation of *DNMT1*, *DNMT3A*, *MECP2*, *HDAC1*, and *EZH2* genes. Moreover, the pre-treatment with N-acetylcysteine, an antioxidant molecule, prevented these events.

Overall, the present study points to a possible link between OS and DNA methylation, which besides the relevance in the development and progression of these MN, could also constitute new diagnostic and prognostic markers as well as new potential therapeutic targets.

Keywords: Acute myeloid leukemia; Disease susceptibility; DNA methylation; Genetic variants; Biomarkers; Mitochondrial membrane potential; Myelodysplastic syndrome; Oxidative stress; Prognosis; Tumor suppressor genes.

Resumo

As neoplasias mieloides (NM), de que são exemplo a síndrome mielodisplásica (SMD), as neoplasias mieloproliferativas (NMP) e a leucemia mieloide aguda, são um grupo heterogéneo de doenças clonais hematopoiéticas. A patogénese destas doenças pode envolver simultaneamente alterações nos níveis de stresse oxidativo (SO) e no padrão de metilação do ADN. O SO resulta do desequilíbrio entre a produção de espécies reativas de oxigénio (ROS), geradas principalmente na mitocôndria, e a sua eliminação pelas defesas antioxidantes. Além disso, estas doenças apresentam frequentemente hipermetilação nos genes supressores de tumor (GST), como os genes *P15* e *P16*, e alguns casos podem também apresentar hipometilação nas sequências repetitivas, nomeadamente as *long interspersed nucleotide elements 1* (LINE-1).

O presente estudo teve como objetivo analisar o envolvimento do stress oxidativo e da metilação do DNA no desenvolvimento e na progressão das neoplasias mieloides, de modo a avaliar o seu papel como marcadores de diagnóstico e prognóstico, e identificar variantes genéticas de susceptibilidade em genes envolvidos nesses mecanismos.

Inicialmente, efetuou-se um estudo de prova de conceito de modo a avaliar o envolvimento do SO e a disfunção mitocondrial na patogénese das SMD, o seu valor como marcadores de diagnóstico e de prognóstico, e a relação dos parâmetros OS (níveis intracelulares de peróxidos, anião superóxido e glutationa reduzida – GSH) com o perfil de metilação dos promotores dos genes *P15* e *P16*. Para este efeito, recorreu-se a duas técnicas: citometria de fluxo e PCR específico de metilação (MSP). Os resultados demonstram que as células de medula óssea dos doentes com SMD apresentam aumento dos níveis de peróxidos e diminuição de GSH, relativamente às células dos controlos. No entanto, estes resultados dependem do subtipo de SMD e do grupo de risco. Além disso, a GSH revelou-se um bom marcador de diagnóstico, enquanto os ROS, a GSH e a razão superóxido/peróxidos mostraram-se marcadores de sobrevivência. Estes doentes apresentam frequências de metilação dos genes *P15* e *P16* mais elevadas do que os controlos.

Por outro lado, os doentes com SMD que apresentam metilação nos gene *P15*, *P16*, e *P15* ou *P16* apresentam níveis de SO mais elevados do que os doentes sem metilação.

De modo a confirmar estes resultados, realizou-se uma avaliação alargada dos parâmetros de SO (várias defesas antioxidantes enzimáticas e não enzimáticas; lesão oxidativa) e de metilação do DNA [metilação localizada – perfil de metilação dos genes *P15*, *P16*, *TP53*, *MGMT*, *DAPK* e KEAP1; metilação global – níveis de metilação da 5-metilcitosina (5-mC), da 5-hidroximetilcitosina (5-hmC) e da LINE-1] não só em doentes com SMD, mas também em doentes com NMP. Estes parâmetros biológicos foram avaliados em amostras de sangue periférico por colorimetria, fluorimetria, MSP e COBRA (*combined bisulfite restriction analysis*). Os resultados demonstraram que os doentes com SMD apresentam diminuição dos níveis de GSH e capacidade antioxidante total (CAP), assim como aumento dos níveis de peróxido e das razões peróxido/GSH e peróxido/CAP comparativamente aos controlos. Por outro lado, observou-se aumento dos níveis de 5-mC e diminuição da razão 5-mC/5-hmC nos doentes com SMD e NMP. Além disso, este doentes apresentam aumento da frequência de metilação de pelo menos um gene (*P15*, *P16*, *DAPK*, ou *KEAP1*). No entanto, não se observou metilação dos genes *TP53* e *MGMT* nestes doentes. Os doentes com genes metilados apresentam aumento dos níveis de peróxido e da razão peróxido/GSH relativamente aos doentes sem metilação. Por fim, os níveis de metilação da LINE-1 e da 5-mC correlacionam-se com os níveis de peróxido e da razão peróxido/GSH.

Seguidamente, avaliou-se os polimorfismos em genes envolvidos no SO, no metabolismo do folato, na reparação e na metilação do DNA e o seu papel na susceptibilidade e prognóstico dos doentes com SMD e LMA. Com essa finalidade, genotiparam-se 16 SNPs (um por gene: *CAT*, *CYBA*, *DNMT1*, *DNMT3A*, *DNMT3B*, *GPX1*, *KEAP1*, *MPO*, *MTRR*, *NEIL1*, *NFE2F2*, *OGG1*, *SLC19A1*, *SOD1*, *SOD2* e *XRCC1*) por técnicas de PCR. Analisaram-se, igualmente, os níveis de OS (ROS/CAP), da lesão (8-hidroxi-2'-desoxiguanosina, 8-OHdG) e da metilação do DNA (5-mC) num subgrupo de doentes com SMD e controlos. Os resultados sugerem que os genes *GPX1*, *NEIL1*, *NFE2L2*, *OGG1* e *SOD2* influenciam a susceptibilidade para SMD, os

genes *DNMT3B* e *SLC19A1* a susceptibilidade para LMA e os genes *CYBA* e *DNMT1* o desenvolvimento das duas doenças. Além disso, os níveis de SO estão relacionados com o genótipo dos genes *CYBA*, *GPX1* e *SOD2*, a lesão do DNA com os genes *NEIL1* e *OGG1*, e os níveis de 5-mC com os genes *DNMT1*, *DNMT3A*, *DNMT3B* e *MTRR*. Os polimorfismos nos genes *DNMT3A*, *MTRR*, *NEIL1* e *OGG1* influenciam a evolução para LMA dos doentes com SMD, enquanto os polimorfismos nos genes *DNMT3A*, *OGG1*, *GPX1* e *KEAP1* influenciam a sobrevivência dos doentes com SMD e LMA.

Por fim, investigou-se a influência da exposição crónica e aguda ao peróxido de hidrogénio (H_2O_2) na metilação de células hematológicas normais e malignas. Para o efeito, utilizaram-se quatro linhas celulares de LMA e uma linha celular de linfócitos B normal. A variação do número de cópias e o perfil de metilação de vários GST, os níveis de metilação da LINE-1, da 5-mC, 5-hmC, de 8-OHdG, de ROS e da GSH, bem como a expressão dos genes *DNMT1*, *DNMT3A*, *DNMT3B*, *MECP2*, *MBD1*, *HDAC1*, *EZH2*, *EP300* e *TET2* foram avaliados por *methylation-specific multiplex ligation-dependent probe amplification* (MS-MLPA), COBRA, colorimetria, fluorimetria e PCR em tempo real. A exposição aguda e crónica ao H_2O_2 induziu hipermetilação nos GST (nas células com aumento da razão ROS/GSH) e hipometilação nas sequências LINE-1 (nas células com aumento da GSH). A hipermetilação dos GST foi acompanhada da sobre-expressão dos genes *DNMT1*, *DNMT3A*, *MECP2*, *HDAC1* e *EZH2* de modo dependente da linha celular. Além disso, o pré-tratamento com N-acetilcisteína, uma molécula antioxidante, preveniu estas alterações moleculares.

Em conclusão, o presente estudo sugere uma possível relação entre o stresse oxidativo e a metilação do DNA, os quais, além da relevância no desenvolvimento e na progressão das neoplasias mieloides, poderão igualmente constituir novos marcadores de diagnóstico e prognóstico, bem como potenciais alvos terapêuticos.

Palavras-Chave: Leucemia mieloide aguda; Susceptibilidade genética; Metilação do DNA; polimorfismos; Biomarcadores; Potencial de membrana mitocondrial; Síndrome mielodisplásica; Stresse oxidativo; Prognóstico; Genes supressores tumorais.

Chapter 1

Introduction

1.1. The hematopoietic system

The hematopoietic system is a hierarchical structure, and hematopoietic stem cells (HSCs) are at the apex of the hierarchy of numerous progenitor cell stages with increasingly restricted lineage potentials that give rise to all blood cell lineages (Figure 1) [Rieger & Schroeder, 2012]. The HSCs reside mainly in the bone marrow, the major site of adult hematopoiesis, and sustain the lifelong production of all blood lineages through a series of proliferation and differentiation events that culminate in the production of mature blood cells [Dzierzak, 2011; Rieger & Schroeder, 2012]. The blood contains different blood cell types with various functions. Leukocytes represent many specialized cell types involved in innate and acquired immunity, erythrocytes provide oxygen (O_2) and carbon dioxide (CO_2) transport, whereas megakaryocytes generate platelets for blood clotting [Rieger & Schroeder, 2012].

HSCs are defined by their high proliferative potential, ability to self-renew, and potential to give rise to all hematopoietic lineages [Rieger & Schroeder, 2012]. Their multilineage potential declines with differentiation ending before unilineage commitment [Dzierzak, 2011; Rieger & Schroeder, 2012]. The production of mature blood cells progresses down through the activation of specific pathways leading to the distinct cell types [Ho *et al.*, 2015]. Upon commitment to proliferation, the pluripotent HSC may differentiate into lymphoid or myeloid restricted progenitors and, subsequently, into one of the mature cell lineages. The mature hematological cells are incapable to divide and after their cell-characteristic lifespan undergo apoptosis [Lensch & Daley, 2004]. Although initially it was thought that progenitor cell populations were committed irreversibly to their downstream lineages, recent studies show that these developmental stages are not strictly binary branch points [Kaufman & Scadden, 2013].

A complex network of signaling pathways regulates HSC self-renewal, lineage commitment, and differentiation. In order to produce mature blood cells, the multipotent HSCs differentiate into multipotent progenitors (MPPs). These

immature cells lose self-renewal potential but differentiate in multipotent myeloid (CMPs) and lymphoid progenitor cells (CLPs). Next, these progenitor cells give rise to more-differentiated and committed cells: the T and natural killer progenitor cells (TNKs), the granulocytes and macrophages progenitor cells (GMPs), and the megakaryocytes and erythroid progenitor cells (MEPs). Further in the differentiation process, these cells differentiate into unilineage-committed progenitors for B cells (Pro-Bs), NK cells (Pro-NKs), T cells (Pro-Ts), granulocytes (GPs), monocytes (MPs), erythrocytes (EPs), and megakaryocytes (MKs). Finally, the unilineage-committed myeloid progenitors differentiate in myeloid mature cells: neutrophils, eosinophils, basophils, erythrocytes, monocytes, and platelets; while the unilineage-committed lymphoid progenitors cells into natural killer (NK) cells, B and T lymphocytes [Kaushansky, 2006; Wadhwa & Thorpe, 2008]. The dendritic cells have a particular differentiation pathway, and may arise from common myeloid progenitor cells or from common lymphoid progenitor cells [Rieger & Schroeder, 2012].

The regulatory pathways that control blood cell differentiation are mediated largely by cytokines and growth factors, such as interleukins (Ils), stem cell factor (SCF), colony-stimulating factors (CSFs), interferons (IFNs), erythropoietin (EPO), and thrombopoietin (TPO), as well as their receptors, such as granulocyte colony-stimulating factor receptor (GCSFR), erythropoietin receptor (EPOR), thrombopoietin receptor (TPOR or c-MPL). For example, during the initial stages of hematopoiesis SCF, TPO, IL-3, and GM-CSF induce HSCs differentiation into CMPs [Kaushansky, 2006; Robb, 2007; Wadhwa & Thorpe, 2008].

To continuously regenerate the hematopoietic system, the right number of specific cell types must be permanently generated at the right time and place [Rieger & Schroeder, 2012]. The correct fate decisions constantly have to be chosen by HSCs, and the exact timing and sequential order of all choices in each cell support normal hematopoiesis. To achieve this, HSCs depend on their microenvironment – the bone marrow hematopoietic niche – for regulation of self-renewal and differentiation [Ho *et al.*, 2015; Orkin & Zon, 2008; Rieger & Schroeder, 2012].

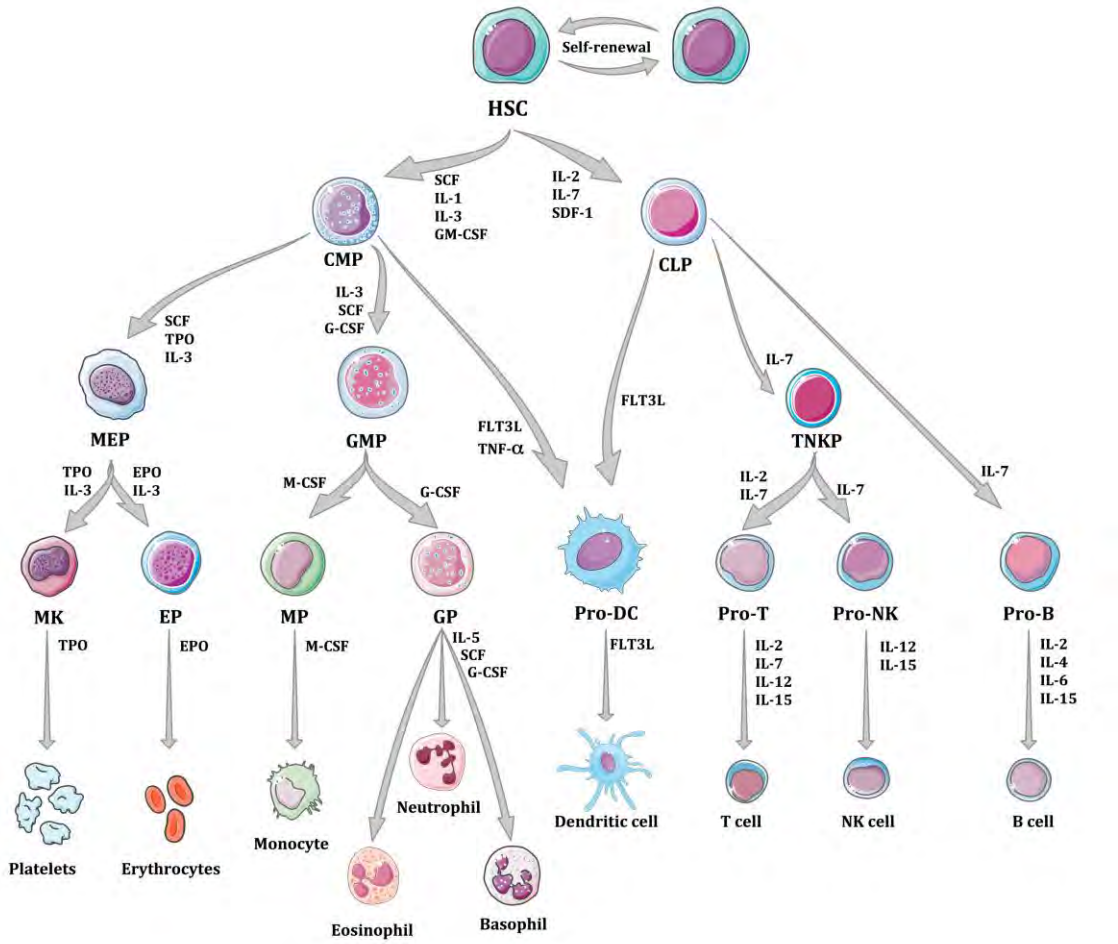


Figure 1. Hematopoiesis – the adult hematopoietic differentiation hierarchy. Long-term self-renewing hematopoietic stem cells (HSCs) are at the apex of a hierarchy of multiple progenitor cell stages giving rise to all blood cell lineages. Blood-cell development progresses from a HSC, which can undergo either self-renewal or differentiation into multilineage committed progenitor cells: the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). These cells then give rise to more-differentiated progenitors, comprising those committed to two lineages that include T cells and natural killer cells (TNKs), granulocytes and monocytes (GMPs), and megakaryocytes and erythroid cells (MEPs). Ultimately, these cells give rise to unilineage-committed progenitors for B cells (Pro-Bs), NK cells (Pro-NKs), T cells (Pro-Ts), granulocytes (GPs), monocytes (MPs), erythrocytes (EPs), and megakaryocytes (MKs). The dendritic progenitor cells can arise from a CMP or a CLP. Some cytokines and growth factors that support the survival, proliferation, or differentiation of each type of cell are represented. EPO, erythropoietin; GM-CSF, granulocyte-macrophage; IL, interleukin; FLT3L, FLT3 ligand; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; SDF1, stromal cell-derived factor 1; TNF-α, tumor necrosis factor alpha; TPO, thrombopoietin.

The niche serves several important functions for hematopoiesis: 1. regulates HSCs self-renewal; 2. controls the number of HSCs; 3. coordinates the regulation of proliferation and differentiation of HSCs; and 4. organizes cell localization [Dzierzak, 2011; Kaufman & Scadden, 2013]. The elements from bone marrow hematopoietic niche establish reciprocal relationships through several factors (such as cytokines, extracellular matrix components, and signal transducers), modulating each element fate [Ho *et al.*, 2015].

The differentiation of a multipotent cell to a specific lineage involves a global change of gene expression. Lineage choice and commitment are accompanied by the induction and maintenance of lineage-affiliated genetic programs. These include not only the expression of lineage-specific genes but also the repression of those specific for other lineages. Stable gene expression requires the presence and activity of a set of distinct transcription factors, which are integrated in complex networks with other transcription factors, modulating cofactors, chromatin modifiers, microRNAs, and other regulatory RNAs [Rieger & Schroeder, 2012].

The hematopoietic cell hierarchy is tightly regulated at every step, from HSCs self-renewal to differentiation into progenitors. Deregulation of such homeostasis can lead to severe diseases, including cancer. Given the observation that cancers are generally monoclonal in nature with heterogeneous cell populations at varying degrees of differentiation, it is not surprising that hematopoietic neoplasms would be organized in a cellular hierarchy similar to hematopoiesis [Chao *et al.*, 2008]. Indeed, almost all hematopoietic transcription factors are intimately associated with hematopoietic malignancies. The disturbance of the homeostatic balance of the critical transcriptional regulators, namely by somatic mutations or chromosomal translocations, is a defining feature of hematopoietic neoplasms. However, these malignancies are not consequence of nonspecific transcriptional effects but rather the end result of attacks at vulnerable points in the hematopoietic network. In addition to somatic mutation in the major hematopoietic transcription factors, lesions in signaling pathways that control specific lineage differentiation, cell death, and survival may underlie hematopoietic malignancies [Orkin & Zon, 2008].

1.2. Hematological malignancies

The myeloid malignancies are a group of heterogeneous clonal disorders with diverse clinical and pathological manifestations affecting hematopoietic stem cell function and lineage-specific differentiation [Lindsley & Ebert, 2013]. This group of malignancies comprises myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), and acute myeloid leukemia (AML) [Murati *et al.*, 2012]. All these malignancies are more incident and prevalent in elderly individuals [Carbonell *et al.*, 2015].

According to the 2008 World Health Organization (WHO) classification, myeloid neoplasms include several entities (Table 1): myeloproliferative neoplasms (MPN), myeloid and lymphoid neoplasms with eosinophilia and abnormalities of platelet-derivated growth factor receptor alpha (*PDGFRA*), beta (*PDGFRB*), or fibroblast growth factor receptor (*FGFR1*), myelodysplastic syndrome (MDS), myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN), and acute myeloid leukemia [Vardiman *et al.*, 2008]. This classification define that myeloid neoplasms include diseases of cells that belong to granulocytic (neutrophil, eosinophil, and basophil), monocytic, erythroid, megakaryocytic, and mast cell lineages [Vardiman *et al.*, 2009]. The 2008 WHO classification is based on a combination of morphologic, cytochemical, immunophenotypic, and genetic features of neoplastic cells that allow the establishment of neoplastic cell lineages and the assessment their maturation [Vardiman *et al.*, 2008].

A myeloid neoplasm with 20% or more blasts in the peripheral blood or bone marrow is considered to be an AML [Vardiman *et al.*, 2009]. In some cases, the diagnosis of AML can also be made when the blast percentage is lower than 20% if: 1. a specific genetic abnormalities is present; 2. erythroid precursors account for 50% or more of the bone marrow cells; or 3. blasts account for 20% or more of the non-erythroid marrow cells [Vardiman *et al.*, 2008; Vardiman *et al.*, 2009].

The landscape of recurrent somatic alterations in the coding genome of many myeloid malignancies has been extensively characterized, revealing a central role

for specific mutations in driving the distinctive features of disease biology [Lindsley & Ebert, 2013]. Recent studies have demonstrated significant genetic similarities on a series of recurrently affected cellular pathways in myeloid neoplasms that are morphologically distinct diseases.

Table 1
WHO classification of myeloid neoplasms (2008)

Myeloproliferative neoplasms (MNP)
Chronic myelogenous leukemia, *BCR-ABL1*-positive
Chronic neutrophilic leukemia
Polycythemia vera
Primary myelofibrosis
Essential thrombocythemia
Chronic eosinophilic leukemia, not otherwise specified
Mastocytosis
Myeloprolipherative neoplasms, unclassifiable
Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*
Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement
Myeloid neoplasms with *PDGFRB* rearrangement
Myeloid and lymphoid neoplasms with *FGFR1* abnormalities
Myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN)
Chronic myelomonocytic leukemia
Atypical chronic myeloid leukemia, *BCR-ABL1*-negative
Juvenile myelomonocytic leukemia
Myelodysplastic syndrome/myeloproliferative neoplasms, unclassifiable
Myelodysplastic syndrome (MDS)
Refractory cytopenia with unilineage dysplasia
Refractory anemia with ring sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory anemia with excess blasts
Myelodysplastic syndrome with isolated del(5q)
Myelodysplastic syndrome, unclassifiable
Childhood myelodysplastic syndrome
Acute myeloid leukemia and related neoplasms (AML)
Acute myeloid leukemia with recurrent genetic abnormalities
Acute myeloid leukemia with myelodysplastic-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

ABL1, ABL proto-oncogene 1, non-receptor tyrosine kinase; *BCR,* breakpoint cluster region; del(5q), deletion of the long arm of chromosome 5; *PDGFRA,* platelet-derived growth factor receptor alpha; *PDGFRB,* platelet-derived growth factor receptor beta; *FGFR1*, fibroblast growth factor receptor 1. Adapted from Swerdlow *et al.* [2008].

The functional categories of these pathways include: 1. cell signaling; 2. transcription; 3. cell cycle regulation; 4. regulation of DNA methylation and histone modification; 5. RNA splicing; and 6. components of the cohesin complex (Figure 2) [Matynia *et al.*, 2015]. Other mechanisms that likely contribute to disease pathogenesis comprise mutations in noncoding genome regions, epigenome abnormalities, and changes in bone marrow microenvironment [Lindsley & Ebert, 2013].

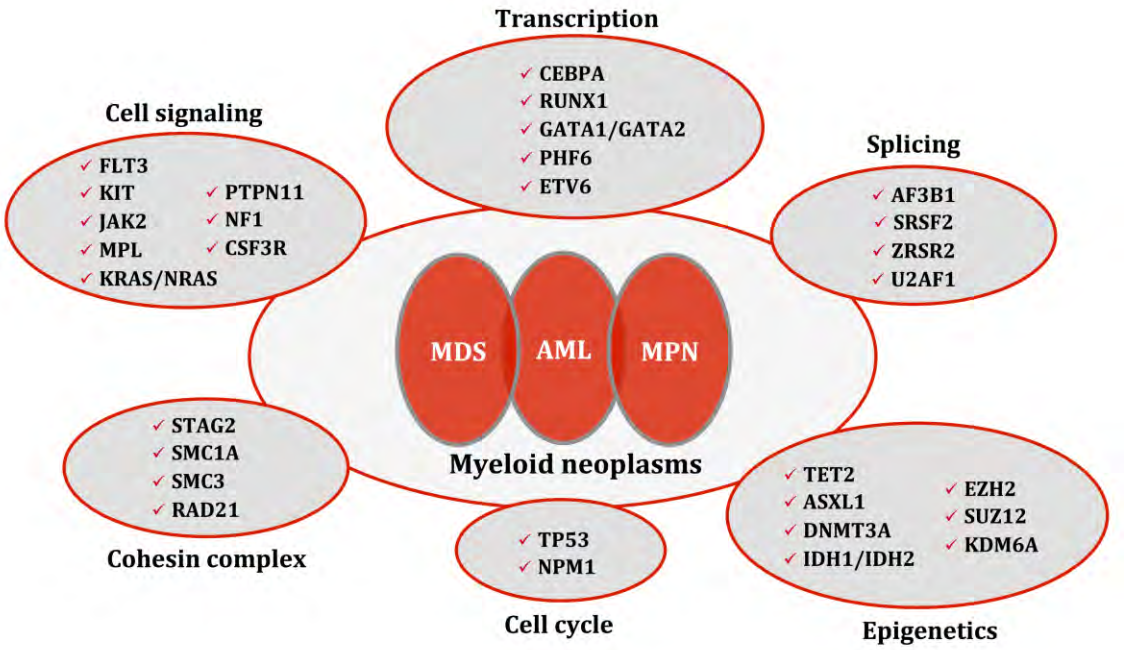


Figure 2. Pathways affected by recurrent mutations in myeloid malignancies. Adapted from Matynia *et al.* [2015].

The molecular basis of myeloid malignancies development and progression is a complex and multistep mechanism involving abnormal changes in cell proliferation, survival, differentiation, and death [Chung *et al.*, 2012]. These abnormalities are mainly determined by altered gene expression profiles induced by gene mutations and/or epigenetic modifications (Figure 3). The development of myeloid neoplasms requires the collaboration of at least three classes of mutations: class I mutations that activate signal-transduction pathways and confer a proliferation advantage to hematopoietic cells; class II mutations that affect transcription factors and primarily impair hematopoietic differentiation; and class

III mutations that interfere with epigenetic regulation [Gutiyama *et al.*, 2012]. The combination of proliferation and cell death evasion (or immortalization) can lead to the development of MPN, while the combination of immortalization with impaired differentiation may lead to the development of MDS. However, the combination of mutations that impair differentiation and promote proliferation can induce the development of AML [Chung *et al.*, 2012].

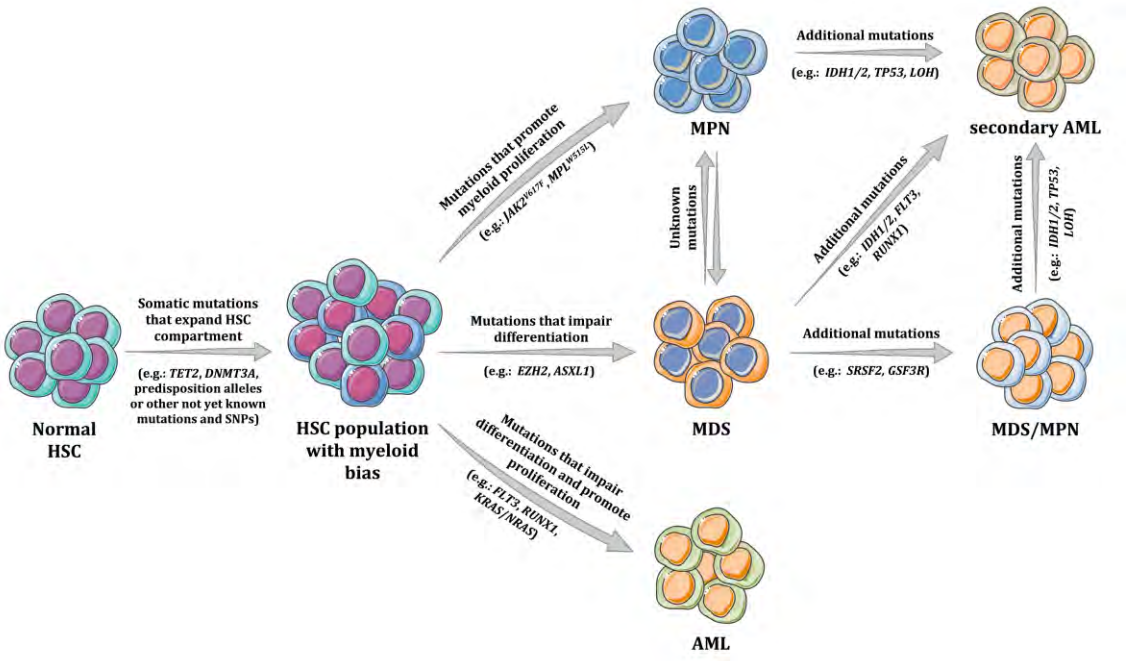


Figure 3. Multistep model of myeloid malignancies pathogenesis. Normal hematopoiesis proceeds from a small pool of hematopoietic stem cells (HSCs) with limitless self-renewal to produce more differentiated lymphoid and myeloid progenitors. During ageing HSC's acquire somatic mutations that leads to a HSC population with myeloid-bias and a decreased in lymphoid progenitor cells, resulting in clonal myeloid proliferation. The acquisition of additional genetic mutations following these early events may result in a clearer malignant phenotype, such as myeloproliferative neoplasms (MPN), myelodysplastic syndrome (MDS), or acute myeloid leukemia (AML). Adapted from Chung *et al.* [2012].

1.2.1. Myeloproliferative neoplasms

Myeloproliferative neoplasms comprise several clonal diseases that arise from the transformation of HSCs, and are characterized by increased proliferation of one

myeloid cell lineage: erythroid, megakaryocytic, or granulocytic [Campbell & Green, 2010; Skoda *et al.*, 2015]. Another feature of MPN is the possible spontaneous transform into MDS, AML, or even into a different subtype of MPN [Titmarsh *et al.*, 2014]. The polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis are the classic MPN subtypes (*BCR-ABL1* negative), and these malignancies share some clinical and bone marrow morphological similarities [Ranjan *et al.*, 2013; Skoda *et al.*, 2015; Tefferi & Vainchenker, 2011]. The chronic myeloid leukemia is another MPN subtype. However, this MPN subtype have been studied separately from the classic MPNs since the discovery of Philadelphia chromosome (produced by the reciprocal translocation t(9:22)(q34;q11) that results in the *BCR-ABL* chimeric gene, currently renamed *BCR-ABL1*). Additionally to these four disorders, the 2008 WHO classification of MPNs also includes mastocytosis, chronic eosinophilic leukemia-not otherwise specified, chronic neutrophilic leukemia, and MPN unclassifiable (Table 1) [Tefferi & Vainchenker, 2011].

The central pathological feature of PV is an expansion in total red cell mass. However, elevated counts of platelets and/or neutrophils are also commonly observed [Campbell & Green, 2011]. In contrast with other classic MPNs, megakaryocytes in PV are described as normal appearing to slightly enlarge, with a minimal amount of clustering [Klco *et al.*, 2010]. The median age at onset of PV is 55–60 years, and the annual incidence of this malignancy rounds 2–3 new cases per 100 000 population, increasing with age, and with a male to female ratio of 1.2:1 [Campbell & Green, 2011]. Almost all patients with PV harbor a *JAK2* (9p24) mutation: 96% displaying somatic activating mutations in exon 14 ($JAK2^{V617F}$) and 3% in exon 12 of *JAK2* [Tefferi & Barbui, 2015]. The overactivation of JAK2 by these mutations autonomously activates down-stream pathways, including JAK/STAT, leading to deregulated hematopoiesis [Griesshammer *et al.*, 2015]. Moreover, the erythropoiesis in PV patients is normally autonomous and erythropoietin independent [Campbell & Green, 2011].

The major characteristic of ET is the persistent elevation in the platelet count [Campbell & Green, 2011]. This malignancy is characterized by deregulated

proliferation of megakaryocytes and platelets in the bone marrow and peripheral blood [Klco *et al.*, 2010]. This clonal stem cell disorder is associated with reduced quality of life, risk of thrombohemorrhagic complications, and risk of transformation to myelofibrosis and acute myeloid leukemia [Sirhan *et al.*, 2015]. The median age at onset of this malignancy is 50–55 years, and the annual incidence rounds 1.5–2 new cases per 100 000 population [Campbell & Green, 2011]. Beside increased in number, platelets are often morphologically and functionally abnormal and, therefore, ET patients have an increased risk of thrombosis and bleeding. The bone marrow is frequently hypercellular due to the increase in megakaryocytes with minimal to no expansion of the granulocytic and erythroid lineages, as well as minimal fibrosis [Klco *et al.*, 2010; Campbell & Green, 2011]. However, ET patients display several differences in their clinical and pathological features, suggesting that ET is a heterogeneous disease [Klco *et al.*, 2010].

The disruption of hematopoiesis is a hallmark feature of MPNs [Cleary & Kralovics, 2013]. Despite their phenotypic diversity, all MPNs exhibit clonal hematopoiesis driven by acquired point mutations or chromosomal aberrations [Cleary & Kralovics, 2013; Milosevic & Kralovics, 2013]. The monoclonal or oligoclonal architecture and hierarchy in these diseases is complex and not always predictable. In general, the somatic mutations found in MPN patients can be functionally classified into three groups: mutations providing clonal advantage but not inducing disease phenotype, mutations capable of inducing clonal growth as well as disease phenotype, and mutations causing acute disease phenotype, such as acute leukemia (Figure 4).

Currently, the known MPN associated mutations involve *JAK2* (exon 14 and exon 12), *MPL* (exon 10), *CALR*, *GSF3R*, *TET2*, *DNMT3A*, *ASXL1*, *IDH1*, *IDH2*, *CBL*, *IKZF1*, *SH2B3*, *SF3B1*, *SRSF2*, and *EZH2* [Milosevic & Kralovics, 2013; Skoda *et al.*, 2015; Tefferi & Vainchenker, 2011]. Most of these mutations originate at progenitor cell level but they do not necessarily represent the primary clonogenic event and are not mutually exclusive, except *CALR* and *JAK2* mutations that are almost mutually exclusive [Skoda *et al.*, 2015; Tefferi & Vainchenker, 2011].

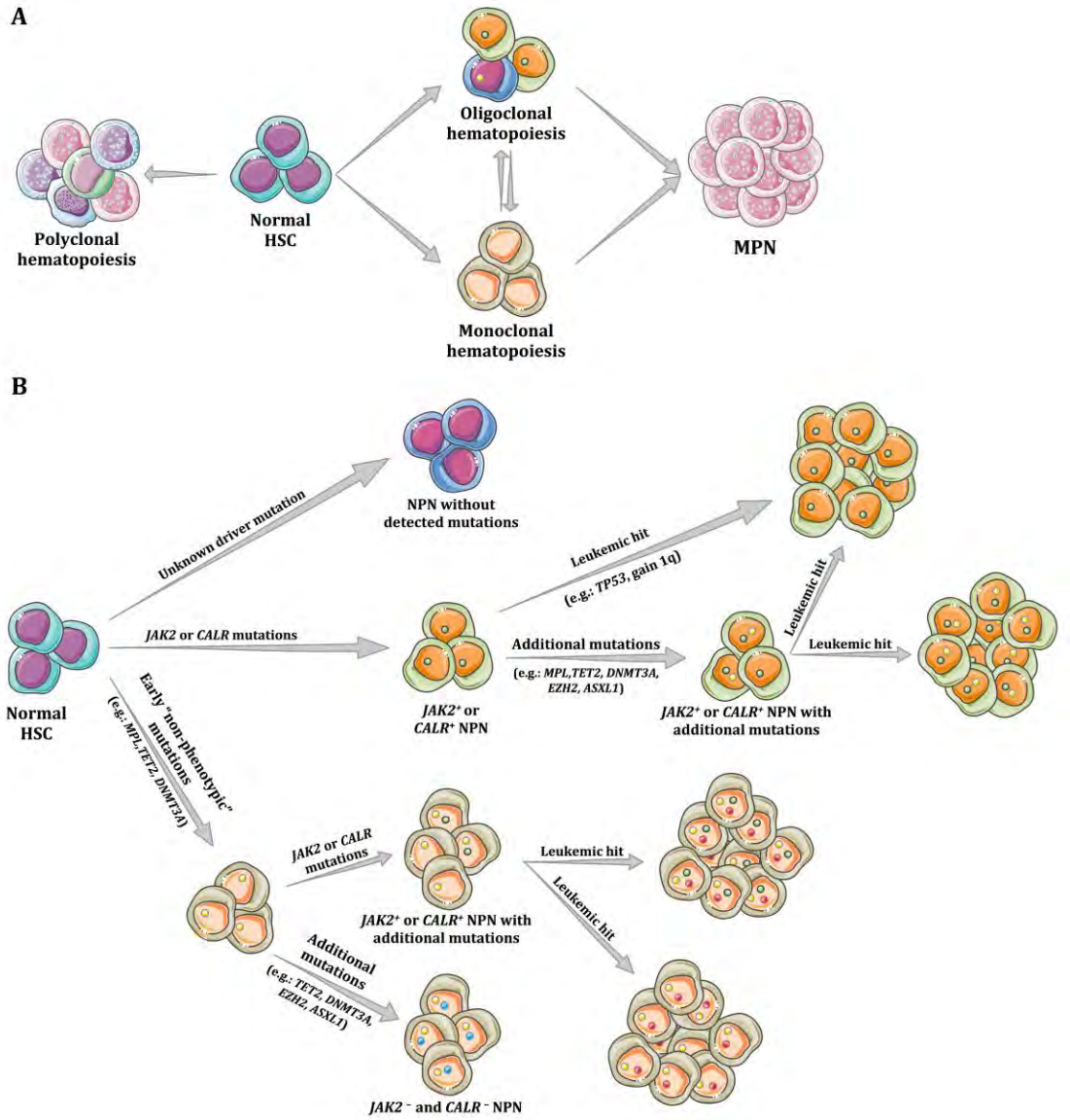


Figure 4. Clonal origin (A) and evolution in *BCR-ABL1*–negative myeloproliferative neoplasms (B). The myeloproliferative neoplasms (MPNs) may emerge from multiple abnormal clones (oligoclonal proliferation) or from an abnormal clone that gives rise to multiple subclones (monoclonal proliferation). Several mutations involving *JAK2* (janus kinase 2), *CALR* (calreticulin), *MPL* (myeloproliferative leukemia virus), *TET2* (tet methylcytosine dioxygenase 2), *ASXL1* (additional sex combs-like 1), *CBL* (casitas B-lineage lymphoma proto-oncogene), *IDH1/2* (isocitrate dehydrogenase 1/2) have been described in chronic- and/or blast-phase MPN. HSC, hematopoietic stem cell; AML, acute myeloid leukemia.

The presence or absence of the *JAK2*^{V617F} (Janus kinase 2; 9p24; c.1849G>T) mutation divides ET into two biologically distinct disorders [Campbell & Green,

2011]. *JAK2*^{V617F}-positive patients display multiple PV features (such as significantly increased hemoglobin levels, neutrophil counts, bone marrow erythropoiesis, and venous thrombosis) and a higher incidence of polycythemia transformation. On the other hand, *JAK2*^{V617F}-negative patients exhibit the clinical and laboratory characteristic features, such as the presence of endogenous erythroid colonies and an increased risk of acute leukemia transformation. ET Patients with *MPL* mutations (exon 10) have a phenotype closely related to *JAK2*-negative ET patients, presenting isolated thrombocytosis and less hypercellular bone marrow [Campbell & Green, 2011].

The *JAK2*^{V617F} is the most prevalent mutation in *BCR-ABL1*–negative MPN. The frequency of this mutation rounds 90–99% in PV and 50–70% in ET [Klco *et al.*, 2010; Matynia *et al.*, 2015; Milosevic & Kralovics, 2013; Skoda *et al.*, 2015; Tefferi *et al.*, 2009; Tefferi & Vainchenker, 2011]. JAK2 is a member of JAK family of tyrosine kinases, which also includes JAK1, JAK3, and TYK2, and mutations in *JAK2* gene lead to the constitutive activation of JAK2/STAT signaling pathway [Milosevic & Kralovics, 2013]. *CALR* (calreticulin; 19p13.2) mutations are rare in PV but occur in 15–24% with ET. CALR is a multi-functional Ca^{2+} binding protein chaperone mostly localized in the endoplasmic reticulum. It is not clear how *CALR* mutations cause MPN; however, they also ultimately lead to hyperactivity of the JAK2/STAT signaling pathway in megakaryocytic and granulocytic progenitor as well as in precursor cells [Skoda *et al.*, 2015]. *MPL* (MPL proto-oncogene, thrombopoietin receptor; 1p34) mutations occur in approximately 4% of ET patients and are rare in PV [Tefferi & Barbui, 2015]. *MPL* mutations, such as W515L and W515K in exon 10, were found to be gain-of-function mutation, leading to thrombopoietin-independent growth and JAK2 constitutive activation [Cleary & Kralovics, 2013; Vainchenker & Constantinescu, 2012]. However, some MPN patients do not carry mutations in *JAK2*, *CALR*, or *MPL* genes (so-called ‘‘triple-negative’’ MPN), but they also have hyperactive JAK2 signaling. Therefore, it seems appropriate to consider that *BCR-ABL1*-negative MPNs are diseases arising from JAK2/STAT signaling hyperactivation [Skoda *et al.*, 2015].

1.2.2. Myelodysplastic syndrome

Myelodysplastic syndromes (MDS) are a highly heterogeneous group of hematopoietic malignancies, arising from hematopoietic stem cells, generally characterized by inefficient hematopoiesis, dysplasia in one or more myeloid cell lineages, variable degrees of cytopenias, and increased risk of AML development [Brunning *et al.*, 2008; Chevassut & Mufti, 2011; Jhanwar, 2015; Visconte *et al.*, 2014]. The abnormal clone expansion is characterized by morphological dysplasia, impaired differentiation, defective cellular functions, and genetic instability. The consequences of ineffective hematopoiesis are peripheral cytopenias that frequently involve erythroid, granulocytic, and megakaryocytic lineages [Mufti, 2004]. MDS patients display several histologic hallmarks of aberrant hematopoietic cell differentiation, such as abnormal nuclear/cytoplasmic ratio, altered nuclear shape, agranularity or persistence of granules when they should be absent at that particular stage of differentiation, among others features (Figure 5). One of the major differences between MDS and more classically proliferative neoplasms, such as AML, is that myelodysplastic cells have a high rate of apoptosis, presumably as a result of the differentiation defects [Issa, 2013].

According to the 2008 World Health Organization classification system for hematologic neoplams, the primary myelodysplastic syndromes are subdivided in six major subtypes (Table 1): 1. refractory cytopenia with unilineage dysplasia, including the subtypes refractory anemia (RA), refractory neutropenia, and refractory thrombocytopenia; 2. refractory anemia with ring sideroblasts (RARS); 3. refractory cytopenia with multilineage dysplasia (RCMD); 4. refractory anemia with excess of blasts (RAEB), subdivided in RAEB-1 (5–9% of blasts in bone marrow) and RAEB-2 (10–19% of blasts in bone marrow); 5. myelodysplastic syndrome with isolated 5q deletion [del(5q)]; and 6. unclassifiable myelodysplastic syndrome [Brunning *et al.*, 2008].

The overall annual incidence rate for MDS in Europe is 3–4 per 100 000 individuals, increasing markedly with age. In individuals over the age of 80 years, the MDS incidence exceeds 30 per 100 000 individuals [Chevassut & Mufti, 2011].

The majority of patients (60%) are over the age of 70 at diagnosis, with males more likely to be diagnosed with MDS than females, with a male to female ratio of 1.4:1, with the exception of isolated del(5q) which predominate in women [Adès *et al.*, 2014; Chevassut & Mufti, 2011].

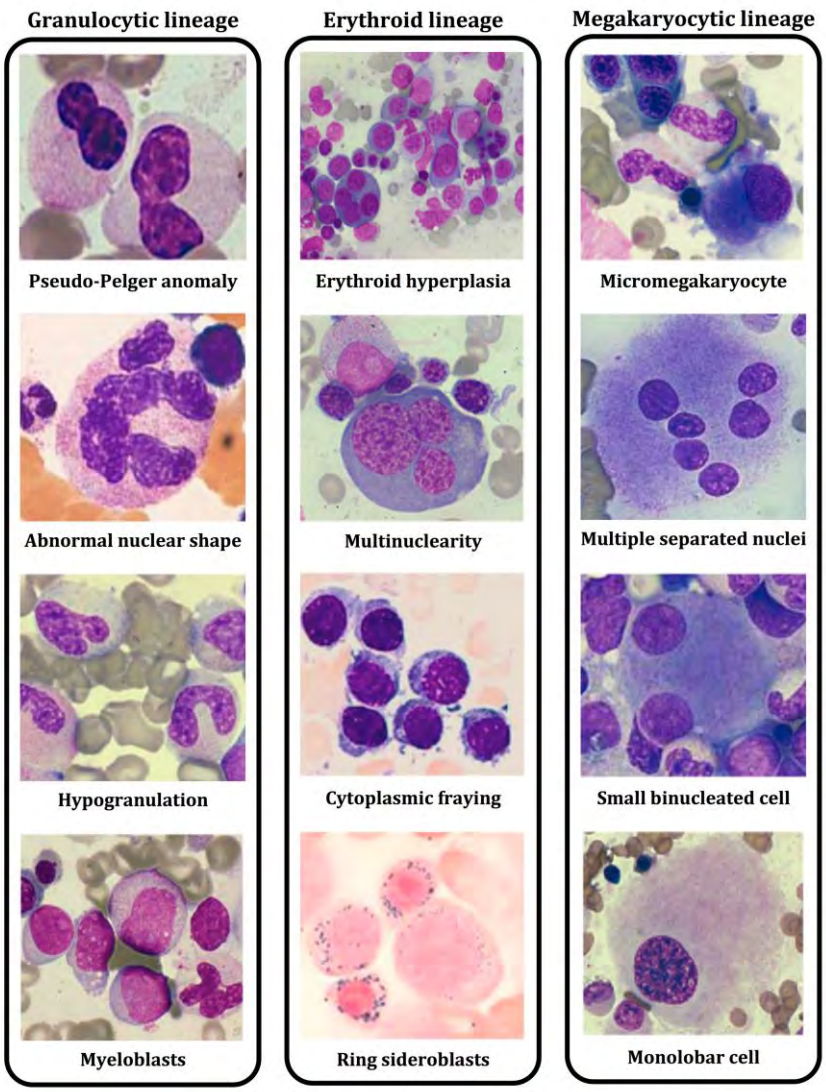


Figure 5. Representative examples of morphologic abnormalities of myelodysplasia. Amplification from 200x to 1000x. Adapted from Cazzola *et al.* [2013].

In the past decades, many prognostic factors have been identified in myelodysplastic syndromes. The most widely used prognostic classification system for MDS is the International Prognostic Scoring System (IPSS). The IPSS is based on a small number of features with independent prognostic value (Table 2

and 3), routinely available in all centers, and classify MDS patients in four subgroups with different risks of acute myeloid leukemia progression and survival [Adès *et al.*, 2014; Garcia-Manero, 2010; Greenberg *et al.*, 1997]. According to the bone marrow blast, peripheral cytopenias, and cytogenetic abnormalities, MDS patients are stratified in low-risk, intermediate-1 (int-1), intermediate-2 (int-2), and high-risk [Greenberg *et al.*, 1997]. Several attempts have been made to refine the IPSS. These refined systems include: the WHO-based prognostic scoring system (WPSS), which takes into account WHO classification and requirements for transfusion of red blood cells; and the revised IPSS (R-IPSS), which also uses cytogenetic abnormalities, cytopenias, and blast count for scoring, but with new thresholds [Adès *et al.*, 2014].

Table 2

The international prognostic scoring system (IPSS) score values

Score (points)	Prognostic variables		
	Bone marrow blasts	Karyotype*	Cytopenias**
0.0	0 – 5%	Good	0 – 1
0.5	5 – 10%	Intermediate	2 – 3
1.0		Poor	
1.5	11 – 19%		
2.0	20 – 30%§		

§This group is recognized as acute myeloid leukemia (AML) in the World Health Organization (WHO) classification.
*Karyotype: Good = normal, Y, del(5q), del(20q); Poor = complex ≥3 abnormalities, chromosome 7 abnormalities; Intermediate = other abnormalities.
**Cytopenias: Platelet count $<100\times10^9$/l; Hemoglobin <10 g/dl; Absolute neutrophil count $<1.8\times10^9$/l.

Table 3

International prognostic scoring system (IPSS) risk category and clinical outcomes

	Low	Int-1	Int-2	High
Risk score	0.0	0.5 – 1.0	1.5 – 2.0	≥2.5
Median survival (years)	5.7	3.5	1.2	0.4
Time to 25% AML evolution (years)	9.4	3.3	1.1	0.2

AML, acute myeloid leukemia; int-1, intermediate-1; int-2, intermediate-2.

The majority of MDS cases are sporadic, or primary MDS, and their pathogenesis remain largely unknown. In contrast, secondary MDS, a less frequent type of MDS,

is frequently associated with cytotoxic exposures, such as alkylating agents and topoisomerase inhibitors, radiation, as well as with certain environmental and occupational toxins such as benzene, agricultural chemicals and solvents [Babushok & Bessler, 2015]. The incidence of MDS development is increased in certain genetic disorders, such as Bloom syndrome, Diamond-Blackfan syndrome, Shwachman-Diamond syndrome, dyskeratosis congenita, Fanconi's anemia, Down syndrome, megakaryocytic thrombocytopenia, severe congenital neutropenia, and neurofibromatosis [Mufti, 2004; Tefferi & Vardiman, 2009]. Moreover, there is also some evidence suggesting that gene variants involved in carcinogens metabolism (e.g. *glutathione S-transferases theta 1 – GSTT1*), oxidative stress (e.g. *NAD(P)H dehydrogenase quinone 1 – NQO1*), and DNA damage repair (e.g. *8-oxoguanine DNA glycosylase – OGG1*) may increase MDS susceptibility [Chen *et al.*, 1996; Chevassut & Mufti, 2011].

MDS is considerate a prototypical epigenetic disease, and patients with this malignancy can display abnormalities in every aspect of epigenetic regulation. Epigenetic abnormalities in MDS include abnormal methylation pattern, modification of histone code, and microRNA (miRNA) expression [Issa, 2013]. Single locus and genome-wide reports indicate that several individual genes and pathways are silenced by DNA methylation, including genes involved in cell cycle control (*P15* and *P16*), apoptosis (*FHIT*, *TRAIL*, and *DAPK*), DNA damage repair (*MGMT*, *MLH1*, and *MSH2*), and cell differentiation (*GATA1*, *RARB*, and *WT1*), indicating their contribution to MDS pathogenesis [Hofmann *et al.*, 2006; Hopfer *et al.*, 2012; Sheikhha *et al.*, 2002; Solomon *et al.*, 2008; Valencia *et al.*, 2011; Wu *et al.*, 2011].

Contrarily to DNA methylation, the implication of histone modifications in the pathogenesis of MDS has been less studied. However, recent reports have implicated the overexpression of polycomb group of proteins genes, such as *EZH2*, *RING-1*, and *BMI1* genes, in the pathogenesis of MDS [Santini *et al.*, 2013]. Moreover, miRNAs play key roles in cell growth, differentiation, and oncogenic transformation. Several research studies demonstrated that miRNA expression is deregulated in MDS patients. These patients have down-regulation of miR-145,

miR-146a, and miR-150 and up-regulation of miR-125b, miR-181, and miR-206, among other miRNA expression abnormalities [Liao *et al.*, 2013; Rhyasen & Starczynowski, 2012].

Epigenetic anomalies in MDS patients coexist with cytogenetic changes in more than half of the cases and with somatic mutations in virtually all cases. In this context, disease development appears to require concurrent genetic and epigenetic damages [Issa, 2013]. The common cytogenetic abnormalities found in MDS vary from a single chromosome abnormality, such as monosomy of chromosomes 5 and 7, to complex karyotype. However, in half of MDS patients a normal chromosome pattern is observed [Jhanwar, 2015; Nimer, 2008]. None of these abnormalities is specifically associated with MDS, since they are also detected in AML and in myeloproliferative diseases [Nimer, 2008]. Interstitial deletion within the long arm of chromosome 5 [del(5q)] as well as the monosomy of chromosome 7 or del(7q) [–7/del(7q)] are among the most frequently reported cytogenetic abnormalities in MDS patients, followed by del(20q), del (17p), and del(11q) [Pellagatti & Boultwood, 2015]. However, it is not clear whether these chromosomal abnormalities are initial events able to induce MDS development.

Most MDS patients have a detectable gene mutation. However, in contrary to MPN in which more than 85% of cases have a *JAK2*, *CALR*, or *MPL* mutations, no dominant mutation is known in MDS [Steensma *et al.*, 2015]. The most common driver mutations detected in MDS patients occur in genes involved in RNA splicing (*PRPF40B, SF1, SF3A1, SF3B1, SRSF2, U2AF1, U2AF35, U2AF65*, and *ZRSR2*), DNA methylation (*TET2*, *DNMT3A*, and *IDH1*/*IDH2*), chromatin modification (*ASXL1* and *EZH2*), as well as transcription (ETV6, *RUNX1*, *BCOR*, *NPM1*, and *TP53*) and cell cycle regulation (*P16* and *PTEN*) [Jhanwar, 2015; Nikoloski *et al.*, 2012]. Moreover, some MDS display mutations in genes that encode signal transduction proteins, such as *BRAF*, *CBL*, *GNAS*, *JAK2*, *PTPN11*, *NRAS*, *KRAS*, and *NF1* genes [Cazzola *et al.*, 2013; Jhanwar, 2015; Lindsley & Ebert, 2013; Nikoloski *et al.*, 2012]. Until now more than 60 mutated genes have been identified in patients with this disease, but only six of these genes are consistently mutated in 10% or more cases (Table 4).

Table 4
Recurrently mutated genes in myelodysplastic syndrome

Biological pathway Gene	Frequency	Prognostic significance
Chromatin modification		
ASXL1	11–20%	Adverse
DNA methylation and hydroxymethylation		
TET2	20–30%	Neutral
DNMT3A	10–15%	Adverse
Myeloid transcription factors		
RUNX1	10–15%	Adverse
Spliceosome complex		
SF3B1	15–30%	Favorable
SRSF2	10–20%	Adverse

ASXL1, additional sex combs like transcriptional regulator 1; *TET2*, tet methylcytosine dioxygenase 2; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3 alpha; *RUNX1*, runt-related transcription factor 1; *SF3B1*, splicing factor 3b, subunit 1; *SRSF2*, serine/arginine-rich splicing factor 2.
Data are derived from the following references: Bejar *et al.* [2014], Cazzola *et al.* [2013], Chen *et al.* [2014], Haferlach *et al.* [2014], Itzykson *et al.* [2013], Jhanwar [2015], Kim *et al.* [2015], Larsson *et al.* [2013], Lin *et al.* [2014a], Lin *et al.* [2014b], Lindsley & Ebert [2013] Nikoloski *et al.* [2012], Skokowa *et al.* [2014], Thol *et al.* [2011], Tsai *et al.* [2015].

Although genetic and epigenetic abnormalities are frequent in MDS, the consequences of these events in MDS pathogenesis have not been clarified, and do not explain all molecular and cellular features of these malignancies. In the past few years, some reports indicated that individuals with normal blood counts and without any apparent disease (normal elderly individuals) also present clonally restricted somatic mutations in MDS-associated genes [Busque *et al.*, 2012; Shlush *et al.*, 2014; Xie *et al.*, 2014]. These mutations confer an increased risk of subsequent hematological malignancy diagnosis and higher all-cause mortality [Steensma *et al.*, 2015]. Recently, Steensma and collaborators proposed the term clonal hematopoiesis of indeterminate potential (CHIP) to describe individuals with a hematologic malignancy-associated somatic mutation in blood or bone marrow, but without other diagnostic criteria for a hematologic malignancy.

According to these authors, the rate of CHIP progression to a hematologic malignancy (0.5 – 1.0% *per* year) appears to be similar to those observed in other known clonal pre-malignant disorders, such as the transition of monoclonal gammopathy of undetermined significance to multiple myeloma and monoclonal

B-cell lymphocytosis to chronic lymphocytic leukemia and other B-cell lymphomas [Steensma *et al.*, 2015]. The current understanding of the multistep pathogenesis of myeloid malignancies suggests that individuals with clonal mutations may already be partway along the path to evolution of a myeloid malignancy (Figure 6). Although individuals with somatic mutations show a higher rate of myeloid neoplasms diagnosis, the mutations by themselves do not currently define MDS diagnosis [Steensma *et al.*, 2015].

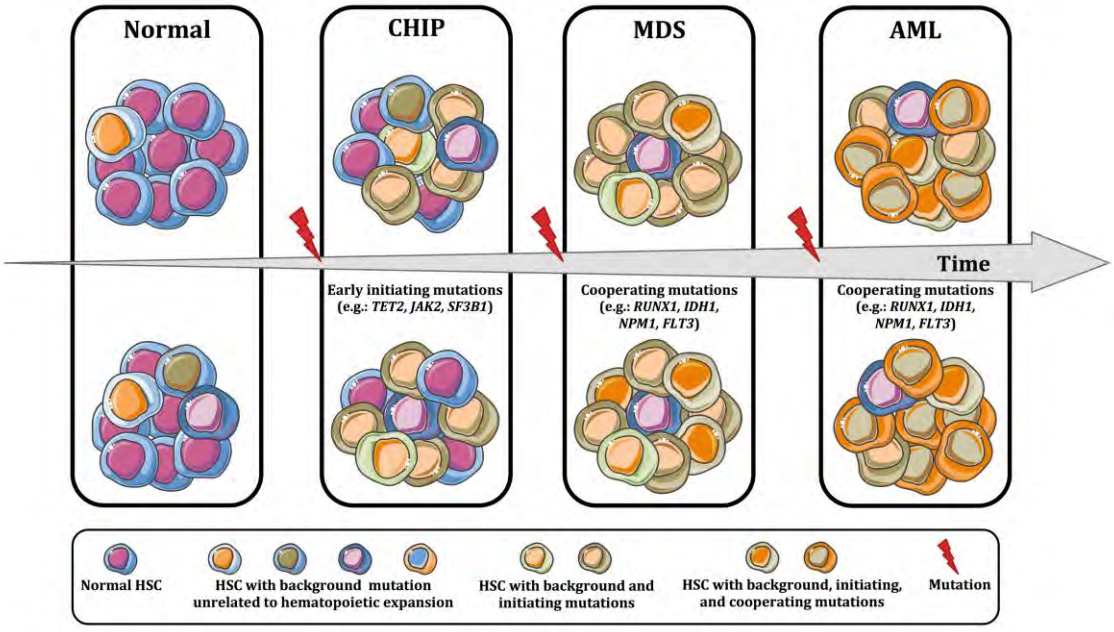


Figure 6. Clonal hematopoiesis of indeterminate potential (CHIP) as a precursor state for hematological neoplasms. A model for evolution from normal hematopoiesis to CHIP and then, in some cases, to MDS or AML. Hematopoietic progenitor or stem cells commonly acquire passenger mutations throughout lifespan, and these mutations that have no consequence for hematopoiesis. However, certain mutations confer survival advantage and allow clonal expansion, a condition defined as "clonal hematopoiesis of indeterminate potential (CHIP)". The occurrence of early and cooperating mutations in these cells may lead to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). The majority of patients with CHIP will never develop an overt neoplasm, and patients will eventually die of unrelated causes. Adapted from Steensma *et al.* [2015].

One of the major hallmarks of MDS is ineffective hematopoiesis. The ineffective hematopoiesis observed in MDS patients results from the increased susceptibility

of clonal myeloid progenitors to apoptosis, which leads to cytopenias [Tefferi and Vardiman, 2009]. This apoptotic process in hematopoietic precursors is present in the initial disease stages but decrease with progression, allowing abnormal clone expansion [Greenberg, 2012]. Other mechanisms have been implicated in the ineffective hematopoiesis and in MDS development, including bone marrow microenvironment, immunologic abnormalities, oxidative stress, and telomere length (Figure 7) [Adès *et al.*, 2014; Chevassut & Mufti, 2011; Farquhar & Bowen, 2003; Visconte *et al.*, 2014]. Additionally, several studies have demonstrated functional mitochondrial abnormalities in MDS patients, such as mitochondrial DNA mutation [Bowen *et al.*, 2002; Matthes *et al.*, 2000; Shin *et al.*, 2003]. The mitochondrial dysfunction induces cellular oxidative stress and, consequently, mitochondrial and nuclear DNA damage [Farquhar and Bowen, 2003].

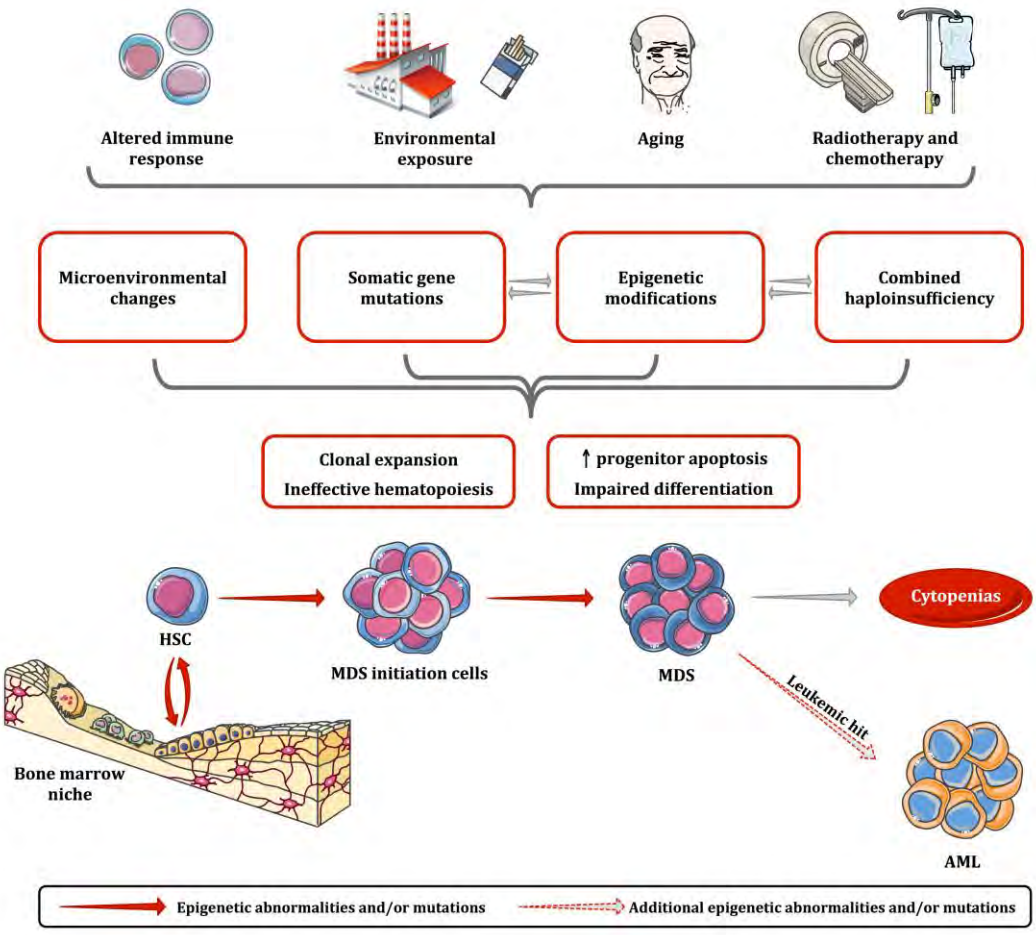


Figure 7. Clonal architecture of myelodysplastic syndromes (MDS) and progression to acute myeloid leukemia (AML).

In summary, age-induced genetic, epigenetic, and immune-mediated changes in hematopoietic stem cells lead to oligoclonal or monoclonal expansion of myelodysplastic initiating cells. These cells display a defective differentiation, without inducing proliferation, and are characterized by increased apoptosis of erythroid and myeloid progenitors, leading to cytopenias. The evolution to AML occurs when these myelodysplastic cells accumulate additional genetic lesions that promote proliferation.

1.2.3. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a clonal malignant disease of hematopoietic stem and/or progenitor cells characterized by severe block in myeloid differentiation, increased proliferation, and accumulation of immature myeloid cells in bone marrow, peripheral blood, and other tissues (mainly the liver and spleen, and to a lesser extent the lymph nodes, central nervous system, and testicles) [Burnett & Venditti, 2011; Eriksson *et al.*, 2015; Mehdipour *et al.*, 2015; Smith & Sung, 2013]. These abnormalities lead to inhibition of normal hematopoiesis, which is characterized by neutropenia, anemia, thrombocytopenia, and clinical features of bone marrow failure [Smith & Sung, 2013]. A considerable heterogeneity between AML patients is observed in respect to morphology, immunophenotype, cytogenetic, and molecular abnormalities, as well as aberrant gene expression patterns [Burnett & Venditti, 2011].

Currently, AML is defined by the presence of 20% or more myeloblasts, monoblasts, promonocytes, erythroblasts, or megakaryoblasts in the peripheral blood or bone marrow. The diagnosis of AML is independent of blasts count in patients with t(8;21)(q22;q22), inv(16)(p13q22), t(16;16)(p13;q22), or t(15;17)(q22;q12) cytogenetic abnormalities [Smith & Sung, 2013). The worldwide annual incidence of AML is 3–4 *per* 100 000 individuals and increases with age, presenting its peak incidence in the seventh decade [Burnett & Venditti, 2011; Smith & Sung, 2013]. It is considered to be the most common acute leukemia

in adults, accounting for 90% of all acute leukemias in this group of individuals. The median age at diagnosis is 67 years, being approximately one third of newly diagnosed patients older than 75 years [Klepin *et al.*, 2014; Smith & Sung, 2013]. The 2008 World Health Organization classifies AML based on clinical history, morphologic findings, and presence or absence of specific genetic abnormalities (Table 5).

Table 5
WHO classification of acute myeloid leukemia (2008)

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
Acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplastic-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

RUNX1, runt-related transcription factor 1; *RUNX1T1*, runt-related transcription factor 1 translocated to 1 (cyclin D-related); *CBFB*, core-binding factor (beta subunit); *MYH11*, myosin (heavy chain 11, smooth muscle); *PML*, promyelocytic leukemia; *RARA*, retinoic acid receptor alpha; *MLLT3*, myeloid/lymphoid or mixed-lineage leukemia translocated to 3; *MLL*, myeloid/lymphoid or mixed-lineage leukemia [alias *KMT2A* (official symbol provided by HGNC), lysine (K)-specific methyltransferase 2A]; *DEK*, DEK proto-oncogene; *NUP214*, nucleoporin 214 kDa; *RPN1*, ribophorin 1; *EVI1*, ecotropic viral integration site 1 [alias *MECOM* (official symbol provided by HGNC), MDS1 and EVI1 complex locus]; *RBM15*, RNA binding motif protein 15; *MKL1*, megakaryoblastic leukemia (translocation) 1; *NPM1*, nucleophosmin (nucleolar phosphoprotein B23, numatrin); *CEBPA*, CCAAT/enhancer binding protein (C/EBP), alpha. Adapted from Swerdlow *et al.* [2008].

Several factors have been associated to an increased risk of AML. These factors include previous hematological diseases, hereditary syndromes, as well as

environmental and drug exposures; however, most patients who present *de novo* AML have no identifiable risk factor [Gutiyama *et al.*, 2012]. The first insights into AML pathogenesis were originated from recurrent chromosomal translocations. The most observed chromosomal abnormalities in AML are balanced translocations, inversions, monosomies, trisomies, insertions, and deletions; and one or more chromosomal abnormalities can be found in approximately 55% of AML cases [Meyer & Levine, 2014]. Other pathogenic events in AML include environmental factors (smoking and benzene exposure), chemotherapy or radiotherapy treatment, and preceding MDS or MPN [O'Brien *et al.*, 2014]. As mentioned before, AML is a highly heterogeneous disease, since patients may show different biological and clinical features among which genetic and epigenetic abnormalities are the most important [Gutiyama *et al.*, 2012].

Chromosomal abnormalities are strong independent outcome predictors and key features in the WHO classification of AML (subclassification of AML with recurrent genetic abnormalities), being the basis of AML risk groups [Meyer & Levine, 2014; Swerdlow *et al.*, 2008]. This current AML classification incorporates only somatic mutation of two genes (*NPM1* and *CEBPA*), being these subclassifications provisionally. The epigenetic changes and mutations in epigenetic modifier genes have not been included yet [Ohgami & Arber, 2015]. The AML with myelodysplastic-related changes is diagnosed in patients with a history of MDS or based on the presence of significant morphologic dysplasia in two cell lineages at the time of AML diagnosis. Moreover, the presence of MDS-related cytogenetic abnormalities and absence of specific genetic abnormalities of AML with recurrent genetic abnormalities are also characteristics of this AML subtype [Swerdlow *et al.*, 2008].

The genomic complexity of AML continues to be disclosed as the genetic and epigenetic landscape is unraveled [Rowe, 2014]. In the past few years, the most common somatic gene mutations detected in AML patients are included into the following functional categories: RNA splicing (*SF3B1, SRSF2,* and *U2AF1*), DNA methylation (*TET2*, *DNMT3A*, and *IDH1*/*IDH2*), chromatin modification (*ASXL1* and *EZH2*), myeloid transcription factors (*CEPBA* and *RUNX1*), tumor suppressor genes

(*TP53, WT1,* and *PHF6*), cohesins (*SMC1A*, *SMC3, RAD21*, and *STAG2*), and signal transduction (*NPM1*, *FLT3*, *NRAS*, *c-KIT*, and *PTPN11*) [Grove & Vassiliou, 2014]. However, only nine of these genes are recurrent mutations (i.e. consistently present in 10% or more of the AML cases – Table 6).

Table 6
Recurrently mutated genes in acute myeloid leukemia

Biological pathway Gene	Frequency	Prognostic significance
Chromatin modification		
ASXL1	10–30%	Adverse
DNA methylation and		
DNMT3A	15–30%	Adverse
IDH1/2	15–30%	Unclear
Myeloid transcription factors		
RUNX1	10–15%	Adverse
CEPBA	10–20%	Favorable
Tumor suppressor genes		
WT1	10–15%	Unclear
Nucleophosmin 1		
NPM1	25–30%	Favorable
Activating signal transduction		
FLT3 (ITD)	20–30%	Adverse
NRAS	12–30%	Neutral

ASXL1, additional sex combs like transcriptional regulator 1; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3 alpha; *IDH1*, isocitrate dehydrogenase 1; *IDH2*, isocitrate dehydrogenase 2; *RUNX1*, runt-related transcription factor 1; *CEBPA*, CCAAT/enhancer binding protein (C/EBP), alpha*; WT1,* Wilms tumor 1; *NPM1,* nucleophosmin 1; *FLT3,* fms-related tyrosine kinase 3; *NRAS,* neuroblastoma RAS viral (v-ras) oncogene homolog. Data are derived from the following references: Addel-Wahab *et al.* [2012], Baldus *et al.* [2006], Boissel *et al.* [2010], Borger *et al.* [2012], Cazzola *et al.* [2010], Eriksson *et al.* [2015], Fathi & Addel-Wahab [2012], Gelsi-Boyer *et al.* [2012], Gutiyama *et al.* [2012], Holz-Schietinger *et al.* [2011], Hou *et al.* [2013], Langemeijer *et al.* [2011], Ley *et al*. [2010], Mardis *et al.* [2009] Mehdipour *et al.* [2015], Milosevic *et al.* [2012], Odenike *et al.* [2011], Thol *et al.* [2011], Ohgami *et al.* [2015], O'Brien *et al.* [2014], Paschka *et al.* [2010], Shih *et al.* [2012], Zang *et al.* [2012].

The AML leukemia stem cells (LSC) share a common immunophenotype ($CD34^+/CD38^-$) with normal HSCs [Martinez-Climent *et al.*, 2010]. The similarities between HSCs and LSCs strongly suggest that LSCs can arise from HSC targeted by oncogenic lesions. However, the origin of leukemic clone remains controversial and the inference about AML cell origin based on the LSC cellular phenotype may be misleading [Horton & Huntly, 2012; Martinez-Climent *et al.*, 2010], since it is unclear whether driver mutations occur in HSCs, in progenitor cells (that

accumulation of appropriate mutations that restore the critical stem-cell abilities of self-renewal and multi-differentiation), or in both (Figure 8) [Horton & Huntly, 2012; Martinez-Climent *et al.*, 2010].

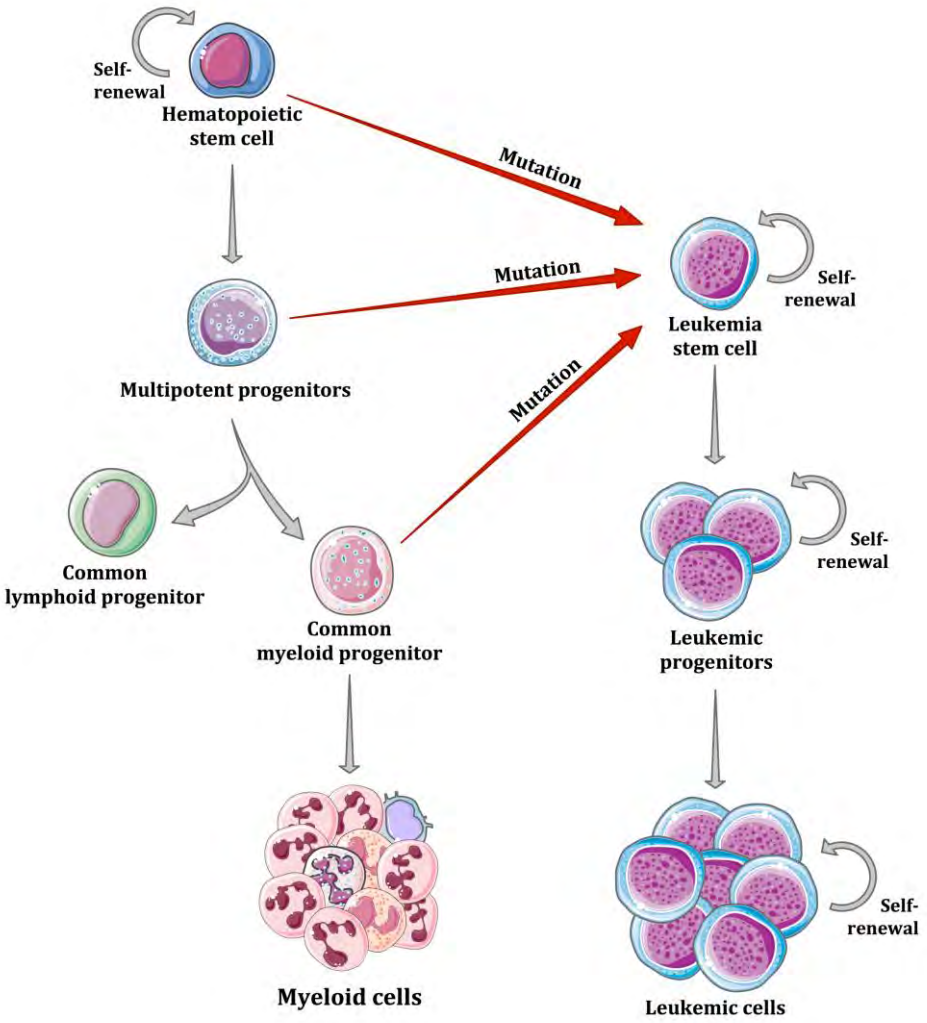


Figure 8. A model of acute myeloid leukemia stem cell origin. Leukemia stem cells (LSCs) can derive from HSCs, multipotent progenitors, committed progenitor cells or even from more differentiated cells that accumulate mutations that reprogram the necessary stem-cell features.

Until 2008, the accepted model of leukemogenesis was the "two-hit hypothesis". According to this model, two different types of genetic mutation (class I and II mutations) were required for malignant transformation of a HSC or myeloid precursor [Meyer & Levine, 2014; O'Brien *et al.*, 2014]. Class I mutations were thought to constitutively activate signaling pathways through to tyrosine kinases

receptors or downstream signaling molecules (in genes such as *BCR-ABL1*, *FLT-3*, *c-KIT*, and *RAS*), leading to uncontrolled cellular proliferation. This class of mutations also included inactivation of tumor suppressor genes, such as *TP53* and *NF1*, which induce apoptosis evasion [Kitamura *et al.*, 2014; Meyer & Levine, 2014; O'Brien *et al.*, 2014]. On the other hand, class II mutations include inactivation of transcription factor, such as *MLL* fusions and *PML-RARα*, and proteins that are involved in transcriptional regulation, such as p300, CBP, MLL, and TIF2. All these class II mutations were associated with impaired hematopoietic differentiation and deregulation of self-renewal capacities of progenitor cells [Meyer & Levine, 2014; O'Brien *et al.*, 2014].

In AML, the phenotype is a consequence of a proliferative advantage and differentiation impair, and class I and II mutations frequently occur together in AML blasts [Burnett & Venditti, 2011; Meyer & Levine, 2014]. On the contrary, mutations of the same class are rarely detected in the same patients [Meyer & Levine, 2014]. These facts suggest that class I and II mutations are required in the leukemic development and are functionally complementary of each other [Burnett & Venditti, 2011; Meyer & Levine, 2014].

However, the "two-hit hypothesis" cannot explain all biological features and clinical manifestations observed in AML patients, and the belief that most leukemias are a result of "multiple-hits" and molecular changes had spread in the scientific community [Burnett & Venditti, 2011]. The implementation of high-speed sequencing techniques, allow the identification of a variety of new mutations in myeloid neoplasms, including AML (Figure 9) [Kitamura *et al.*, 2014]. These genomic methods improve the understanding about genetic changes that drive AML pathogenesis, leading some researchers to divide functional gene mutations in three classes: classical class I and II mutations, and a new class III (epigenetic modifiers).

Recently, according to Cancer Genome Atlas Project analysis of AML genomes, nine functional groups were designated (Figure 9): Class 1 – transcription factor fusions (e.g. t(8;21), t(16;16), t(15;17), MLL fusions); Class 2 – nucleophosmin 1 (*NPM1*

mutations); Class 3 – tumor suppressor genes (e.g. *TP53*, *WT1*, *PHF6* mutations); Class 4 – DNA-methylation-related genes (e.g. DNA hydroxymethylation – *TET2*, *IDH1*/2; DNA methyltransferases – *DNMT3A*,); Class 5 – activated signaling genes (e.g. *FLT3*, *KIT*, *RAS* mutations); Class 6 – chromatin-modifying genes (e.g. *ASXL1*, *EZH2* mutations, *MLL* fusions, *MLL* partial tandem duplications); Class 7 – myeloid transcription factor genes (e.g. *CEBPA*, *RUNX1* mutations); Class 8 – cohesin-complex genes (e.g. *STAG2*, *RAD21*, *SMC1*, *SMC2* mutations); and Class 9 – spliceosome-complex genes (e.g. *SRSF2*, *U2AF35*, *ZRSR2* mutations) [Meyer & Levine, 2014].

Figure 9. Evolution of functional classes of genetic mutation that co-occur during acute myeloid leukemia development. The grey boxes indicate the frequency of occurrence of each mutation class in acute myeloid leukemia. Adapted from Meyer & Levine [2014].

The study of AML genomes revealed that patients with AML display more than one LSC population. The finding that multiple populations of LSC may co-occur within the same AML patients suggested that LSC population is not static and uniform, and could evolve from one phenotype to another depending on the acquired genetic and/or epigenetic abnormalities [Horton & Huntly, 2012]. This stepwise acquisition of genetic changes leads to a clonal heterogeneity with a subclonal architecture [Grove & Vassiliou, 2014]. In some AML cases, multiple genetic subclones of leukemia-initiating cells co-exist within a complex clonal architecture, usually consisting of distinct subpopulations with a dominant leukemic clone (Figure 10).

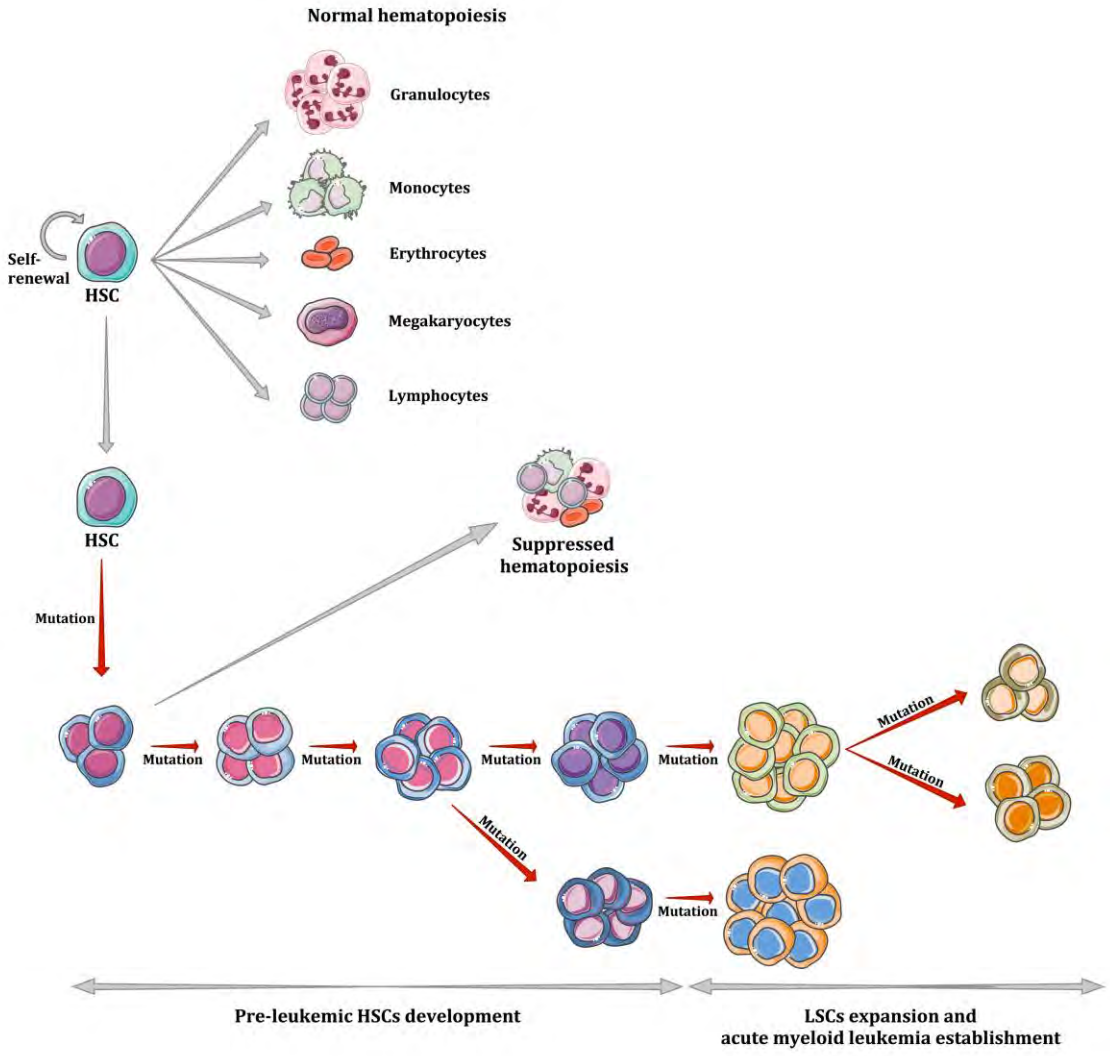


Figure 10. Clonal heterogeneity of acute myeloid leukemia cells.

Additional leukemic clones can arise when the dominant clone undergo additional genetic and/or epigenetic events. Alternatively, initiating mutations in a normal HSC may establish one or more pre-LSCs with multilineage differentiation potential. Upon the acquisition of further mutations or epigenetic changes, the initiated clone evolves to produce a subclone of lineage-restricted malignant myeloblasts. Ultimately, the leukemic pool consists of multiple clones or subclones with a complex genetic and/or epigenetic relationship, exhibiting common and divergent mutations [Jan & Majati, 2012; Wiseman *et al.*, 2013].

Although genetic abnormalities are common events in myeloid neoplasms, MNP, MDS, and AML share other non-genetic mechanisms such as oxidative stress and abnormal DNA methylation. Moreover, it is well recognized that interindividual genetic variability can predispose do myeloid neoplasm and that genetic susceptibility studies may serve to clarify important disease mechanisms.

1.3. Oxidative stress in myeloid neoplasms

The reactive oxygen species (ROS) are sub-products of normal cellular metabolism of living organisms [Birben *et al.*, 2012]. These oxygen free radicals are recognized for it dual role as deleterious and beneficial species, since they can be either harmful or beneficial to living systems [Valko *et al.*, 2007]. ROS are free radical and non-free radical oxygenated molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), and the hydroxyl radical ($^{\bullet}OH$) [Birben *et al.*, 2012; Valko *et al.*, 2007; Pisoschi & Pop, 2015]. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological cell processes, for example in defense against infectious agents and in normal function of a number of cellular signaling systems. However, at high concentrations, they have harmful effect causing potential biological damage to cell components (such as lipids, proteins, and DNA) and inhibiting their normal function.

These free radical molecules can be produced by both endogenous and exogenous sources. The endogenous sources include oxidative phosphorylation, P450

metabolism, peroxisomes, and inflammatory cell activation. The cellular ROS are mainly generated in three sites: mitochondria, endoplasmic reticulum, and NADPH oxidase complex (Figure 11).

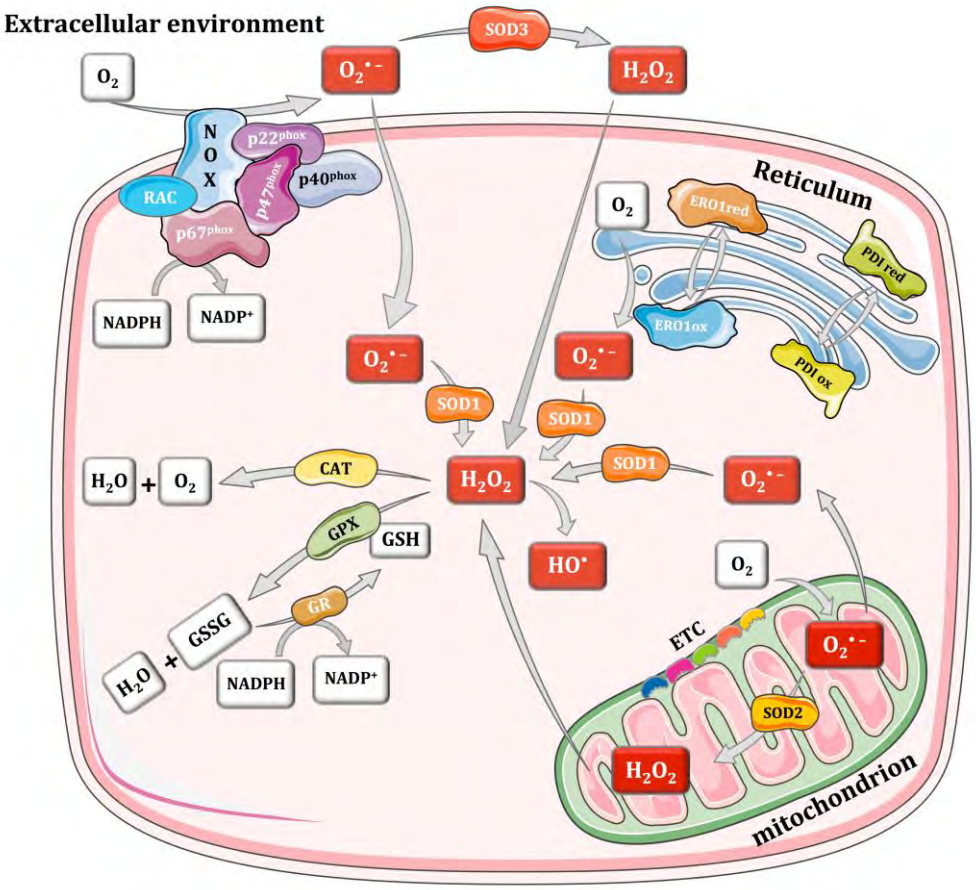


Figure 11. Role of mitochondria, NADPH oxidase, and endoplasmic reticulum in reactive oxygen species production. Cellular ROS are constitutively produced in mitochondria, endoplasmic reticulum, and NADPH oxidase complex. Under normal conditions, ROS are eliminated by enzymatic (SOD, catalase, and GPX) and non-enzymatic defenses (NADPH and GSH). ERO1, endoplasmic reticulum oxidoreductin; GSH, reduced glutathione; GSSG, oxidized glutathione; H_20, water; H_2O_2, hydrogen peroxide; O_2, oxygen; $O_2^{-\bullet}$, superoxide anion; SOD1, copper-zinc superoxide dismutase SOD2, manganese superoxide dismutase; SOD3, extracellular superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; ox, oxidized; red, reduced; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; p22phox, cytochrome b-245 alpha subunit; p40phox, neutrophil cytosolic factor 4; p47phox, neutrophil cytosolic factor 1; p67phox, neutrophil cytosolic factor 2; PDI, protein disulfide isomerase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; RAC, RAC GTPase. Adapted from Ye *et al.* [2015].

In the aerobic process, which employs oxygen to produce energy and heat, molecular oxygen is stepwise reduced to a series of intermediate species producing ROS [Klauning & Kamedulis, 2004; Pisoschi & Pop, 2015]. During mitochondrial oxidative metabolism, approximately 5% of molecular oxygen is converted to ROS, primarily superoxide anion [Lin & Beal, 2006; Ye *et al.*, 2015]. As the primary cellular consumers of oxygen, mitochondria contain numerous redox enzymes capable of transferring single electrons to oxygen, generating superoxide anion through the tricarboxylic acid cycle enzymes, electron-transport chain complexes I, II and III, among others enzymes [Lin & Beal, 2006]. Other endogenous sources of ROS are the neutrophils, eosinophils, and macrophages. They are major contributors of ROS through the respiratory burst, which elicit a rapid but transient increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, hydrogen peroxide, and nitric oxide [Klauning & Kamedulis, 2004]. Exogenous sources of ROS include the action of pollutants/toxins such as cigarette smoke, alcohol, ionizing and UV radiations, pesticides, hyperoxia, as well as ozone and heavy metal ions exposure [Birben *et al.*, 2012; Pisoschi & Pop, 2015].

ROS are able to interconvert into each other through several reactions. The endogenous and exogenous superoxide anion is dismutated by superoxide dismutases, producing hydrogen peroxide. The hydrogen peroxide can be produced by any system yielding superoxide, such as xanthine oxidase, amino acid oxidase, and NADPH oxidase, as well as in peroxisomes by consumption of molecular oxygen in metabolic reactions [Birben *et al.*, 2012]. The direct action of hydrogen peroxide involves the attack on heme proteins with release of iron and enzyme inactivation, as well as oxidation of DNA, lipids, thiol (-SH) groups, and keto-acids [Pisoschi & Pop, 2015]. Moreover, hydrogen peroxide easily diffuses across the plasma membrane, and although it low reactivity, in the presence of reduced metal ions, particularly iron, is converted to a hydroxyl radical – a more reactive and damaging ROS – through Fenton and Haber-Weiss reactions. The hydroxyl radical interact with nucleic acids, lipids, and proteins, inducing oxidative lesions in these macromelecules [Klauning & Kamedulis, 2004].

Fenton reaction

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + Fe^{3+} + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\cdot-} + 2H^+$$

$$2H_2O_2 \xrightarrow[Fe^{2+}/Cu^{+}]{} {}^{\cdot}OH + OH^- + O_2^{\cdot-} + 2H^+$$

Haber-Weiss reaction

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O \rightarrow {}^{\cdot}OH + Fe^{3+}$$

$$O_2^{\cdot-} + H_2O \rightarrow O_2 + {}^{\cdot}OH + OH^-$$

Under normal physiological conditions, cells are capable of counterbalancing ROS production with scavengers (Figure 12). The antioxidant defenses can be divided in two categories: enzymatic and non-enzymatic [Birben *et al.*, 2012]. These types of antioxidant defense systems against ROS prevent their occurrence and block their formation [Pisoschi & Pop, 2015]. The major antioxidant enzymes directly involved in the neutralization of ROS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GRx) [Birben *et al.*, 2012; Klauning & Kamedulis, 2004; Pisoschi & Pop, 2015, Valko *et al.*, 2007]. SOD is the first line of defense against free radicals, catalyzing the superoxide anion radical into hydrogen peroxide. Then, CAT or GPX convert the hydrogen peroxide into water and oxygen. The GPX removes the hydrogen peroxide through the oxidation of reduced glutathione (GSH) into oxidized glutathione (GSSG). Additionally, glutathione reductase regenerates GSH from GSSG, using NADPH as reducing power [Birben *et al.*, 2012; Klauning & Kamedulis, 2004; Pisoschi & Pop, 2015; Valko *et al.*, 2007].

The GSH/GSSG ratio reflects the oxidative state and is responsible for the maintenance of appropriate redox balance in the cell [Handy & Loscalzo, 2012; Jones, 2006; Ribas *et al.*, 2014]. Additionally to GSH, cells have other non-enzymatic antioxidants including vitamins (vitamins C and E), β-carotene, uric acid, melatonin, polyphenols (flavonoids and nonflavonoids), and *N*-acetylcysteine (NAC). Some nutrients such as minerals, fibres, fatty acids or amino acids, are also source of exogenous antioxidants [Birben *et al.*, 2012; Pisoschi & Pop, 2015; Valko *et al.*, 2007].

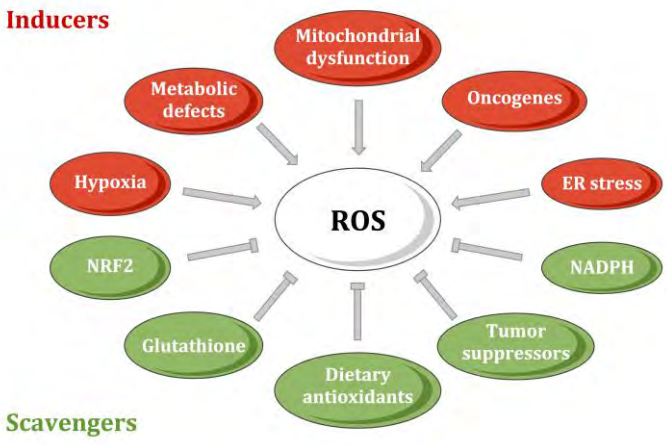


Figure 12. Cellular redox status equilibrium. Reactive oxygen species (ROS) production can be induced, among others, by mitochondria, hypoxia, metabolic defects, endoplasmic reticulum (ER) stress, and oncogenes. However, ROS are eliminated by the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), the production of glutathione and NADPH, the activity of tumor suppressors [such as p53 and ataxia telangiectasia mutated (ATM)] and the action of dietary antioxidants (such as vitamin C and selenium).

Cellular repair processes represent another important cellular antioxidant system. These molecular mechanisms remove damaged biomolecules, repair oxidatively damaged nucleic acids, remove oxidized proteins, and repair oxidized lipids [Pisoschi & Pop, 2015]. The most studied repair system is the complex and coordinated DNA repair system. DNA damage repair mechanisms comprise DNA repair systems and cell cycle checkpoints that manage DNA damage induced by endogenous and exogenous sources (Figure 13). Upon detection of DNA damage, the cell cycle progression is stopped by these checkpoints allowing the DNA repair machinery to correctly repair the damage [Maynard *et al.*, 2009; Curtin, 2012].

DNA repair mechanisms include the following systems: direct repair pathway, base excision repair mechanism (BER), nucleotide excision repair pathway (NER), non-homologous end joining (NHEJ), homologous recombination repair (HRR), and DNA mismatch repair pathway (MMR) [Economopoulou *et al.*, 2011]. The simplest form of DNA repair is the direct reversal of the DNA lesion [Curtin, 2012; Economopoulou *et al.*, 2011]. DNA lesions induced by ROS are mainly repair by NER, BER, and NHEJ systems. The NER system repair bulky DNA lesions with

potential to block DNA replication or transcription, while BER is the pathway that removes damaged bases from DNA and repairs single-strand breaks (SSBs) [Curtin, 2012; Economopoulou *et al.*, 2011]. SSBs are a common endogenous lesions, arising directly from ROS damage, and indirectly from BER-mediated enzymatic excision of damaged bases following their spontaneous deamination, ROS oxidation, or SAM alkylation [Curtin, 2012].

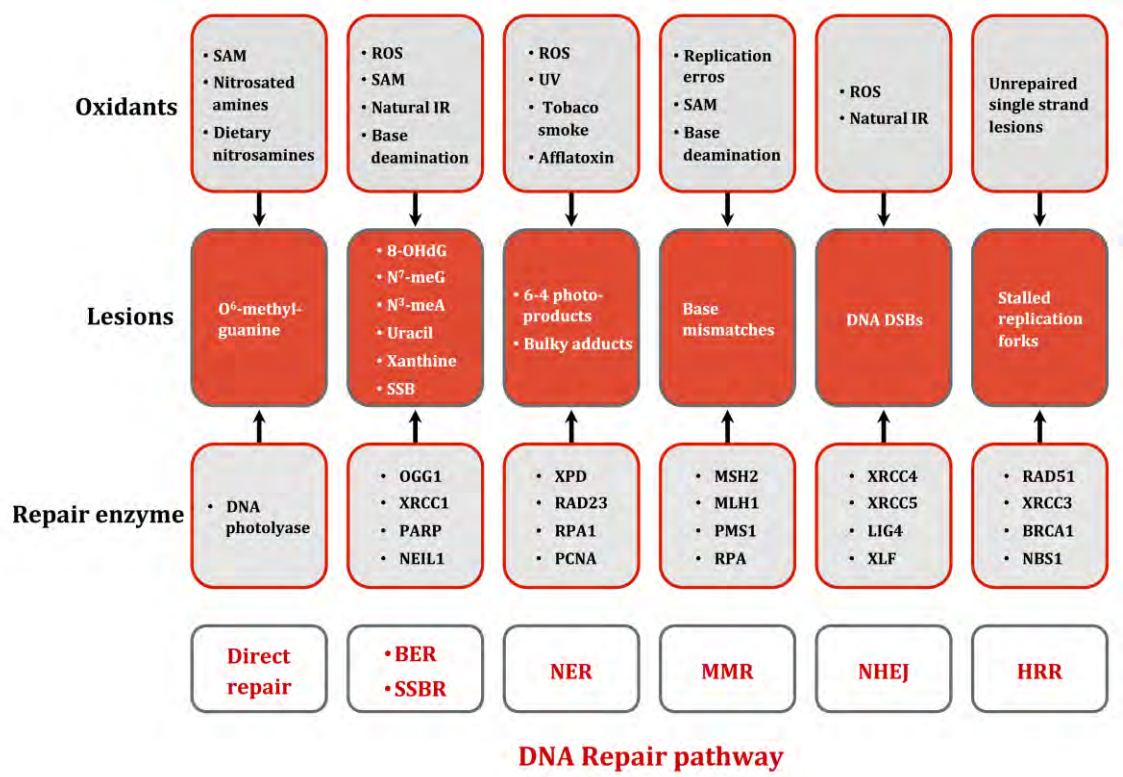


Figure 13. Sources of DNA damage and their correspondent repair pathways. BER, base excision repair; HRR, homologous recombination repair; IR, ionizing radiation; MMC, mitomycin C; NER, nucleotide excision repair; NHEJ, non-homologous end joining; ROS, reactive oxygen species; SAM, S‑adenosyl methionine; SSB, single-strand break; SSBR, SSB repair; UV, ultraviolet.

The most common and widely studied oxidative DNA lesion is the 8-oxo-7,8-dihydroguanine (8-OHdG), and the BER pathway is the primary repair mechanism of this oxidative base lesions. Unless repaired prior to DNA replication, 8-OHdG lesions lead to GC → TA transversions, and consequently to point mutations [Maynard *et al.*, 2009; Olinski *et al.*, 2003]. Cells have different specific glycosylases for certain lesions. For example, the 8-oxoguanine DNA glycosylase

(OGG1) is a DNA repair enzyme with highly specificity for 8-OHdG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) lesions repair, while nei-like DNA glycosylase 1 (NEIL1) is more efficient in the repair of FapyG and 4,6-diamino-5-formamidopyrimidine lesions. Moreover, individual glycosylases may recognize more than one type of damage, and each specific lesion may be recognized by more than one type of glycosylase, giving a degree of redundancy in the process [Economopoulou *et al.*, 2011; Maynard *et al.*, 2009; Olinski *et al.*, 2003].

Human cells have numerous regulators, both positive and negative, that have a significant impact on the expression of antioxidant genes [Gorrini *et al.*, 2013]. To coordinate the transcription of cytoprotective genes, cells have antioxidant response elements (ARE sequences) that are essential for cellular protection against xenobiotic and oxidative stress [Mitsuishi *et al.*, 2012; Niture *et al.*, 2014]. The activation of ARE sequences and, consequently, the transcription of target genes involved in cell adaptation to oxidative stress is controlled by the cap 'n' collar (CNC) family of transcription factors. This family comprises four members: nuclear factor erythroid 2-like 1 (NRF1), NRF2, NRF3, and p45 NF-E2. NRF1 and NRF2 are ubiquitously expressed, while NRF3 is expressed in placenta and liver, and p45 NF-E2 expression is restricted to erythrocytes [Niture *et al.*, 2014]. The NRF2 protein, a basic-region leucine zipper transcription factor, responds to changes in cellular stimuli induced by oxidants and electrophiles, and is considerate the most important regulator of the expression of molecules that have antioxidant functions within the cell [Rushworth & MacEwan, 2011; Gorrini *et al.*, 2013; Stepkowski & Kruszewski, 2011]. This transcriptional factor regulates a transcriptional program that maintains cellular redox homeostasis and protects cells from oxidative insults. The NRF2 activates the transcription of more than 100 genes, mainly cytoprotective and detoxification genes, including antioxidants (e.g. superoxide dismutase and heme oxygenase-1), xenobiotic metabolism enzymes (e.g. NADPH quinone oxireductase 1 and glutathione-S-transferase), and ATP-dependent drug efflux pumps [Rushworth & MacEwan, 2011; Rushworth *et al.*, 2012; Singh *et al.*, 2006; Stepkowski & Kruszewski, 2011].

Under physiological conditions, the NRF2 is constitutively inhibited by KEAP1 (kelch-like ECH-associated protein 1), which mediates the proteasomal degradation of NRF2 [Mitsuishi *et al.*, 2012; Niture *et al.*, 2014; Rushworth & MacEwan, 2011]. Upon exposure to oxidative or electrophilic stresses, KEAP1 is inactivated and NRF2 translocates into the nucleus (Figure 14). In the nucleus, NRF2 forms a heterodimer complex with MAF proteins and bind to ARE sequences located in the enhancer regions of NRF2-inducible genes. However, the nuclear activation of NRF2 is regulated by BACH1 (BTB and CNC homolog 1). Under normal physiological conditions, this transcriptional repressor bound to ARE sequences and block NRF2 binding. Upon pro-oxidant stimuli, BACH1 becomes deactivated and translocates to the cytosol, allowing the transcriptional activation of antioxidant genes targeted by NRF2 [Rushworth & MacEwan, 2011].

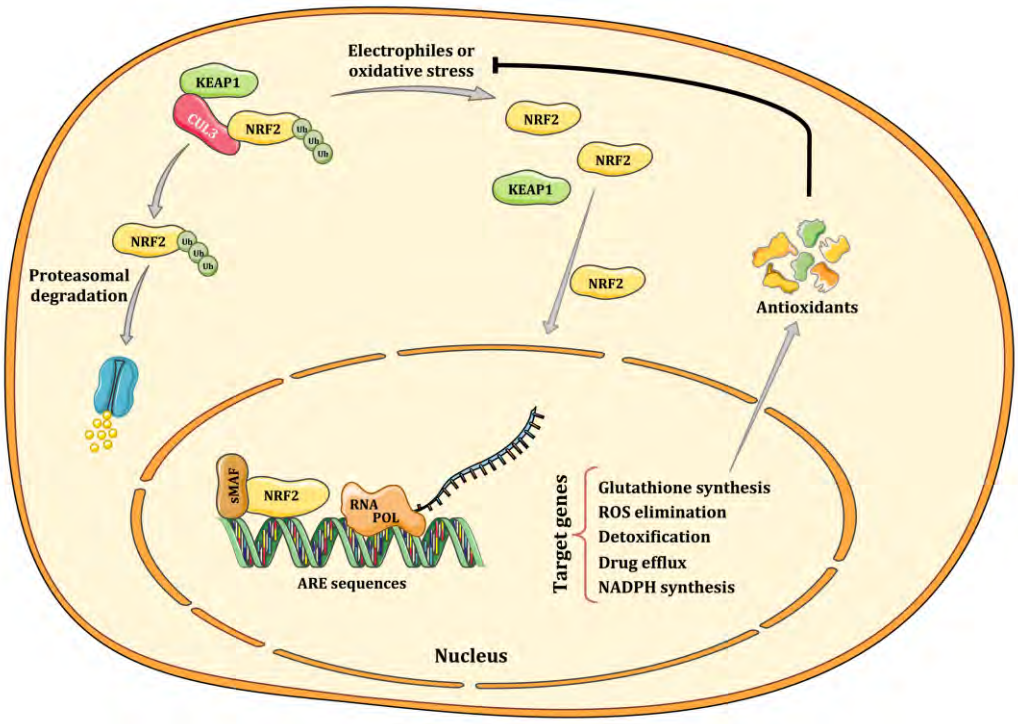


Figure 14. The KEAP1–NRF2 system. Under normal physiological conditions, NRF2 is constantly inhibited by KEAP1 and, consequently, degraded in the proteasome. Following exposure to electrophiles or oxidative stress, KEAP1 is inactivated and the NRF2 accumulates in the nucleus activating cytoprotective genes. Adapted from Mitsuishi *et al.* [2012].

ROS are predominantly implicated in cell damage, but they also play a major physiological role in the induction and maintenance of signal transduction pathways involved in cell growth and differentiation [Valko *et al.*, 2007]. Redox-responsive signaling pathways regulate several physiological functions, namely: 1. nitric oxide production (NO); 2. ROS production by NAD(P)H oxidase in phagocytic (oxidative burst) and in nonphagocytic cells; 3. vascular tone; 4. cell adhesion; 5. immune responses; 6. ROS-induced apoptosis; among other mechanisms [Valko *et al.*, 2007]. These signal transduction pathways rely on ROS as signaling molecules that act on different levels in the signaling cascade, functioning as secondary messengers. To induce and maintain signal transduction pathways responsible for cell growth and differentiation, cells constitutively produce ROS [Thannickal & Fanburg, 2000; Valko *et al.*, 2007]. Most of the growth factor receptors, namely epidermal growth factor receptor, protein tyrosine phosphatases, as well as tyrosine kinase receptors and serine/threonine kinases are targeted and regulated by ROS. These oxidant molecules also regulate other signaling transducers, such as extracellular signal-regulated kinases, JNK, and p38, which are members of mitogen-activated protein kinase (MAPK) family [Bigarella *et al.*, 2014; Birben *et al.*, 2012; Klaunig *et al.*, 2010; Valko *et al.*, 2007]. Overall ROS are involved in several cellular processes including proliferation, differentiation, apoptosis, and even HSC homeostasis, lineage commitment, and self-renewal.

In hematological cells, ROS can also influence several functions in HSCs as well as in mature blood cells, including self-renewal, differentiation, senescence, and cell death [Bigarrela *et al.*, 2014; Bourgeais *et al.*, 2013; Ghaffari, 2008; Hole *et al.*, 2011]. Moreover, ROS are involved in the inhibition of tyrosine phosphatases through the oxidation of cysteine residues in these proteins. Cytokines and growth factors activate intracellular regulation of redox processes through ROS production. In this context, ROS act as second messengers to regulate the activity of redox-sensitive enzymes including phosphatases [Bourgeais *et al.*, 2013]. Moreover, phosphatases inhibition mediated by ROS contributes to the activation and/or maintenance of signaling pathways driven by kinases. Additionally, ROS inhibit several kinases, such as MAPK/ERK, AKT, PKA, and PKC, through the

oxidation of these enzymes [Hole *et al.*, 2011; Rodrigues *et al.*, 2008; Sardina *et al.*, 2012].

The establishment of an imbalance between oxidant/antioxidant, in favor of the former, is termed "oxidative stress" [Birben *et al.*, 2012]. This condition occurs when an overproduction of ROS and/or a deficiency of enzymatic and non-enzymatic antioxidants are established [Birben *et al.*, 2012; Valko *et al.*, 2007]. Oxidative stress has been implicated in ageing and in various pathological conditions including cardiovascular disease, cancer, neurological disorders, and diabetes [Valko *et al.*, 2007]; and a close significant correlation between chronic oxidative stress and carcinogenesis has been demonstrated in several human epidemiological studies [Toyokumi, 2008]. Chronic exposure to ROS leads to genomic instability, involving SSBs, DSBs, DNA bases modifications, DNA crosslinks, and epigenetic modifications. These abnormalities influence various cellular processes, such as silence or induction of transcription, activation of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Figure 15). Moreover, it has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways. ROS contribute to carcinogenesis not only through genetic mutations but also through cell signaling deregulation [Glebova *et al.*, 2013; Imbesi *et al.*, 2013; Klauning *et al.*, 2010; Sallmyr *et al.*, 2008; Toyokumi, 2008; Valko *et al.*, 2007].

Several studies suggested a relationship between oxidative stress and hematological malignancies, and excessive ROS production, oxidative DNA damage, and/or deficient DNA damage repair have been frequently observed in these neoplasms. As mentioned above, several of the recurring mutations, such as those affecting the tyrosine kinases, observed in myeloid malignancies result in aberrant activations of signal transduction pathways. These oncogenic tyrosine kinases are often associated with an increase of intracellular ROS, which can in turn promote chromosomal instability. Elevated ROS levels have been detected in cell expressing the oncogenes *JAK2*^{V617F}, *NPM-ALK*, *FLT3-ITD*, *c-SRC*, *TEL-PDGFR*, or *BCR-ABL* [Gianni *et al.*, 2008; Hurtado-Nedelec *et al.*, 2013; Marty *et al.*, 2013; Sallmyr *et al.*,

2008; Slupianek *et al.*, 2002]. The mechanisms by which oncogenic tyrosine kinases regulate the intracellular level of ROS are not fully determined. For example, constitutively activating mutations in the *FLT3* gene, such as *FLT3-ITD* mutations, are observed in AML and MDS patients. This genetic abnormality was associated with increased levels of ROS, DSBs, and compromised NHEJ repair system in *FLT3-ITD* hematopoietic cell lines [Sallmyr *et al.*, 2008]. According to this study, the constitutive activation of STAT5 and RAC1 appear to be responsible for the increased ROS production [Sallmyr *et al.*, 2008].

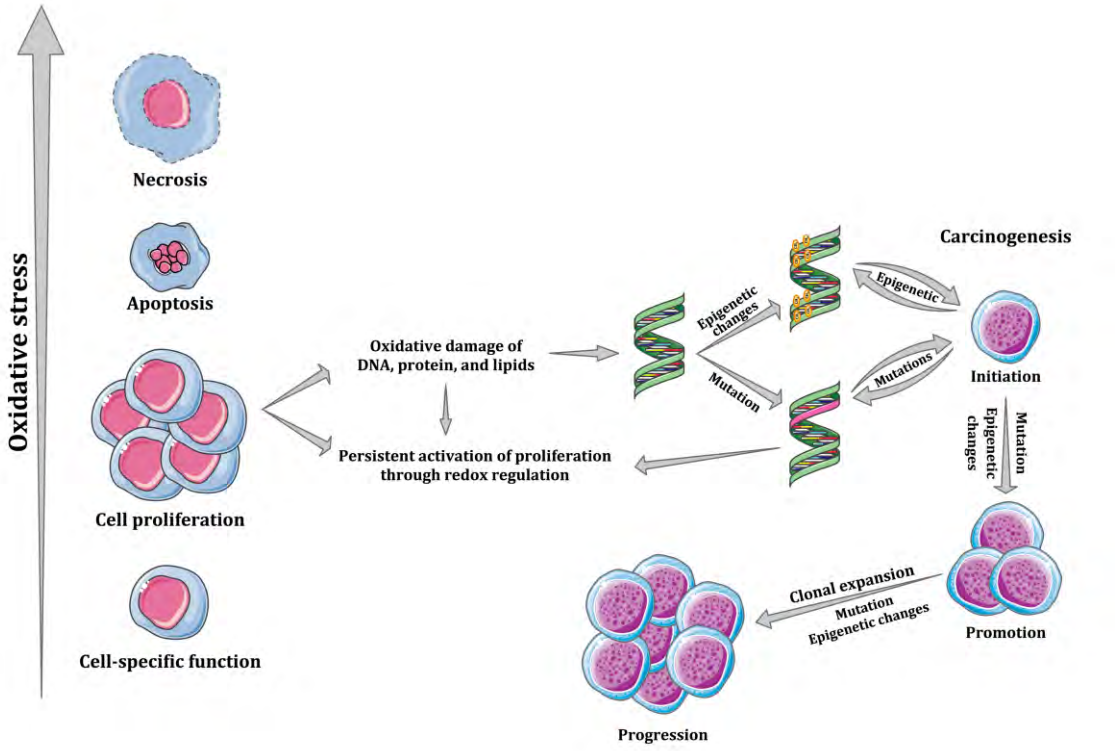


Figure 15. The fundamental role oxidative stress in carcinogenesis.

The persistence of oxidative stress, due to an increased production of sub-lethal amounts of ROS or to a decrease of antioxidant defenses, has been implicated in malignant cell proliferation and chemotherapy susceptibility in hematological neoplasms [Sarmento-Ribeiro *et al.*, 2012]. Patients with these malignancies display increased levels of oxidants (such as hydrogen peroxide and NO), DNA damage (such as 8-OHdG) and lipid damage (such as malonyldialdehyde – MDA), as well as decreased levels of antioxidants (such as GSH and thioredoxin) and DNA

repair activity (such as lower activity and expression of OGG1) [Ghoti *et al.,* 2007; Hole *et al.,* 2013; Jankowska *et al.,* 2008; Pimková *et al.,* 2014; Vener *et al.,* 2010]. An overview of oxidative stress markers observed in myeloid neoplasm patients is summarized in Table 7.

Table 7
Oxidative stress markers observed in myeloid neoplasms patients

Disease Oxidative stress markers	References
Myeloproliferative neoplasms	
↑ Oxidants	Vener *et al.,* 2010; Durmus *et al.,* 2013; Durmus *et al.,* 2014
↓ Antioxidants	Vener *et al.,* 2010; Durmus *et al.,* 2013
↑ Protein damage	Musolino *et al.,* 2012
↑ Lipid damage	Durmus *et al.,* 2013
Myelodysplastic syndrome	
↑ Oxidants	Ghoti *et al.,* 2007; Saigo *et al.,* 2011;
↓ Antioxidants	Ghoti *et al.,* 2007; Pimková *et al.,* 2014
↑ DNA damage	Peddie *et al.,* 1997; Novotna *et al.,* 2009
↑ Lipid damage	de Souza *et al.,* 2015; Pimková *et al.,* 2014
↓ DNA repair activity	Jankowska *et al.,* 2008
Acute myeloid leukemia	
↑ Oxidants	Hole *et al.,* 2013
↓ Antioxidants	Hole *et al.,* 2013; Rasool *et al.,* 2015; Zhou *et al.,* 2010
↑ DNA damage	Honda *et al.,* 2000; Zhou *et al.,* 2010
↑ Lipid damage	Rasool *et al.,* 2015; Zhou *et al.,* 2010
↓ DNA repair activity	Liddiard *et al.,* 2010

↑, increase;↓, decrease.

1.4. Epigenetics in myeloid neoplasms – Focus on DNA methylation

Even before DNA was identified as the molecule responsible for the transmission of genetic information, scientists knew that not every gene in an organism could be active in each cell at all times, since all cells in an organism share the same genetic background [Portela & Esteller, 2010]. Contrarily to genetics, which is the study of heritable changes that directly alter the DNA sequence, epigenetics is the study of heritable changes in gene activity or function that is not associated with any change in the DNA sequence itself [Moore *et al.,* 2013]. On a molecular level, the

mechanisms involved in epigenetic include covalent modifications in cytosine bases and histones, as well as changes in the chromatin remodeling. These mechanisms regulate gene and microRNA expression, DNA-protein interactions, transposable element suppression, differentiation, embryogenesis, X-chromosome inactivation, and genomic imprinting [Mazzio & Soliman, 2012; Portela & Esteller, 2010]. The epigenetic mechanisms are not static, and the regulation of gene transcription results from the cooperation between the different epigenetic modifications and from positive and negative regulatory feedback mechanisms.

In human cells, DNA is wrapped around nucleosomes, which is comprised of two copies of H2A, H2B, H3, and H4 histones [Dawson & Kouzarides, 2012; Mazzio & Soliman, 2012]. Histone modifications involve several post-transcriptional changes that occur in histone tails: acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation, among others [Dawson & Kouzarides, 2012; Mazzio & Soliman, 2012; Portela & Esteller, 2010]. Histone acetylation opens up the chromatin allowing the recruitment and binding of transcription machinery. This process is executed by histone acetyltransferases that inserts acetyl groups on lysines of histone, and histone deacetylases that remove acetyl groups [Mazzio & Soliman, 2012; Ropero & Esteller, 2007].

Additionally, the degree of methylation (mono-, di- or tri-methylation) at specific lysine residues also regulates the outcome of methylation [Dawson & Kouzarides, 2012; Rinaldi & Benitah, 2014]. These modifications may occur in all histones, and have been demonstrated that histone modification levels can predict gene expression [Karlic *et al.,* 2010]. Actively transcribed genes have high levels of H3K4me3, H3K27ac, H2BK5ac, and H4K20me1 in their promoters, simultaneously with H3K79me1 and H4K20me1 in gene bodies [Karlic *et al.,* 2010]. Histone modifications play essential roles in several cellular mechanisms, such as transcriptional regulation, DNA repair, DNA replication, alternative splicing, and chromosome condensation [Portela & Esteller, 2010].

Nucleosomes regulate transcription by blocking the access of activators and transcription factors to their sites on DNA, and at the same time they inhibit the

elongation of the transcripts by engaging polymerases. The packaging of DNA into nucleosomes also affects transcription [Chodavarapu *et al.,* 2010, Portela & Esteller, 2010]. The position of nucleosomes has an important influence on the initiation of transcription. The nucleosome displacement of a few base pairs of transcription start sites and the nucleosome-free regions at 5' and 3' ends of genes have been implicated in the assembly of transcription machinery. The loss of a nucleosome upstream of transcription start sites is correlated with gene activation, while the nucleosome occlusion at these sites is associated with gene repression. Moreover, chromatin remodelers, such as the SWI/SNF family, regulate the location and spacing of nucleosomes [Clapier & Cairns, 2009; Brookes & Shi, 2014].

The methylation of cytosine residues is an ancient epigenetic modification that plays major biological roles in bacterial antiviral response, eukaryotic genome stability, and transcription repression [Bracht, 2014]. The DNA methylation corresponds to the addition of methyl groups from S-adenylmethionine (SAM) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5-mC), being this process executed by DNA methyltransferases (DNMTs). Currently, the following DNMTs are known: DNMT1, DNMT1B, DNMT1O, DNMT1P, DNMT2, DNMT3A, DNMT3L, and DNMT3B with its isoforms [Akhavan-Niaki & Samadani, 2013; Das & Singal, 2004]. DNMT3A and DNMT3B are responsible for the establishment of new methylation patterns in unmethylated DNA, and display a powerful *de novo* DNMT activity. On the other hand, DNMT1 is mainly a maintenance DNMT, being responsible for the copy of DNA methylation pattern in hemimethylated DNA. The DNMT1 also have *de novo* methyltransferase activity; however, it has been shown that this DNMT have a 10-fold preference for maintenance activity [Akhavan-Niaki & Samadani, 2013; Taby & Issa, 2010].

DNA methylation can be found in different genomic regions, regulating gene expression in a sequence-dependent manner. DNA methylation occurs almost exclusively in the context of CpG dinucleotides, which tend to cluster in regions called CpG islands, and are defined as regions of 200-1000 bases with a G+C content of at least 50% [Moore *et al*., 2013; Portela & Esteller, 2010]. The majority of cytosine methylation occurs on CpG dinucleotides; however, non-CG

methylation events have been also observed, being the CA dinucleotides methylation more common than CT or CC [Moen *et al.*, 2015; Portela & Esteller, 2010]. Under normal physiological conditions, the majority of gene promoters are associated with CpG islands. However, approximately 6% of gene promoters become methylated in a tissue-specific manner during early development and tissue differentiation (Figure 16), and methylated CpG islands are associated with stable silencing of gene expression [Akhavan-Niaki & Samadani, 2013; Moore *et al.*, 2013; Portela & Esteller, 2010; Saxonov *et al.*, 2006]. The methylation of CpG islands located at gene promoters impairs binding of transcription factors, recruits repressive methyl-binding proteins, and silences gene expression [Das & Singal, 2004; Wajed *et al.*, 2001].

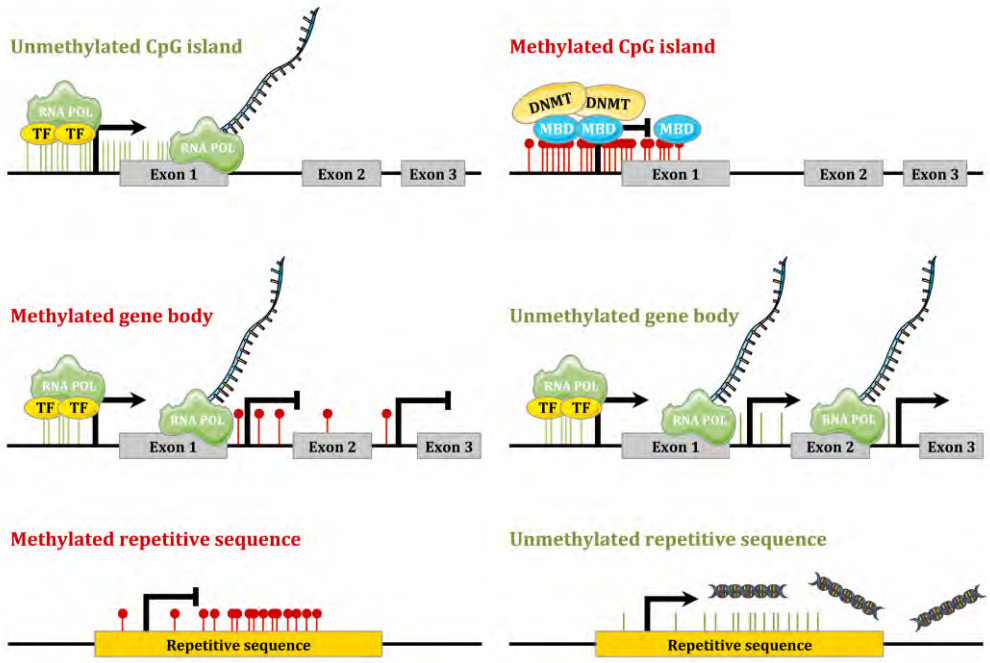


Figure 16. DNA methylation patterns. DNA methylation can occur in different regions of the genome. Unmethylated CpG islands at promoters of genes allow transcription, and their methylation leads to transcriptional inactivation. However, when methylation occurs at the gene body, it facilitates transcription, preventing incorrect transcription initiations. The repetitive sequences are usually hypermethylated, preventing chromosomal instability, translocations, and gene disruption through the reactivation of endoparasitic sequences. Adapted from Portela & Esteller [2010].

However, CpG islands are not only located in gene promoters but also within genes. Similarly to methylation at gene promoters, methylation at first exon of a gene is associated with gene silencing [Brenet *et al*., 2011], and DNA methylation in gene body (gene region immediately after the first exon) is associated with a high gene expression levels in dividing cells, being commonly methylated in ubiquitously expressed genes [Moore *et al*., 2013]. The mechanisms by which gene body methylation regulates gene transcription remains not fully understood, but it has been proposed that this type of DNA methylation regulates elongation efficiency and prevents improper transcription [Portela & Esteller, 2010].

Additionally, a significant fraction of methylated CpGs is found in repetitive elements, such as DNA satellites (Sat2 and Satα) and retrotransposons (long interspersed nuclear elements – LINEs, and short interspersed nuclear elements – SINEs). This DNA methylation pattern protects chromosomal integrity by preventing reactivation of repetitive elements that, if expressed, are potentially harmful since they can induce chromosomal instability, genetic translocation, and gene disruption [Wilson *et al*., 2007]. Recently, it was found that intron-exon boundaries also have a distinct methylation pattern that cooperates with nucleosomes and methylated H3K36me3 histone to ensure appropriate splicing of transcripts [Choi & Friso, 2010; Moen *et al*., 2015]. Moreover, the methylation of alternative promoters has been shown to regulate the expression of alternative transcripts at a tissue specific level [Maunakea *et al*., 2010].

DNA methylation was historically considered to be a relatively stable chromatin modification [Dawson & Kouzarides, 2012], but the establishment of specific genomic methylation pattern and its edition seems to be particularly relevant in several stages of embryogenesis and cell differentiation [Kohli & Zhang, 2013]. DNA demethylation takes place as a passive process due to lack of maintenance methylation during DNA replication or as an active DNA demethylation pathway initiated by the ten-eleven translocation (TET) protein family. The TET family of Fe(II)- and α-ketoglutarate (α-KG) dependent dioxygenases comprises three proteins (TET1, TET2, and TET3) with different expression levels according to cell

type. TET1 and TET2 are relatively highly expressed in embryonic stem cells and early embryogenesis, and their levels decrease with differentiation. However, TET2 regains its highly expression in the hematopoietic system. TET3 is overexpressed in differentiated cells, such as germ cells/oocyte, brain tissue, and somatic cells [Delatte *et al*., 2014; Ficz & Gribben, 2014]. The TET proteins are responsible for the conversion of 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) through three consecutive oxidation reactions. The cytosine modifications 5-fC and 5-caC are then recognized by thymine DNA glycosylase (TDG), a BER pathway enzyme, which replaces the modified cytosine by an unmodified cytosine [Dao *et al*., 2014; Delatte *et al*., 2014; Kroeze *et al*., 2015]. The abundance of cytosine modifications tends to vary between tissues in the following order: 5-mC > 5-hmC > 5-fC > 5-caC [Delatte *et al*., 2014].

Gene expression can also be regulated at post-transcriptional level by microRNAs (miRNAs) – a class of noncoding RNAs. Since miRNAs induce heritable changes in gene expression without altering the DNA sequence, they are also considered epigenetic modifications [Kanwal & Gupta, 2012]. These small single-stranded RNAs can bind to specific sites within the 3'UTR of the targeted mRNA [Chuang & Jones, 2007; Iorio & Croce, 2012; Zaidi *et al*., 2010]. These non-coding RNAs often act in concert with components of chromatin and with DNA methylation machinery to establish and/or sustain gene silencing [Zaidi *et al*., 2010]. miRNAs exert it function by mRNA degradation or prevention of its translation [Iorio & Croce, 2012], through a complex network in which several miRNAs work together to lower the expression of a shared target mRNA, and individual miRNA can target numerous different mRNAs [Chuang & Jones, 2007].

The major role of epigenetics in normal development and biology is suggested by the observation that several diseases, including cancer, are initiated and/or promoted when an inadequate epigenetic mark is added at the wrong time or at the wrong place. It is well documented that tumor cells are characterized by an abnormal pattern of DNA methylation, with many non–CpG island regions, such as LINE and ALU elements, becoming demethylated and some CpG islands, such as

tumor suppressor genes, undergoing *de novo* methylation (Figure 17) [Bergman & Cedar, 2013]. The timeline and coverage of aberrant DNA methylation in cancer is not yet clear. However, considering the genes targeted in cancer cells, it is likely that multiple signaling pathways are affected, including differentiation, proliferation, DNA repair, and/or apoptosis [Bergman & Cedar, 2013].

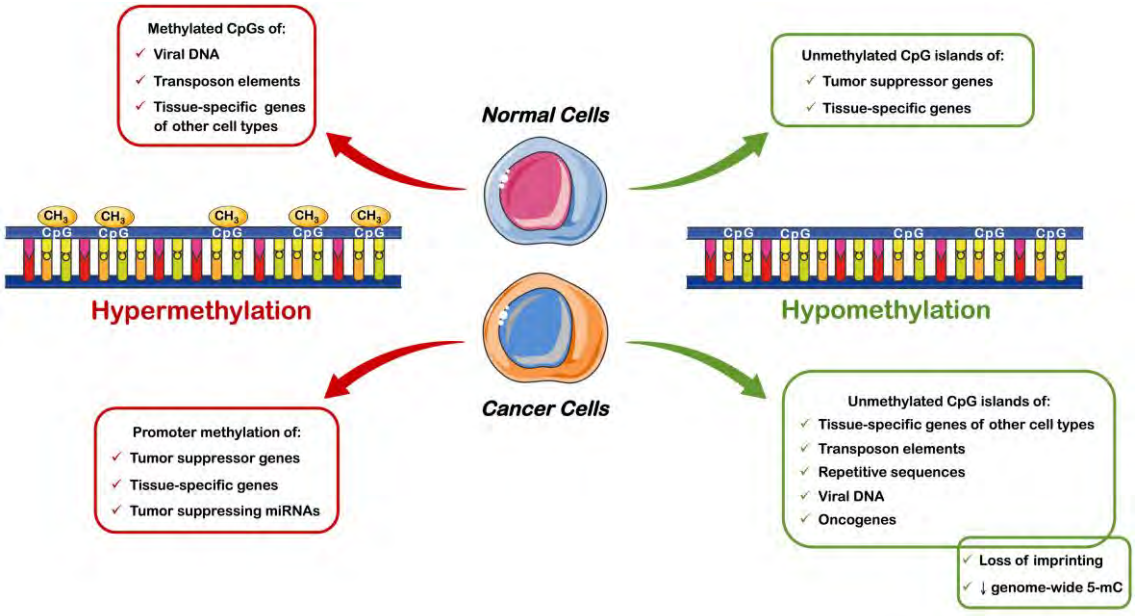


Figure 17. Normal *versus* neoplastic cells methylome.

Cancer cells often have silenced tumor suppressor genes and tissue-specific genes due to hypermethylation of their promoters [Das & Singal, 2004; Esteller, 2008; Taby & Issa, 2010]. Inactivation of those genes results in dysfunction of a variety of pathways, such as DNA repair and cell cycle control, which contributes to carcinogenesis. In the past years, several studies have reported promoter hypermethylation at tumor suppressor genes, for example at *P16*, *RB* (retinoblastoma), *MLH1* (mutL homolog1), *BRCA1* (breast cancer 1), among others [Das & Singal, 2004; Luczak & Jagodziński, 2006; Taby & Issa, 2010]. A wide spectrum of other genes involved in cell cycle control, such as *RARβ* or *ESR1*, and stress response, such as *KEAP1*, are silenced by methylation in cancer cells [Das & Singal, 2004; Hanada *et al*., 2012].

Generally, cancer cells demonstrate a global decrease in 5-mC and increased in 5-hmC levels [Bhattacharyya *et al*., 2013; Moen *et al*., 2015; Murata *et al*., 2015]. Moreover, DNA hypomethylation can promote carcinogenesis through the activation of transposable elements (such as LINEs, SINEs, and ALU sequences), activation of proto-oncogenes, loss of imprinting, and activation of genes specific of other cell types [Akhavan-Niaki & Samadani, 2013; Ehrlich, 2002; Ehrlich, 2009; Ross *et al*., 2010]. The hypomethylation of repetitive sequences are important to cancer development and progression, since they can lead to chromosomal rearrangements, mitotic recombination, and aneuploidy [Kitkumthorn & Mutirangura, 2011; Rodic & Burns, 2013; Ross *et al*., 2010].

The aberrant epigenetic landscape, including deregulated DNA methylation patterns, is a hallmark of many myeloid malignancies, including MPN, MDS, and AML. The hypermethylation of tumor suppressor genes (e.g. *P15*, *P16*, and *DAPK*), transcription factors (e.g. *GATA1*), and other genes involved in the regulation of signaling transduction pathways (e.g. *ESR1*, *SHP1*, and *SOCS1*) are frequent events in these neoplasms [Meldi & Figueroa, 2014]. An overview of common hypermethylated genes in myeloid neoplasm patients is summarized in Table 8.

Additionally, myeloid neoplasms exhibit global DNA hypomethylation; however, hypomethylation of repetitive sequences are not fully addressed in these hematological malignancies. Patients with AML and MDS have LINE-1 hypomethylation [Bujko *et al*., 2014; Kim *et al*., 2015] as well as lower levels of 5-mC and 5-hmC in comparison to normal individuals [Bujko *et al*., 2014; Calvo *et al*., 2014; Kroeze *et al*., 2014].

1.4.1. Genetic control of DNA methylation

The mechanism behind aberrant DNA methylation remains obscure, and only few studies were able to link recurrent mutations in epigenetic modulators, such as *IDH1/2*, *TET2*, and *DNMT3A*, with distinct DNA methylation profiles [Schoofs et al., 2014]. For example, AML patients with IDH mutations were found to have a very

pronounced hypermethylation signature [Akalin *et al*., 2012; Cancer Genome Atlas Research Network, 2013; Figueroa *et al*., 2010]. According to Figueroa *et al.* [2010] and Schoofs *et al*. [2014], the IDH mutants inhibit TET2 DNA demethylation activity, suggesting that *IDH* mutations lead to DNA hypermethylation in consequence of α-ketoglutarate-dependent dioxygenases inhibition [Figueroa *et al*., 2010; Schoofs *et al*., 2014].

Table 8
Examples of common hypermethylated genes in myeloid neoplasms patients

Gene	Function	Methylation frequency		
		MPN	MDS	AML
Calcitonin	Ca^{2+} bone resorption	–	40–80%	50–90%
DAPK	Apoptosis regulation	0–5%	0–63%	3–83%
CDH1	Cell–cell adhesion	5%	27–53%	13–69%
ESR1	Signal transduction; Cell growth regulation	–	19–60%	30–90%
HIC1	Transcription factor	–	22–32%	10–83%
P15	Cell cycle control	3–10%	6–92%	10–90%
P16	Cell cycle control; Apoptosis regulation	0–3%	6–10%	0–38%
TP73	Cell cycle control; Apoptosis regulation	3%	31%	10–13%
RAR β	Transcription regulation; Signal transduction	0%	–	18–20%
RB1	Cell cycle control	0%	14%	<10%
SHP1	Signal transduction	0%	0–59%	52%
FHIT	Nucleotide metabolism	–	47–50%	14%
SOCS1	Signal transduction	15%	11–31%	39%
BCL2L10	Apoptosis regulation	–	12%	45%

MPN, myeloproliferative neoplasms; MDS, myelodysplastic syndromes; AML, acute myeloid leukemia. Data are derived from the following references: Aggerholm *et al.* [2006], Bennemann *et al.* [2012], Chim *et al.* [2008], Chim *et al.* [2010], Claus & Lübbert [2003], Deneberg *et al.* [2010], Fabiani *et al.* [2010], Jost *et al.* [2007], Karlic *et al.* [2014], Khan *et al.* [2013], Kumagai *et al*. [2005], Oki & Issa [2010]; Santini *et al*. [2013], Zhao *et al*. [2013].

Other studies observed that AML patients with *TET2* mutations also have a specific hypermethylation signature, but the hypermethylation levels were less pronounced than in AML patients with *IDH* mutations [Akalin *et al*., 2012; Figueroa *et al*., 2010]. Since patients with *TET2* mutations show lower 5-hmC levels, it seems likely that DNA hypermethylation in AML patients with mutated *IDH* and *TET2* results from a common mechanism based on TET2 function [Schoofs *et al*.,

2014]. The relationship of DNA methylation pattern with *DNMT3A* mutations was also addressed by some studies. Ley *et al.* [2010] found that 182 DNA regions were differentially hypomethylated in AML patients with *DNMT3A* R882H mutations in comparison to those with wild-type *DNMT3A*. Similarly, Yan *et al.* [2011] found differences in the methylation pattern of AML patients with *DNMT3A* mutations and with wild-type *DNMT3A*; however, more hyper- than hypomethylated genes were found in this study. Additionally, the Cancer Genome Atlas Research Network [2013] found a clear DNA hypomethylation signature in AML patients with *DNMT3A* mutant. The mechanistic base of this difference is not yet clear, but it was proposed that DNMT3A R882H mutants have an impaired function, losing the ability to methylate CpGs clusters [Schoofs *et al*., 2014].

1.4.2. Metabolic control of DNA methylation

DNA methylation pattern is not only modulated by genetic mutations but also by development, lifestyle, and environmental determinants [Cortessis *et al*., 2012]. Among these determinants, nutrition is exceptionally important since nutrients and bioactive food components can modify epigenetic phenomena, altering gene expression at the transcriptional level [Choi & Friso, 2010]. Diet nutrients and bioactive food components can influence epigenetic directly through the modulation of enzymes that catalyze DNA methylation and histone modifications, and indirectly by alter the systemic metabolism that will lead to alterations in epigenetic patterns [Choi & Friso, 2010; Crider *et al*., 2012; Park *et al*., 2012]. Folate, vitamin B-12, methionine, choline, and betaine are nutrients that can affect DNA methylation and histone methylation through the modulation of one-carbon metabolism [Choi & Friso, 2010; Crider *et al*., 2012; Park *et al*., 2012].

DNA methylation relies on one-carbon metabolism to produce SAM and, therefore, this metabolic pathway is able to regulate methylation processes and DNA synthesis [Choi & Friso, 2010; Park *et al*., 2012]. Derivatives of folate and folic acid, which is the synthetic form of folate that is found in supplements and fortified

foods, are utilized in one-carbon metabolism to remethylation of homocysteine to produce methionine, which then will be converted in SAM (Figure 18).

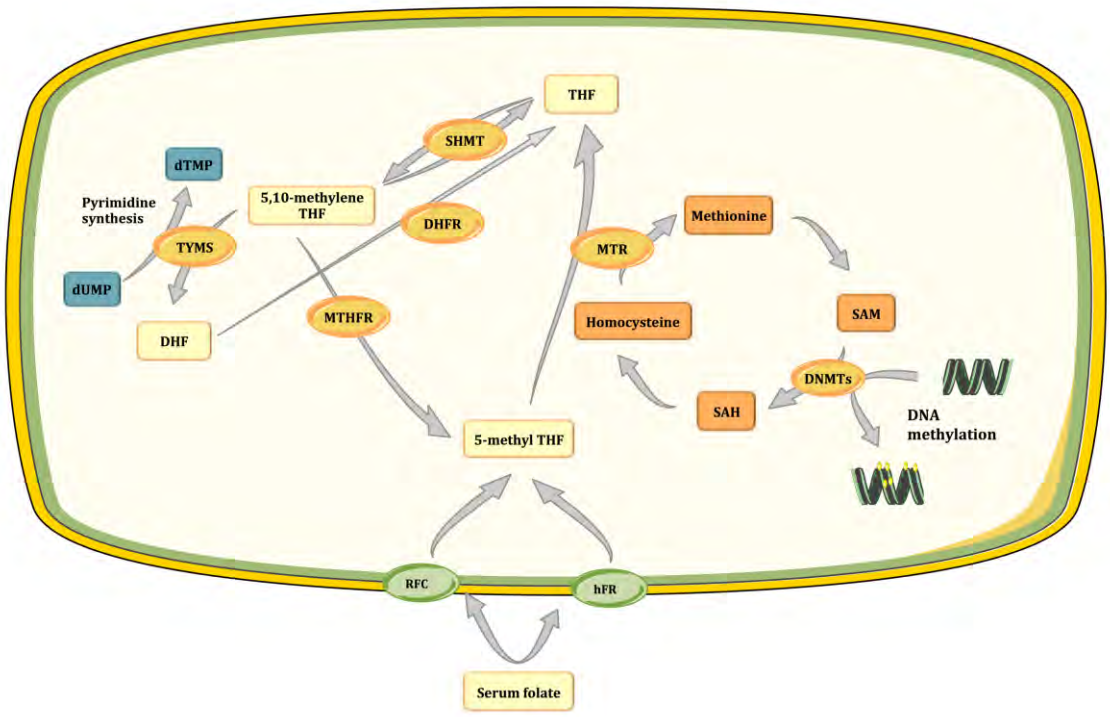


Figure 18. Diagram of folate and one-carbon metabolism in mammalian organisms. The one-carbon transfer reactions are mainly supported by folate. Dietary folates are hydrolyzed to monoglutamates and then absorbed via reduced folate carrier (encoded by *SLC19A1* gene) or by passive diffusion at high concentrations. The 5-methyltetrahydrofolate (5-methyl THF) donates its one-carbon moiety to methylate homocysteine to methionine, yielding tetrahydrofolate (THF). Methionine, from homocysteine or diet, is converted to S-adenosylmethionine (SAM) via methyltransferases (DNMTs). The THF obtains one-carbon moiety from the amino acid serine via serine hydroxymethyltransferase (SHMT) catalysis, yielding 5,10-methylenetetrahydrofolate (5,10-methylene THF), which is an important common substrate to methylation pathway via methylenetetrahydrofolate reductase (MTHFR) or to nucleic acid synthesis pathways via thymidylate synthase (TYMS; pyrimidine synthesis) with production of dihydrofolate (DHF). The DHF is then converted into THF via dihydrofolate reductase (DHFR). dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate.

Additionally, choline provides methyl groups for folate-independent homocysteine remethylation reactions, and ultimately to SAM production [Choi & Friso, 2010;

Moren *et al*., 2015; Park *et al*., 2012]. As mentioned above, SAM is the universal methyl donor for biological methylation reactions, and after transferring the methyl group, SAM is converted to S-adenosylhomocysteine (SAH). SAH binds to methyltransferases with higher affinity than SAM, acting as a potent inhibitor of SAM-dependent methyltransferases, such as DNMT1, DNMT3A and DNMT3B [Castro *et al*., 2003; Crider *et al*., 2012; James *et al*., 2002]. In this context, the ratio SAM/SAH is also able to regulate DNA methylation.

In mid-1990s Michaud *et al.* [1994] reported for the first time the influence of dietary methyl donors on DNA methylation, using *agouti* mice. Thereafter, several studies have been examining the effects of folate deficiency and dietary supplementation on genomic 5-mC levels [Moen *et al*., 2015]. In the majority of studies conducted until now, folate deficiency is associated with global hypomethylation, while increased folate intake is correlated with global hypermethylation [Choi & Friso, 2010; Crider *et al*., 2012; Ly *et al*., 2012; Park *et al*., 2012]. However, these results are inconsistent. Moreover, *in vitro* studies using human tissue culture showed that folate depletion not only leads to global hypomethylation but also to *H-cadherin* hypermethylation [Jhaveri *et al*., 2001; Wasson *et al*., 2006]. Due to the complex relationship of folate with DNA methylation, more studies that determine the clinical, biological, and molecular effects of folate are needed.

The GSH is another molecule biochemically linked to cofactors that influence epigenetic processes. GSH is synthesized in cytosol in virtually all cells by two ATP-requiring enzymatic steps. Under normal physiological conditions, the rate of GSH synthesis is mainly determined by cysteine availability and by glutamate cysteine ligase activity [Lu, 2009]. However, when cysteine levels are low or when high GSH levels are need, glutathione biosynthesis occurs through the transsulfuration pathway (Figure 19) [Hitchler & Domann, 2007]. Under these conditions, the homocysteine, derived from the hydrolysis of SAH, enter into the transsulfuration pathway and, through a series of enzymatic reactions, is converted in cysteine – a GSH precursor [Hitchler & Domann, 2007; Lu, 2009]. By favoring homocysteine's entry into the transsulfuration pathway, the levels of

methionine and SAM decrease leading to genome wide DNA hypomethylation [Hitchler & Domann, 2007; Lertratanangkoon *et al.*, 1997]. Therefore, any cellular condition that leads to biosynthesis of GSH via transsulfuration pathway can directly impact DNA methylation by altering SAM pools. In this context, one-carbon metabolism and transsulfuration pathway may be a mechanistic bridge linking oxidative stress and DNA methylation.

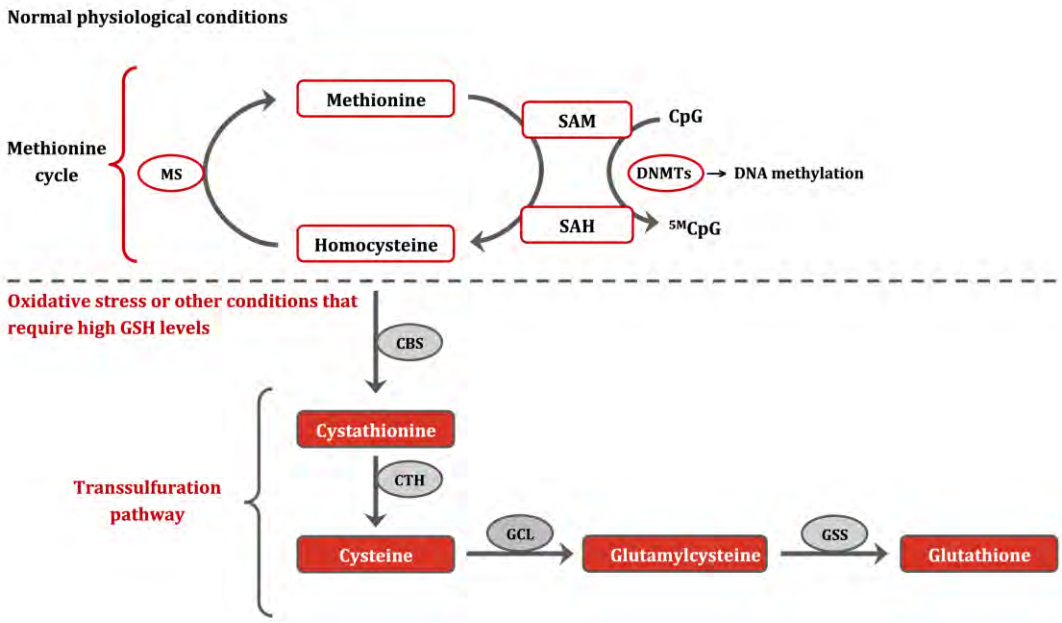


Figure 19. Transsulfuration pathway – A connection between DNA methylation, methionine cycle, and glutathione synthesis.

1.5. The interplay of oxidative stress and DNA methylation

ROS levels and DNA methylation patterns change with age. Moreover, they are common features in age-related diseases, such as myeloid neoplasms [Cerda & Weitzman, 1997; Donkena *et al.*, 2010; Hayes & Knaus, 2013; Rang & Boonstra, 2014; Yara *et al.*, 2015; Ziech *et al.*, 2011]. Indeed, there is a refined interrelationship between oxidative stress and DNA methylation. Excess oxidative stress tends to deplete GSH, impair the one-carbon metabolism, and consequently leading to hypomethylation. On the other hand, hypomethylation of genes involved in metabolic pathways as well as hypermethylation of antioxidant enzymes can

reduce the production of glutathione, cysteine, and metallothionein, causing oxidative stress [Walsh, 2010].

Several studies have demonstrated the ability of ROS to affect DNA methylation under physiologic and pathologic conditions [Campos *et al.*, 2007; Hepburn *et al.*, 1991; Kang *et al.*, 2012; Li *et al.*, 2014a; Mishra *et al.*, 2008; O'Hagan *et al.*, 2011; Rang & Boonstra, 2014; Turk *et al.*, 1995; Valinluck *et al.*, 2004; Weitzman *et al.*, 1994; Zhang *et al.*, 2015]. Oxidative stress induced by metabolic, dietary, environmental, or other means, leads to a wide range of DNA lesions including base modifications, deletions, strand breakage, and chromosomal rearrangements [Donkena *et al.*, 2010]. In addition to causing genetic changes, ROS may lead to epigenetic alterations that affect the genome, especially the DNA methylation patterns [Ziech *et al.*, 2011], which may be one other way by which ROS exert their deleterious effects [Rang & Boonstra, 2014]. Acute and chronic oxidative stress can induce genetic and epigenetic changes. However, their effects are more likely to occur during chronic stress, which leads to the evolution of abnormal cell states that contribute to disease. Moreover, cellular abnormalities established during chronic stress may persist even upon the exposure to stress, resulting in an altered cellular 'memory' that helps to drive disease pathology [Johnstone & Baylin, 2010].

The oxidative DNA lesions, such as 8-OHdG, O^6-methylguanine, and single stranded DNA, contribute to decreased DNA methylation by interfering with the ability of DNA to function as a substrate for DNMTs, and thus resulting in global hypomethylation (Figure 20A) [Hepburn *et al.*, 1991; Kuchimo *et al.*, 1987; Turk *et al.*, 1995; Valinluck *et al.*, 2004; Weitzman *et al.*, 1994]. The N^7 position of guanine acts as a hydrogen bond acceptor during the assembly of methyl binding proteins (MBP)-DNA complex. However, the N^7 position of 8-OHdG lesions acts as hydrogen bond donor, diminishing substantially the ability of MBP bind to the 5-mC [Valinluck *et al.*, 2004; Weitzman *et al.*, 1994]. The replacement of 5-mC by 5-hmC also reverses the binding affinity to MBPs, interfering with subsequent steps in the chromatin condensation cascade, and resulting in potentially heritable epigenetic alterations [Donkena *et al.*, 2010].

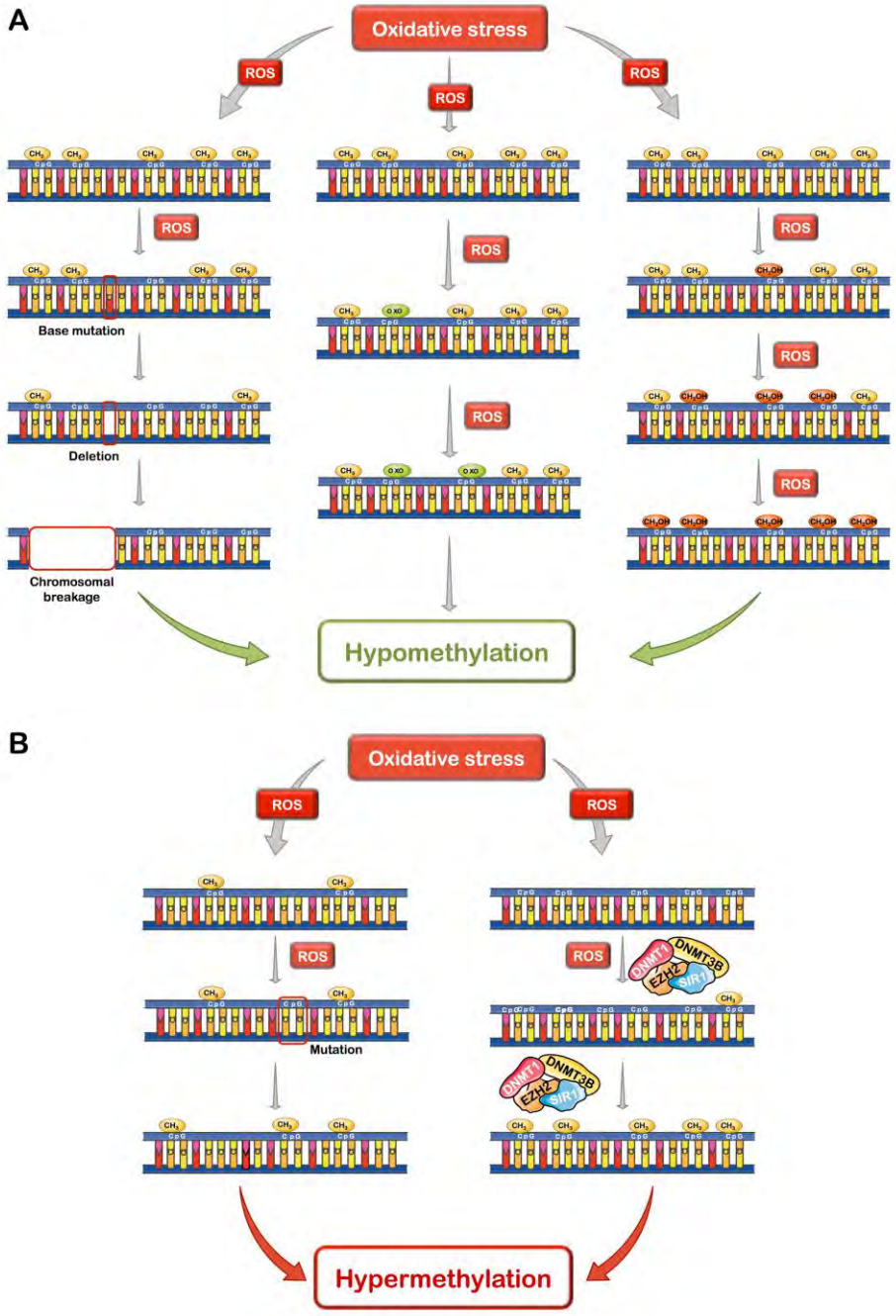


Figure 20. Oxidative stress can induce both DNA hypomethylation (A) and hypermethylation (B).

As previously mentioned the 5-hmC can result from passive demethylation, mediated by ROS, or from active demethylation, mediated by enzymes [Wu & Ni, 2015]. In the active demethylation of 5-mC, oxidative stress decreases NADPH and NADH levels leading to the activation of Sirtuin 3, which deacetylase IDH2 and,

therefore, activating it. Activated IDH2 produce increased amounts of α-ketoglutarate that lead to the conversion of 5-mC into 5-hmC by TET enzymes [Chia *et al*., 2011]. Moreover, the oxidative DNA lesion O^6-methylguanine can also inhibit the binding of DNA methyltransferases to DNA leading to hypomethylation [Hepburn *et al*., 1991]. Finally, single-stranded DNA can signal *de novo* methylation and, thus, it may be possible that formation of single strand breaks by oxidative stress can contribute to the modifications of DNA methylation patterns observed in oxidant-transformed cell lines [Christman *et al*., 1995].

Additionally, ROS are able to directly catalyze DNA methylation. As mentioned above, the conversion of cytosine into 5-mC is generally mediated by DNMTs, which catalyze the transfer of a methyl group from SAM to the nucleotide. However, according to Afanas'ev [2014], superoxide can act as catalyst of DNA methylation inducing hypermethylation in promoter regions of target genes induced by ROS, especially tumor suppression genes [Afanas'ev, 2014; Wu & Ni, 2015]. This author postulated that superoxide molecule could directly deprotonate the five carbon of cytosine, providing nucleophilic properties. Then, the positively charged S-atom of SAM is able to react with the nucleophilic C-5 atom and produce a methylated cytosine [Afanas'ev, 2014].

ROS are also able to induce aberrant hypermethylation of tumor suppressor gene promoter regions by facilitating the formation of polycomb repressive complexes and by the stimulation of DNMTs expression levels (Figure 20B) [Rang & Boonstra, 2014; Wu & Ni, 2015]. ROS exposure leads to the formation of large complexes containing DNMT1, DNMT3b, Sirtuin1, and members of the polycomb repressive complex 4, namely EZH2 (enhancer of zeste protein-2). This complex facilitates the binding of DNMTs to target promoters, and consequently, leads to functional changes, including histone mark changes, reduction of nascent transcription levels and increases in DNA methylation [Li *et al*., 2014b; O'Hagan *et al*., 2011; Rang & Boonstra, 2014; Zhang *et al*., 2015]. As mentioned above, ROS have the ability to stimulate DNMTs expression levels. According to Campos *et al.* [2007], Mishra *et al.* [2008], and Kang *et al.* [2012] the exposure to oxidative stress donors, such as hydrogen peroxide, increases the expression levels of DNMT1 and DNMT3B.

Furthermore, oxidative stress also up-regulates histone deacetylase 1 (HDAC1), and increases the DNMT1 binding affinity to HDAC1 [Fuks *et al*., 2000; Kang *et al*., 2012].

Moreover, oxidative stress influences DNA methylation by changing the activity of SAM synthetase, the enzyme that produces SAM. SAM synthetase, also known as methionine adenosyltransferase (MAT), catalyzes the enzymatic addition of methionine to adenosine to produce SAM. The redox buffering capacity (the GSH/GSSG ratio) modulates MAT activity; and, in the presence of high cellular GSH/GSSG ratio, the MAT activity is markedly increased leading to SAM production. On the other hand, when the GSH/GSSG ratio is low the MAT activity decreases as well as the SAM levels, leading to global hypomethylation [Hitchler and Domann, 2007].

Several studies have showed that exposure to oxidative stress leads to tumor suppressor genes hypermethylation and, consequently, to gene silencing. For example, exposure to hydrogen peroxide induced hypermethylated of *E-cadherin*, *catalase*, and *POU class 2 homeobox 1* (alias *OCT-1*) gene promoters in hepatocellular carcinoma cell lines [Lim *et al*., 2008; Min *et al*., 2010; Quan *et al*., 2011], *RUNX3* hypermethylation in colorectal [Kang *et al*., 2012] and bladder cells [Wongpaiboonwattana *et al*., 2013], and *SP1* hypermethylation in cervical adenocarcinoma [Chuang *et al*., 2011]. Furthermore, Soberanes *et al.* [2012] reported that oxidative stress-induced by particulate matter air pollution results in increased DNMT1 protein expression and *P16* hypermethylation. Other tumor suppressor genes, such as *P16*, *RB*, *VHL*, and *BRAC1*, have also been identified in cancer cells as being inactivated via oxidative-induced hypermethylation [Ziech *et al*., 2011]. In addition, ROS exposure are also able to induce hypomethylation of LINE-1 sequences in bladder and urothelial cancer as well as in normal kidney cells [Kloypan *et al*., 2015; Patchsung *et al*., 2012; Wongpaiboonwattana *et al*., 2013]. However, the relationship between oxidative stress and DNA methylation in normal and/or malignant hematopoietic cells remains to be studied.

On the other side of the coin, DNA methylation influences oxidative stress levels through the repression of enzymatic antioxidant. The hypermethylation of *SOD2* (manganese superoxide dismutase) was observed in human pancreatic carcinoma [Hurt *et al*., 2007] and breast cancer cells [Hitchler *et al*., 2006]. Moreover, several genes involved in xenobiotic metabolism (*GSTP1* and *NQO1*) and DNA repair (*OGG1*, *MLH1*, and *BRCA1*) were also found to be hypermethylated in several types of cancers [Guan *et al*., 2008; Lee *et al*., 2007; Seedhouse *et al*., 2003; Tada *et al*., 2005; Xu *et al*., 2013; Zhong *et al*., 2002].

1.6. Genetic susceptibility and myeloid neoplasms

Complex disease, such as myeloid neoplasms, arises from a combination of inherited and acquired mutations in more than one gene, as well as exposure to environmental factors. These factors act together to modulate disease susceptibility, severity, clinical manifestations, and treatment responses. On contrary to monogenic diseases, none mutation is necessary or sufficient to cause a complex disease. These diseases develop under specific genetic backgrounds in association with exposure to certain environmental factors such as xenobiotics, contaminants, UV radiation, among other [Prokunina & Alarcón-Riquelme, 2004].

The human genetic diversity comprises approximately 0.1% of their genomes, and with the exception of identical twins, no two humans have identical genomes. Genetic polymorphisms are defined as natural genetic variations that occur randomly in the general population and are responsible, at least in part, for the interindividual variability [Buckland, 2006; Marian, 2012]. The most common DNA sequence variants in the genome are single nucleotide polymorphisms (SNPs), which are variations at a single base pair with a frequency of >1% across the genome [Buckland, 2006; Marian, 2012; Tan *et al*., 2010]. Single nucleotide polymorphisms arise from point mutations that are selectively maintained in populations, and their frequencies are determined by: 1. the amount of time since the mutation occurred; 2. evolutionary pressure on biologically significant

functional variants; 3. random genetic drift; and 4. bottleneck events [Erichsen & Chanock, 2004].

Although some of these polymorphisms occur in protein-coding genes (coding SNPs), the vast majority of these polymorphisms lying in noncoding or intronic regions (non coding SNPs) (Figure 21). The coding SNPs can lead to a change in the translated amino acids (missense variants), and are likely to contribute to phenotypic changes [Tan *et al.*, 2010]. The non coding SNPs are located in promoters, introns, UTRs, among others locations, and can disrupt gene expression by several mechanisms: 1. altering transcription factor binding sites and microRNAs binding sites; 2. influencing the strength of enhancers and promoters, and 3. changing methylation sites [Buckland, 2006; Marian, 2012; Patnala *et al.*, 2013; Preskill and Weildhaas, 2013; Prokunina & Alarcón-Riquelme, 2004].

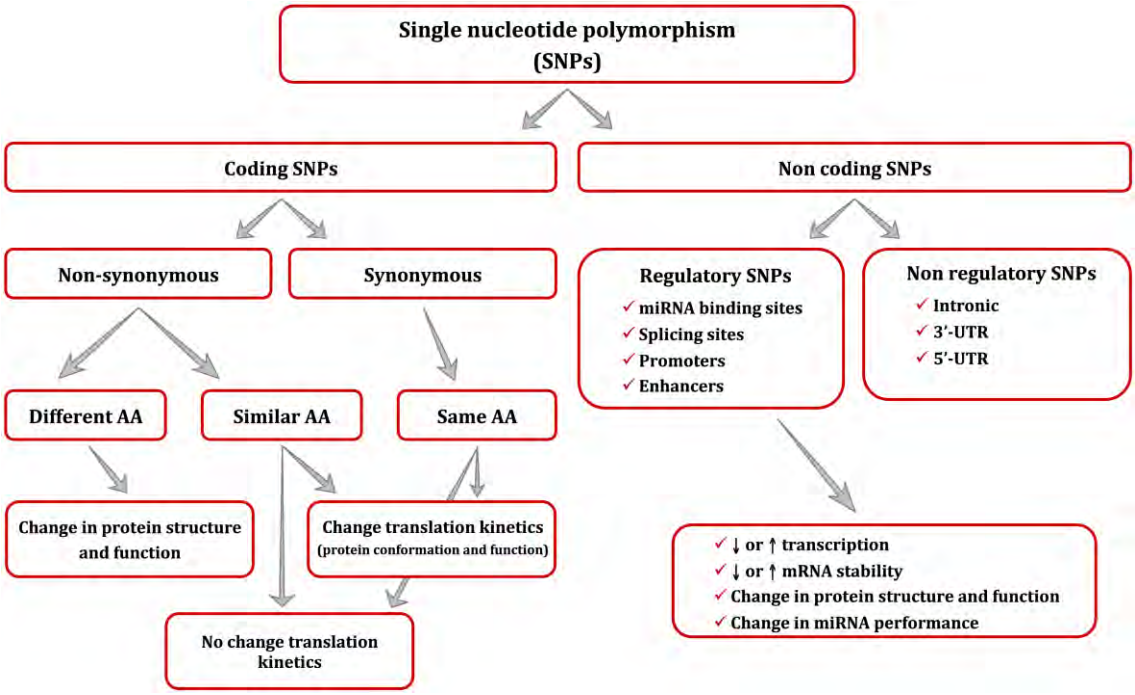


Figure 21. Types of single nucleotide polymorphisms (SNPs) and their biological consequences.

On the past years, extensive effort has been invested in identifying sources of genetic susceptibility to cancer. The International Human Genome Sequencing

Project and the International HapMap Project have generated a very large amount of data on the location, quantity, type, and frequency of genetic variants in the human genome [Dong *et al*., 2008]. The human genome is very diverse, and each genome contains approximately 50 to 100 variants associated with complex diseases, such as cancer, and approximately 30 *de novo* variants [Marian, 2012].

The etiology of a specific cancer is probably associated with a set of genetic variants that can adversely interact with environmental factors [Erichsen & Chanock, 2004]. As mentioned above, oxidative stress and DNA methylation contribute to the development of myeloid neoplasms. Oxidative stress levels as well as DNA methylation profiles can be modulated by the complex relationship between constitutive risk factors (genotypes) and modifiable factors (diet and environment). Several reports have demonstrated the importance of polymorphisms on myeloid malignancies susceptibility. Genetic variants affecting antioxidant defense mechanisms, DNA repair pathways, DNA methylation, and one-carbon metabolism have been demonstrated to be positively or negatively associated with myeloid neoplasms in at least one association study. Some examples of SNPs associated with the genetic predisposition to MDS and AML are shown in Table 9. Disease-association studies in MPN are rare and none of them studied the above-mentioned pathway.

Currently, several genetic variants have been associated not only with cancer susceptibility, but also with cancer progression. DNA variations were identified as prognostic markers, and some of them improved clinical decision [Erichsen & Chanock, 2004; Savas *et al*., 2013]. For instance, genetic variants of *XRCC3* modulate AML survival [Bănescu *et al*., 2013]. Moreover, the therapy responses and the toxicity levels experienced by cancer patients undergoing chemotherapy treatments led to the association of genetic variants with chemotherapy-induced phenotypes [Wheeler *et al*., 2012]. For example, the standard dose of mercaptopurine (a treatment for acute lymphoblastic leukemia) results in life-threatening toxicity for individuals with certain variant alleles of thiopurine S-methyltransferase. These observations lead the United States Food and Drug Administration (FDA) to recommend the genotyping of thiopurine

S-methyltransferase before treatment initiation. The genotype assessment of this enzyme allows the prevention of myelosuppression of individuals with inactive alleles as well as it successfully treated with reduced doses of mercaptopurine [Relling *et al*., 2011; Yong *et al*., 2006; Wheeler *et al*., 2012]. In this context, the identification of genetic variations that predict drug response is other clinical implication of genetic variability.

Table 9
Examples of genetic variants associated with myeloid neoplasms predisposition

Biological role	Gene	dbSNP	Variant	Associated risk	
				MDS	AML
Oxidative stress					
	NQO1	rs1800566	c.559C>T; p.Pro187Ser	↑	↑
	SOD2	rs4880	c.47T>C; p.Val16Ala	–	↑
	GPX3	rs8177426	c.88-1961A>G	↑	–
DNA repair					
	XRCC1	rs25487	c.1196A>G; p.Gln399Arg	↑	↑
	XRCC3	rs861539	c.722C>T; p.Thr241Met	↑	↑
	OGG1	rs1052133	c.977C>G; p.Ser326Cys	↑	=
DNA methylation					
	DNMT3B	rs1569686	c.-6-1045G>T	–	↑
		rs2424908	c.-6-7741C>T	–	↓
One-carbon metabolism	MTR	rs1805087	c.2756A>G; p.Asp919Gly	↑	↑
	MTHFR	rs1801131	c.1286A>C; p.Glu429Ala	↑	↑
	SHMT	rs1979277	c.1420C>T; p.Leu474Phe	–	↓

↑, increased susceptibility; ↓, decreased susceptibility; =, without influence in susceptibility; –, not determined; MDS, myelodysplastic syndromes; AML, acute myeloid leukemia. Data are derived from the following references: Aktuglu *et al.* [2014], Belickova *et al.* [2013], Dunna *et al.* [2014], Dong *et al.* [2014], Jankowska *et al.* [2008], Hamdy *et al.* [2011], Huang *et al.* [2015]; Naoe *et al.* [2000], Seedhouse *et al.* [2004], Smith *et al.* [2001], Sorour *et al.* [2013], Vineis *et al.* [2007], Yang *et al.* [2011], Zheng *et al*. [2013].

Chapter 2

Framework, hypothesis & objectives

2.1. Framework & Hypothesis

Myeloid neoplasms are a group of heterogeneous diseases, including MDS, MNP, and AML, with a higher incident in elderly individuals [Carbonell *et al*., 2015; Odenike *et al*., 2011]. They are classified by a combination of clinical, morphologic, immunophenotypic, and genetic features [Vardiman *et al*., 2008]. As complex diseases, myeloid neoplasms result from the association of molecular, genetic, environmental, and lifestyle factors. These factors include somatic genetic and epigenetic modifications in genes that are crucial for hematopoietic differentiation, cellular proliferation, and survival pathways [Hole *et al*., 2011; Kim *et al*., 2015; Mehdipour *et al*., 2015].

Reactive oxygen species (ROS) are considered important players in the initiation and progression of hematological malignancies [Sardina *et al*., 2012]. These free radical molecules are mainly produced in mitochondria during oxidative metabolism, and they can have beneficial and deleterious effects depending of their levels [Boland *et al*., 2013; Imbesi *et al*., 2013; Ghaffari, 2008]. In the case of imbalance in redox homeostasis, ROS levels overwhelm cellular antioxidant defenses, and oxidative stress is established [Ghaffari, 2008; Sardina *et al*., 2012]. Many biological processes involved in the activation of signaling pathways, such as proliferation, differentiation, and cell death, are dependent upon appropriate intracellular ROS levels, and are deregulated in cells under oxidative stress conditions [Imbesi *et al*., 2013; Hasselbalch *et al*., 2014]. Several studies implicated oxidative stress in the etiology of myeloid neoplasms [Durmus *et al*., 2013; Ghaffari, 2008; Ghoti *et al*., 2007; Hasselbalch *et al*., 2014; Imbesi *et al*., 2013; Vener *et al*., 2010].

Epigenetic alterations, such as aberrant DNA methylation and mutations in epigenetic regulator genes, are also involved in the development and progression of myeloid neoplasms [Mascarenhas *et al*., 2011; Santini *et al*., 2013; Solomon *et al*., 2008; Woods & Levine, 2015]. The hypermethylation of genes crucial to cell survival, differentiation, and proliferation, for example *CDKN2B* (*P15*) and *CDKN2A* (*P16*) genes, are observed in these myeloid malignancies [Mascarenhas *et al*.,

2011; Santini *et al*., 2013]. Moreover, the genome of cancer cells also displays DNA hypomethylation in repetitive sequences, namely in long interspersed nuclear element 1 (LINE-1) [Patchsung *et al*., 2012].

Inherited genomic variations, such as SNPs or copy number variations, may modulate oxidative stress and DNA methylation and, therefore, influence genetic susceptibility to cancer [Erichsen & Chanock, 2004]. Since myeloid neoplasms are multifactorial disorders, the study of gene variants involved in oxidative stress, DNA repair, DNA methylation, and one-carbon metabolism pathways may contribute to a deeper understanding of their molecular mechanisms. Several reports have demonstrated the importance of polymorphisms on myeloid malignancies susceptibility. Some of them involved gene affecting antioxidant defense mechanisms, DNA repair pathways, DNA methylation, and one-carbon metabolism [Aktuglu *et al*., 2014; Dong *et al*., 2014; Jankowska *et al*., 2008; Huang *et al*., 2015; Sorour *et al*., 2013; Yang *et al*., 2011; Zheng *et al*., 2013].

As mentioned before, myeloid malignancies share some common features, such as: oxidative stress [Hole *et al*., 2011; Sardina *et al*., 2012; Zhou *et al*., 2013], DNA repair deficiency [Economopoulou *et al*., 2011; Esposito & So, 2014; Zhou *et al*., 2015], and aberrant DNA methylation [Meldi & Figueroa, 2014; Schoofs *et al*., 2014; Woods & Levine, 2015]. The relationship of oxidative stress and DNA damage repair with DNA methylation is supported, at least, by four observations: 1. oxidative stress induces DNA damage; 2. DNMTs form a complex with DNA replication and repair factors; 3. DNMT1 is recruited to DNA repair sites; and 4. global inhibition of methylation leads to genome instability [Akhavan-Niaki & Samadani, 2013; Esposito & So, 2014; Sosa *et al*., 2013]. Taken these observations in consideration, it is of interest to study the potential link between oxidative stress and DNA methylation, since they both contribute to development and progression of myeloid neoplasms, and can be potentiated by each other. Furthermore, the understanding of this potential relationship may allow improvements in diagnosis and prognosis of myeloid neoplasm patients, unravel therapeutic targets, provide new insights in preventive strategies and, consequently, improve quality of clinical care.

In regard to this framework, we ***hypothesize*** that oxidative stress may contribute to development and progression of myeloid neoplasms not only through tumor suppressor genes hypermethylation, but also through repetitive sequences hypomethylation (Figure 22).

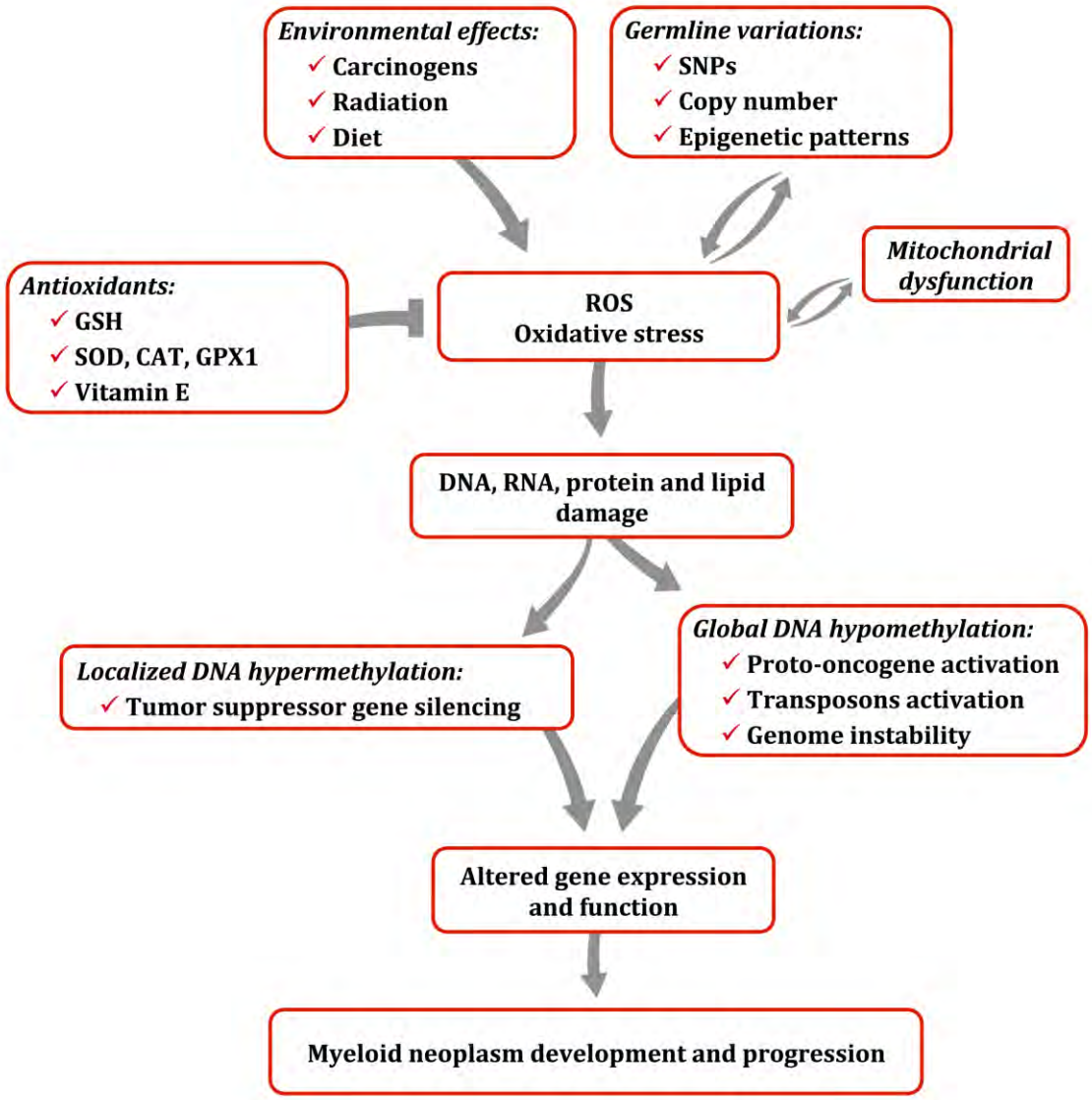


Figure 22. Schematic representation of the hypothesized cross talk between oxidative stress and DNA methylation.

2.2. Objectives

The present study **aimed** to investigate the involvement of oxidative stress and DNA methylation, as well as the role of their genetic variants in the development and progression of myeloid neoplasms. The ultimate goal was to better understand the biology of myeloid neoplasms, through the cross talk between oxidative stress and DNA methylation, in order to identity diagnostic and prognostic biomarkers as well as susceptibility genes to myeloid neoplasms.

For this purpose, we addressed the following **five specific objectives**:

- To investigate the involvement of oxidative stress and mitochondrial dysfunction in the MDS development, as well as their use as diagnostic and prognostic biomarkers – ***Chapter 3***;

- To explore the potential relationship of oxidative stress with methylation of *P15* and *P16* gene promoters in bone marrow leucocytes of MDS patients – ***Chapter 4***;

- To evaluate the association of a broad spectrum of oxidative stress parameters (non-enzymatic and enzymatic antioxidants, free radicals, and oxidative damage) with localized and global DNA methylation in patients with MDS and MPN, as well as the usefulness of these parameters as diagnostic biomarkers of both diseases – ***Chapter 5***;

- To assess whether genetic polymorphisms in genes involved in oxidative stress, DNA damage repair, DNA methylation, and folate metabolism pathways predispose to MDS and AML development, and influence disease progression (rate of MDS transformation into AML and survival) – ***Chapter 6***;

- To address whether acute and chronic exposure to exogenous hydrogen peroxide modulate genetic (copy number changes and DNA damage) and epigenetic events (TSG hypermethylation and global hypomethylation) in normal and malignant hematopoietic cell lines – ***Chapter 7***.

Chapter 3

Oxidative stress and mitochondrial dysfunction play a role in myelodysplastic syndrome development, diagnosis, and prognosis: a pilot study

Gonçalves, A.C., Cortesão, E., Oliveiros, B., Alves, V., Espadana, A.I., Rito, L., Magalhães, E., Lobão, M.J., Pereira, A., Nascimento Costa, J.M., Mota-Vieira, L. & Sarmento-Ribeiro, A.B. (2015)
Free Radic Res, **49,** 1081–94.

3.1. Abstract

The imbalance between reactive oxygen species (ROS) production and their elimination by antioxidants leads to oxidative stress. Depending on their concentration, ROS can trigger apoptosis or stimulate cell proliferation. We hypothesized that oxidative stress and mitochondrial dysfunction may not only participate in apoptosis detected in some myelodysplastic syndrome (MDS) patients, but also in increase proliferation in other patients. We investigated the involvement of oxidative stress and mitochondrial dysfunction in MDS pathogenesis, as well as assessed their diagnostic and prognostic value. Intracellular peroxides, superoxide, superoxide/peroxides ratio, reduced glutathione (GSH), and mitochondrial membrane potential ($\Delta\psi_{mit}$) levels were analyzed in bone marrow cells from 27 MDS patients and 12 controls, by flow cytometry. We observed that all bone marrow cell types from MDS patients had increased intracellular peroxides levels and decreased GSH content, compared with control cells. Moreover, oxidative stress levels were MDS subtype- and risk group-dependent. Low-risk patients had the highest ROS levels, which can be related with their high apoptosis; and intermediate-2-risk patients had high $\Delta\psi_{mit}$ that may be associated with their proliferative potential. GSH levels were negatively correlated with transfusion dependency, and peroxides levels were positively correlated with serum ferritin. GSH content showed to be an accurate parameter to discriminate patients from controls. Finally, patients with high ROS or low GSH levels, as well as high superoxide/peroxides ratio had lower overall survival. Our results suggest that oxidative stress and mitochondrial dysfunction are involved in MDS development, and that oxidative stress parameters may constitute novel diagnosis and/or prognosis biomarkers for MDS.

3.2. Introdution

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell diseases characterized by cytopenias, abnormal myeloid cell differentiation and maturation, as well as increased propensity to acute leukemia transformation. Besides peripheral cytopenias, MDS patients often present hypercellular and dysplastic bone marrow [Adès *et al.,* 2014; Greenberg, 2013; Nimer, 2008; Platzbecker *et al.,* 2007; Shih & Levine, 2011; Tefferi & Vardiman, 2009]. MDS are the most frequent hematopoietic neoplasms with a slight higher male rate and a median age at diagnosis between 65–70 years [Adès *et al.,* 2014; Tefferi & Vardiman, 2009]. In 2008, World Health Organization (WHO) classified the MDS in six categories: 1. refractory cytopenia with unilineage dysplasia, including the subtypes refractory anemia (RA), refractory neutropenia and refractory thrombocytopenia; 2. refractory anemia with ring sideroblasts (RARS); 3. refractory cytopenia with multilineage dysplasia (RCMD); 4. refractory anemia with excess of blasts (RAEB), subdivided in RAEB-1 (5–9% of blasts in bone marrow) and RAEB-2 (10–19% of blasts in bone marrow); 5. myelodysplastic syndrome with isolated del(5q); and 6. unclassifiable myelodysplastic syndrome [Adès *et al.,* 2014; Tefferi & Vardiman, 2009]. Several mechanisms underlay MDS biology but the ineffective hematopoiesis that results in peripheral cytopenias, despite the hypercellular bone marrow, has been attributed to the increased apoptosis susceptibility of myeloid progenitors [Adès *et al.,* 2014; Greenberg, 2013; Platzbecker *et al.,* 2007; Tefferi & Vardiman, 2009].

Apoptosis may be triggered by several factors including intracellular or exogenous reactive species of oxygen (ROS) [Invernizzi, 2010]. ROS, as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), are a group of radical and non-radical molecules that contain one or more unpaired electrons, and a wide range of reactivity [Farquhar & Bowen, 2003; Hole *et al.,* 2011; Valko *et al.,* 2007]. However, in order to defend itself from ROS, eukaryotic cells developed enzymatic and non-enzymatic defense mechanisms. Among cellular enzymatic defenses are superoxide dismutase, glutathione peroxidase, and catalase, while non-enzymatic defenses include reduced glutathione (GSH), vitamin C, and E [Shi *et al.,* 2012;

Valko *et al*., 2007]. The imbalance between ROS production and their elimination by antioxidants conduces to an oxidative state called oxidative stress [Farquhar & Bowen, 2003; Ghaffari, 2008; Hole *et al*., 2011; Invernizzi, 2010; Shi *et al.,* 2012; Valko *et al.,* 2007]. The persistent of this stress induces damage in several macromolecules, such as DNA, proteins, and lipids, contributing to the development of several diseases including cancer [Hole *et al*., 2011; Valko *et al*., 2007]. Beside ROS production, mitochondrion is also a target of free radicals that may induce mitochondrial damage and dysfunction. Furthermore, ROS can trigger apoptosis or stimulate proliferation by activating several cellular pathways [Antico *et al.,* 2012; Pieczenik, 2007]. These molecules influence invasion, metastasis, and angiogenesis pathways [Sosa *et al.,* 2013]. Moreover, ROS appear to be particularly important in hematopoietic cells. These cells seem to be highly vulnerable to free radical accumulation, especially the red blood cells, since deficiencies in several antioxidants are conducive to severe or lethal anemia and/or hematopoietic neoplasms [Ghaffari, 2008].

Oxidative stress markers were already reported in MDS patients, like increased lipid peroxidation [Cortelezz *et al.,* 2000] and presence of oxidized DNA bases, like 8-hydroxy 2'-deoxyguanosine (8-OHdG) [Honda *et al.,* 2000; Novotna *et al.,* 2009; Peddie *et al.,* 1997]. Furthermore, an increase in ROS simultaneously with a decrease in GSH levels were observed in peripheral red blood cells and platelets from low risk MDS patients [Ghoti *et al.,* 2007]. However, the role of oxidative stress and mitochondrial dysfunction in several bone marrow cells from MDS patients is not fully understood. In order to broaden the knowledge about the role of ROS and mitochondria dysfunction in MDS patients, the present study focuses on intracellular peroxides, superoxide anion, reduced glutathione, and mitochondrial membrane potential ($\Delta\psi_{mit}$) levels. These parameters were analyzed in different bone marrow cells: hematopoietic stem cells (blasts), erythroid precursors, monocytes, granulocytes, and lymphocytes. We hypothesized that oxidative stress and mitochondrial dysfunction may participate not only in apoptosis detected in some MDS patients, but also in increased cell proliferation in other patients. Additionally, we investigated the potential of

oxidative stress and mitochondrial dysfunction parameters as diagnosis and prognosis biomarkers.

3.3. Methods

3.3.1. Ethical statement

The present study was conducted in accordance with the Helsinki declaration. The Ethics Committee of Faculty of Medicine of University of Coimbra (Coimbra, Portugal) approved all research procedures. All participants provided their informed consent for participation prior to enrollment.

3.3.2. Study population

Thirty-nine individuals were enrolled in the present study, being 27 patients with *de novo* myelodysplastic syndrome at diagnosis and 12 controls (none of these individuals had known oxidative stress or mitochondrial related diseases). Patients were grouped according to the WHO classification of tumors of hematopoietic and lymphoid tissues (2008) [Brunning *et al*., 2008], and to the International Prognostic Scoring System (IPSS) [Brunning *et al*., 2008]. The main characteristics of these patients are summarized in Table 10.

3.3.3. Sample preparation

Bone marrow samples were obtained from MDS patients and controls at diagnosis by bone marrow aspiration into EDTA tubes. Initially, bone marrow samples were stained with monoclonal antibodies in order to identify hematopoietic cells. Briefly, 1×10^6 cells, in quadruplicate, were incubated with APC-conjugate anti-CD34 (BD Biosystems, San Diego, USA) and PerCP-Cy5.5 conjugate anti-CD45 (BD Biosystems, San Diego, USA) monoclonal antibodies for 15 min at room

temperature (RT), in the dark, according to manufacture's instructions. Then, cells were incubated with FACS lysing solution (BD Biosystems, San Diego, USA) for 15 min (RT in the dark), washed twice with phosphate-buffered saline (PBS), by centrifugation at 300g for 5 min, and used immediately for ROS, GSH and $\Delta\psi_{mit}$ measurements. Bone marrow cells were identified by CD45 expression and side scatter properties in: nucleated erythroid precursors, monocytes, granulocytes, and lymphocytes (Figure 23). Early precursors (blast cells) were defined by positive expression of CD34, dim expression of CD45, and low side scatter [Caldwell *et al*., 1991; Yuan *et al*., 2004].

3.3.4. Assessment of ROS and GSH levels

ROS levels (intracellular peroxides and superoxide) were measured using the dies 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Molecular Probes, Life Technologies Corporation, Carlsbad, USA) and dihydroethidium (DHE; Molecular Probes, Life Technologies Corporation, Carlsbad, USA), respectively, as described by others [Almeida *et al*., 2008; Zielonka *et al*., 2008]. Briefly, 1×10^6 previously stained cells were incubated with 5 μM of $DCFH_2$-DA for 45 min at 37°C in a humidified atmosphere of 5% CO_2 or with 5 μM of DHE for 15 min at RT, in the dark.

The GSH content was measured using mercury orange (MO) dye (Sigma-Aldrich, Sintra, Portugal), by incubating 1×10^6 previously stained cells with 40 μM of MO for 15 min at RT, in the dark [O'Connor *et al*., 1988]. Cells were then washed twice with cold PBS by centrifugation at 300g for 5 min, resuspended in the same buffer and kept on ice for an immediate detection by flow cytometry. Flow cytometry analysis was performed in a FACS Calibur flow cytometer (BD Biosystems, San Diego, USA), and at least 50 000 events were collected using CellQuest software (BD Biosystems, San Diego, USA). Results were analyzed through Paint-a-gate software (BD Biosystems, San Diego, USA).

Table 10
MDS patient's characteristics

Demographic data			Clinical features									
Patient (No)	Age (years)	Sex	Subtypes (WHO)	Hb (g/L)	ANC (10^9/L)	PTLs (10^9/L)	Blasts (%)[a]	Cytogenetic (FISH)	Risk groups[b]	IC therapy	Transf. depend.	Ferritin (ng/ml)
1	68	F	RA	9.3	3.0	225	1	N	Low	No	No	225
2	82	M	RA	5.9	4.3	277	1	N	Low	No	Yes	221
3	61	M	RA	9.4	2.6	276	0	N	Low	No	No	195
4	84	F	RA	8.3	4.3	342	1	N	Low	No	No	476
5	85	F	RA	9.2	2.1	160	0	+8	Int-1	No	No	257
6	72	M	RCMD	7.4	1.9	12	1	N	Int-1	DFO	Yes	1809
7	72	M	RCMD	15.5	2.7	89	1	N	Low	No	No	183
8	82	M	RCMD	10.5	0.4	39	2	+8	Int-1	No	No	224
9	80	F	RCMD	12.7	0.4	140	2	Del(20q)	Low	No	No	177
10	56	M	RCMD	5.6	2.4	253	4	N	Low	No	Yes	497
11	81	F	RCMD	7.9	0.8	118	1	N	Int-1	No	No	128
12	78	F	RCMD	5.8	2.1	233	2	N	Low	No	Yes	168
13	65	F	RCMD	9.4	1.4	13	2	N	Int-1	No	No	357
14	33	F	RCMD	12.3	4.0	29	4	N	Low	No	No	141
15	81	F	RCMD	9.1	2.8	215	1	N	Low	No	No	82
16	81	M	RCMD	6.0	1.9	324	1	+8	Int-1	DFO	Yes	1750
17	79	F	RCMD	13.1	0.9	107	6	Del(7q)	Int-1	No	No	398
18	81	M	RAEB-1	10.4	1.3	170	6	N	Int-1	No	No	451
19	74	M	RAEB-1	6.4	1.2	39	5	N	Int-1	No	Yes	444
20	73	F	RAEB-1	9.7	0.9	76	9	N	Int-2	No	Yes	808
21	84	F	RAEB-1	7.9	2.4	367	5	Del(20q)	Int-1	No	No	134
22	84	M	RAEB-2	7.6	2.4	26	18	N	Int-2	No	No	358
23	76	M	RAEB-2	5.7	1.0	61	10	N	Int-1	No	Yes	83
24	78	F	RAEB-2	4.6	0.8	65	12	N	Int-2	No	Yes	1525
25	71	M	RAEB-2	5.4	2.7	19	16	N	Int-2	No	Yes	538
26	77	M	RAEB-2	9.8	5.1	49	11	N	Int-2	No	No	203
27	77	F	RAEB-2	9.0	0.1	29	10	N	Int-1	No	No	157

[a], bone marrow; [b], IPSS groups. No, sample number; M, male; F, female; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, RA with excess of blasts type 1; RAEB-2, RA with excess of blasts type 2; Hb, hemoglobin; ANC, absolute neutrophil count; PTLs, platelets; IPSS, International Prognostic Scoring System; Low, low risk; Int 1, intermediate risk 1; Int 2, intermediate risk 2; N, normal; +8, trisomy 8; Del(7q), long arm deletion of chromosome 7; Del(20q), long arm deletion of chromosome 20; IC, iron chelation; Transf. depend., transfusion dependency; DFO, deferoxamine.

3.3.5. Assessment of mitochondrial membrane potential

$\Delta\psi_{mit}$ was determined using the fluorescent probe 5,5',6,6'-tethrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Life Technologies Corporation, Carlsbad, USA), as previously described [Almeida *et al.*,

2008]. JC-1 is a lipophilic cationic probe that exists in a monomeric form emitting at 527 nm (green fluorescence) after excitation at 490 nm. However, this dye is able to reversibly form aggregates, which are associated with a large shift in emission (590 nm, greenish orange fluorescence), as the mitochondrial membrane becomes more polarized. JC-1 monomer/aggregate ratio allows comparative measurements of $\Delta\psi_{mit}$, since low JC-1 M/A ratio corresponds to high $\Delta\psi_{mit}$ and vice-versa. To determine $\Delta\psi_{mit}$, 1×10^6 stained cell were incubated with 5 µg/ml of JC-1 for 15 min at 37°C in a humidified atmosphere of 5% CO_2. At the end of the incubation period, the cells were washed twice in cold PBS, resuspended and kept on ice until flow cytometry detection.

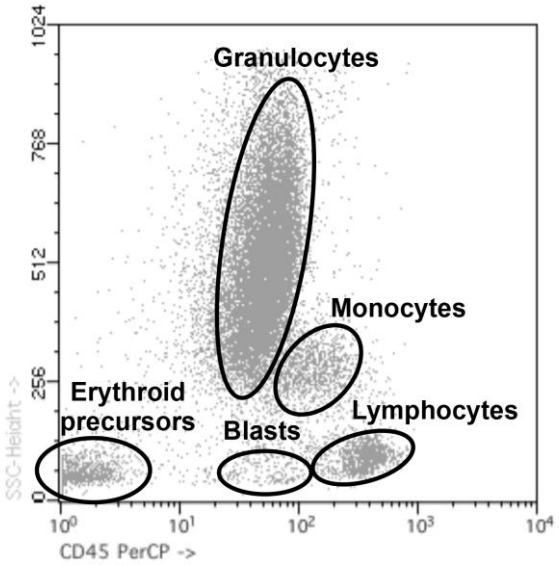


Figure 23. Representative dot-plot of side scatter (SSC) versus CD45 used to identify bone marrow cells. Bone marrow cells were identified by CD45 expression vs side scatter properties, as indicated in the figure. Blast cells were identified by positive expression of CD34, dim expression of CD45, and low side scatter (not shown).

3.3.6. Statistical analysis

Statistical analysis was performed using SPSS version 21.0, and graphics were constructed through GraphPad Prism version 5.0. Data are expressed as mean ± standard error of mean (SEM), unless otherwise specified. Normality was

assessed by Kolmogorov-Smirnov analysis. For normally distributed continuous variables, Student's t-test and ANOVA with Bonferroni Post-Hoc test were performed to assess the statistical significance of the difference between means of two groups (patients *vs.* controls; transfusion independent patients *vs.* transfusion dependent patients) and more than two groups (subtype groups: RA *vs.* RCMD *vs.* RAEB-1 *vs.* RAEB-2; IPSS risk groups: low *vs.* int-1 *vs.* int-2), respectively. When continuous variables did not show normal distribution, Mann Whitney U and Kruskal Wallis tests were used. The association between clinical variables and oxidative stress levels or with $\Delta\psi_{mit}$ was determined by Spearman or Pearson correlation coefficients.

The receiver operating characteristic (ROC) curves analysis was performed to assess the variables accuracy as diagnostic biomarkers. For each ROC curve, an optimal cut-off point was determined as the value of the parameter that maximized the sum of specificity and sensitivity (Youden's J Index). Survival analysis was performed by Kaplan Meier method, using the interval from diagnosis to last contact or death (overall survival). Patients were dichotomized according to the cut-off points obtained from the ROC curves constructed to predict death. Differences in overall survival were tested through log rank statistics, and median survival ratios calculated. All statistical analysis were two-sided, and a $p < 0.05$ was considered statistically significant.

3.4. Results

3.4.1.Demographic and clinical characteristics of MDS patients

Clinical characteristics of MDS patients are shown in Table 10. The present study enrolled 27 MDS patients with a median age of 78 years, ranging from 33 to 85 years, with 52.0% females (*n*=14) and 48.0% males (*n*=13). Patients and controls were recruited between June 2006 and December 2007 in Centro Hospitalar e Universitário de Coimbra (CHUC,EPE) and Hospital Distrital da Figueira da Foz (HDFF,EPE), Portugal. According with WHO classification (2008), five patients

were diagnosed with RA (18.5%), 12 with RCMD (44.4%), four with RAEB-1 (14.8%), and six with RAEB-2 (22.3%).

The IPSS prognostic score were low in 10 patients (37.0%), intermediate-1 (int-1) in 12 (44.4%), and intermediate-2 (int-2) in five (18.6%). Six patients (22.2%) had cytogenetic abnormalities, 10 (37.0%) were transfusion dependent, and the serum ferritin presented an average value of 444±93 ng/ml. The majority of MDS patients died from infection or disease progression. Only patient No 23 transformed to acute myeloblastic leukemia. This patient was treated with azacytidine without response and died in consequence of acute leukemia transformation. The control group consists of 12 individuals without neoplastic malignancies with six females (50.0%) and six males (50.0%), and a median age of 72 years, ranging from 38 to 89 years.

3.4.2. Bone marrow cells from MDS patients are under oxidative stress

In order to analyze the participation of oxidative stress in MDS development, we examined ROS, GSH, and superoxide/peroxides ratio differences between MDS patients and controls. As observed in Figure 24, all cell populations from MDS patients had significant higher levels of intracellular peroxides [blasts: 687±96 MIF (mean intensity of fluorescence); erythroid precursors: 157±31 MIF; monocytes: 313±55 MIF; granulocytes: 274±16 MIF; lymphocytes: 262±11 MIF], when compared with controls (blasts: 320±41 MIF; erythroid precursors: 59±7 MIF; monocytes: 193±15 MIF; granulocytes: 195±14 MIF; lymphocytes: 205±12 MIF).

Intracellular superoxide levels were significantly increased in granulocytes (216±11 MIF), relatively to controls (159±12 MIF; p=0,004). Moreover, superoxide/peroxides ratio was significantly decreased in blasts (0.6±0.1, p=0.001) and erythroid precursors (4.1±0.9, p=0.001), comparatively to controls (1.0±0.1 and 8.2±1.2, respectively). Furthermore, GSH levels were significantly decreased in all cells (blasts: 201±29 MIF; erythroid precursors: 142±9 MIF; monocytes: 345±52 MIF; granulocytes: 116±10 MIF; lymphocytes: 229±18 MIF),

when compared with control ones (blasts: 1057±81 MIF; erythroid precursors: 218±10 MIF; monocytes: 609±21 MIF; granulocytes: 179±4 MIF; lymphocytes: 298±15 MIF).

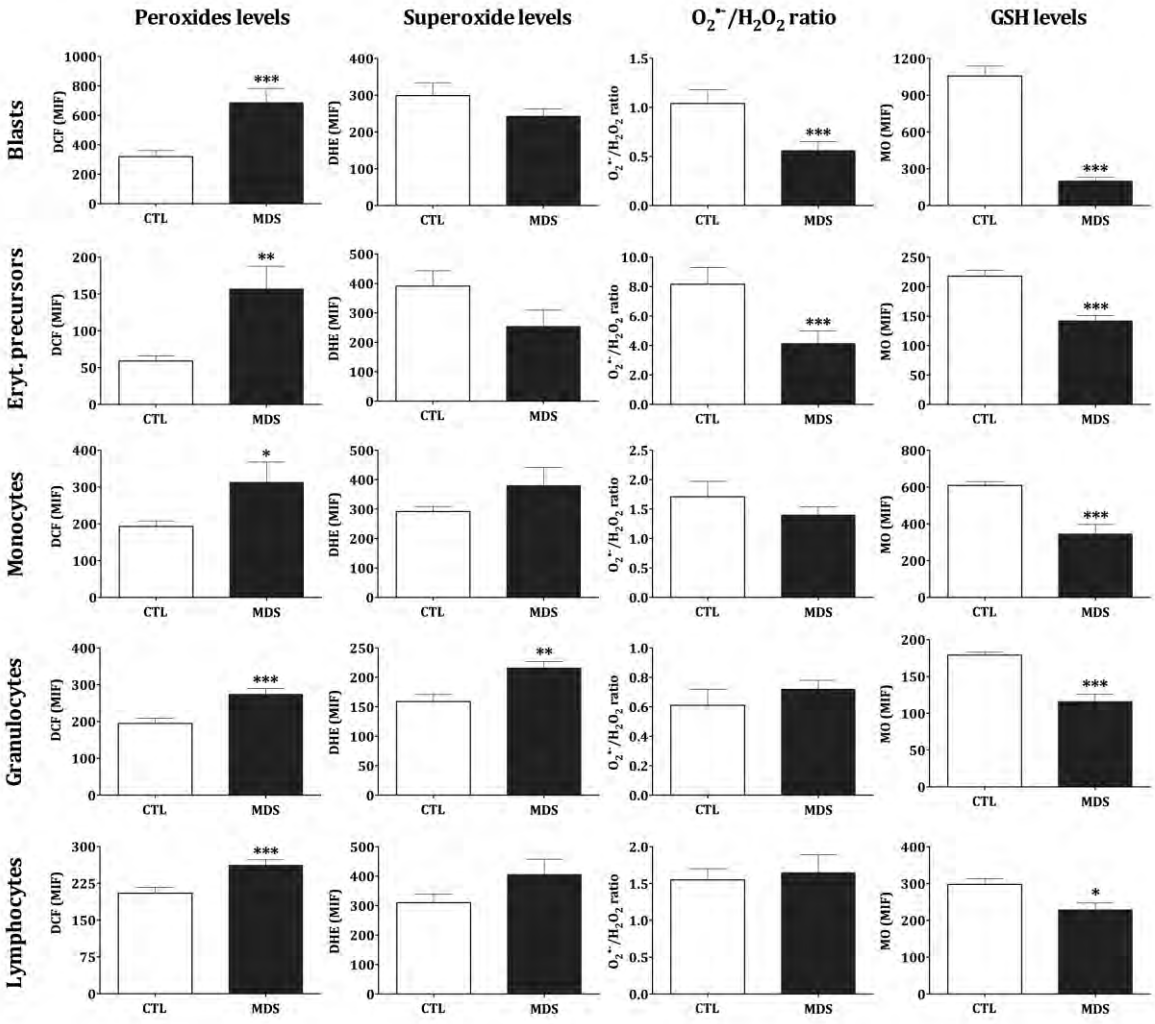


Figure 24. Analysis of oxidative stress parameters in MDS patients and controls. Oxidative stress levels in bone marrow cell populations were analyzed as following: intracellular peroxides and superoxide levels using $DCFH_2$-DA and DHE dies, respectively; and GSH levels through MO dye. The results are represented as mean ± SEM. MDS, myelodysplastic syndrome; CTL, control; Eryt. precursors, erythroid precursors; GSH, reduced glutathione; $DCFH_2$-DA, 2,7-dichlorodihydrofluorescein-diacetate; DHE, dihydroethidium; MO, mercury orange; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.4.3. Oxidative stress is MDS subtype dependent and higher in low risk patients

To explore the possible role of oxidative stress in MDS subtypes, we analyzed these parameters according to WHO classification. As shown in Figure 25, the highest intracellular peroxides levels were observed in blasts (562±136 MIF) and monocytes (1125±106 MIF) from RCMD patients, while the lowest were detected in erythroid precursors from RAEB-1 (202±12 MIF) and RAEB-2 (243±65 MIF). Furthermore, erythroid precursors from RA patients had 3.7-, 9.3-, and 20.7-fold more intracellular peroxides than RCMD, RAEB-1, and RAEB-2, respectively. The intracellular superoxide levels were significantly increased in monocytes from RCMD patients (2.5-, 3.5-, and 3.7-fold, respectively to RA, RAEB-1, and RAEB-2) and decreased in RA granulocytes (approximately 0.6-fold), comparatively to the other subtypes. Furthermore, blasts from RCMD had the lower superoxide/peroxides ratio (0.3±0.0) comparatively with RA (0.5±0.1), RAEB-1 (1.1±0.3), and RAEB-2 (1.9±0.8). In general, all cell types from RAEB-1 and RAEB-2 patients had higher superoxide/peroxides ratios relatively with RA and RCMD patients; however, these differences were only significant in blasts, erythroid precursors, and granulocytes. The lowest GSH levels were noted in blasts from RAEB-1 patients (0.1-fold relatively to RA and RCMD; $p<0.05$) and RAEB-2 (0.2-fold relatively to RA and RCMD; $p<0.05$), as well as in erythroid precursors from RA patients (0.6-, 0.4-, and 0.4-fold, respectively to RCMD, RAEB-1, and RAEB-2; $p<0.05$). Moreover, the monocytes from RCMD patients had 3.8-, 4.0-, and 2.0-fold more GSH content than RA, RAEB-1, and RAEB-2 ($p<0.05$), respectively. GSH levels in granulocytes from RCMD patients (69±6 MIF) were lower than RA (161±5 MIF), RAEB-1 (205±4 MIF), and RAEB-2 (114±3 MIF).

To determine the contribution of oxidative stress in MDS prognosis, patients were grouped according to their IPSS risk (Figure 26). The intracellular peroxides levels were lower in all hematopoietic cells from int-2-risk patients comparatively to low- and int-1-risk patients, except in lymphocytes. Furthermore, monocytes from int-2-risk patients also had the lowers superoxide levels, when compared with low and int-1-risk patients. The superoxide/peroxides ratio was significantly higher in blast (1.0±0.3) and erythroid precursors (10.7±2.8) from int-2-risk patients,

comparatively with low (1.0±0.3 and 10.7±2.8, respectively) and int-1-risk ones (1.0±0.3 and 10.7±2.8, respectively). GSH levels were 0.2- and 0.3-fold lower in blast cells from int-2 risk patients, relatively to low- and int-1-risk patients (p<0.05); while in erythroid precursors from int-2-risk patients, this antioxidant molecule were 1.6- and 1.4-fold higher comparatively to low- and int-1-risk patients (p<0.05).

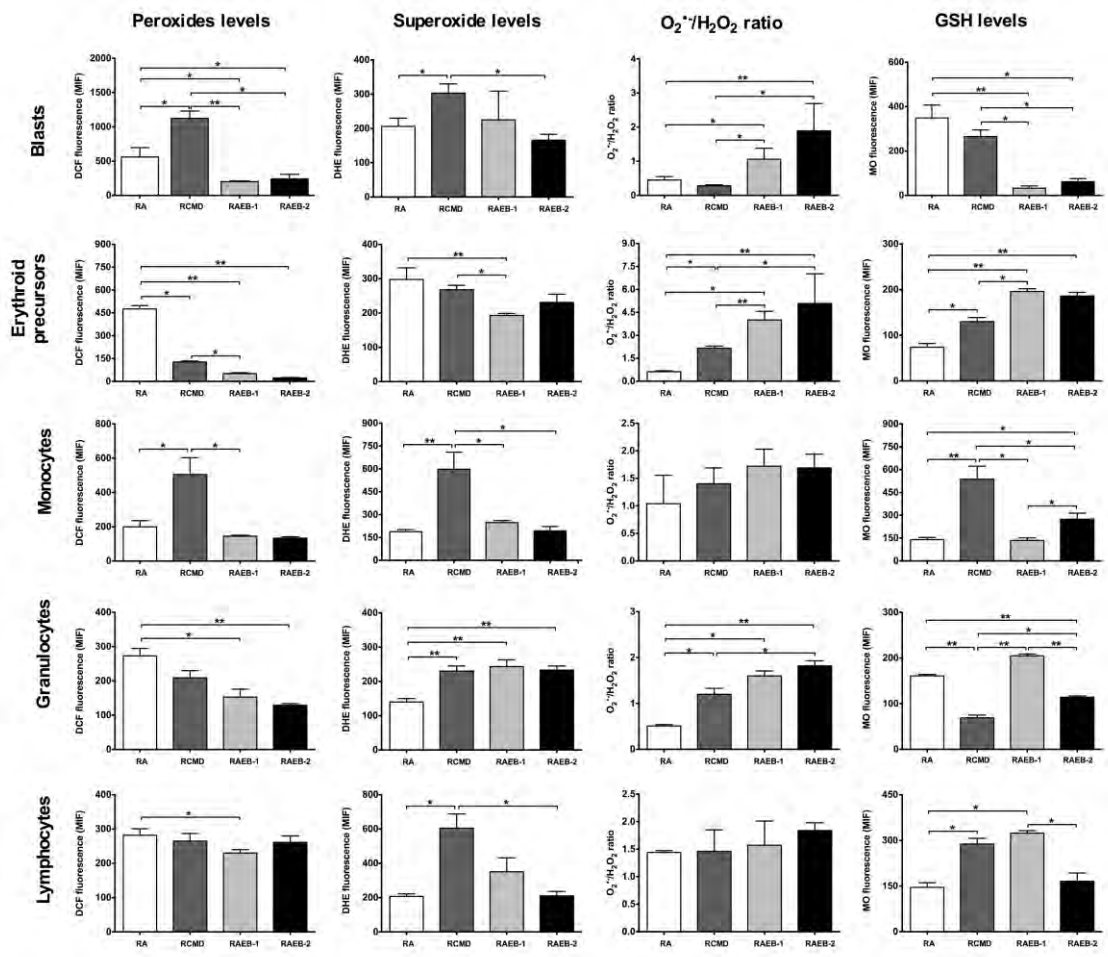


Figure 25. Analysis of oxidative stress parameters in MDS subtypes, according to World Health Organization classification. Oxidative stress levels in bone marrow cell populations were analyzed as following: intracellular peroxides and superoxide levels using $DCFH_2$-DA and DHE dies, respectively; and GSH levels through MO dye. The results are represented as mean ± SEM. MDS, myelodysplastic syndrome; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess of blasts type 1; RAEB-2, refractory anemia with excess of blasts type 2; GSH, reduced glutathione; $DCFH_2$-DA, 2,7-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; MO, mercury orange; *, p<0.05; **, p<0.01; ***, p<0.001.

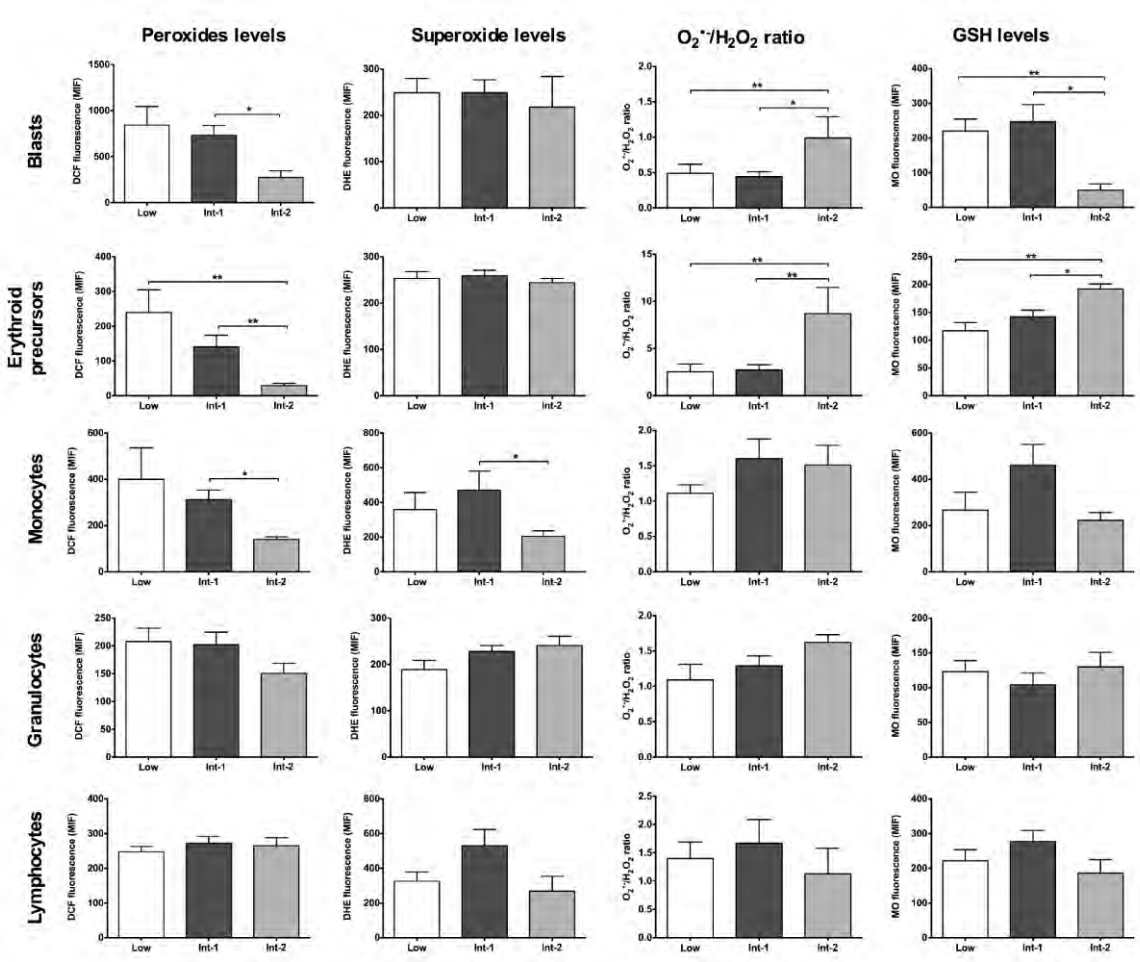


Figure 26. Analysis of oxidative stress parameters in MDS risk groups, according to International Prognostic Scoring System. Oxidative stress levels in bone marrow cell populations were analyzed as following: intracellular peroxides and superoxide levels using $DCFH_2$-DA and DHE dies, respectively; GSH levels through MO dye; and $\Delta\psi_{mit}$ by JC-1 monomer/aggregate ratio. The results are represented as mean ± SEM. MDS, myelodysplastic syndrome; Int 1, intermediate 1; Int 2, intermediate 2; GSH, reduced glutathione; $DCFH_2$-DA, 2,7-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; MO, mercury orange; *, $p<0.05$; **,$p<0.01$; ***, $p<0.001$

3.4.4. $\Delta\psi_{mit}$ is associated with MDS subtypes and IPSS risk groups

In order to analyze the involvement of mitochondrial dysfunction in MDS development and prognosis, we calculate $\Delta\psi_{mit}$ in patients and controls, as well as in patients stratified according to WHO classification and to IPSS risk groups. As observed in Figure 27, MDS patients had a significant increase in JC-1 M/A ratio (high JC-1 M/A ratio corresponding to low $\Delta\psi_{mit}$) in erythroid precursors

(0.901±0.104), comparatively with controls (0.490±0.041; p<0.001). Moreover, a significant increase in JC-1 M/A ratio, which indicates a significant decrease in $\Delta\psi_{mit}$, was detected in RCMD blasts (3.3-, 3.1-, and 3.0-fold when compared with RA, RAEB-1, and RAEB-2, respectively) and in RA lymphocytes (1.6-, 3.5-, and 2.3-fold when compared with RCMD, RAEB-1, and RAEB-2, respectively). Finally, all cell types from int-2 risk patients had significantly lower JC-1 M/A ratio that correspond to a higher $\Delta\psi_{mit}$, ranging from 0.3- and 0.6-fold, comparatively with low and int-1-risk groups.

3.4.5. Reduced glutathione is related with serum ferritin levels and transfusion dependency in MDS patients

Firstly, we correlate the oxidative stress parameters with each other and with $\Delta\psi_{mit}$, in order to detect possible associations between them. We observed that intracellular peroxides levels were positively correlated with intracellular superoxide levels in blasts (r=0.557; p=0.003; Figure 28A), erythroid precursors (r=0.516; p=0.006), and monocytes (r=0.647; p<0.001). In erythroid precursors, we detected a negative correlation of GSH content with intracellular peroxides levels (r=–0.790; p<0.001; Figure 28B), as well as with intracellular superoxide levels (r=–0.443; p=0.021). However, no significant correlations were observed between oxidative stress parameters and $\Delta\psi_{mit}$ (data not shown).Secondly, we correlate the oxidative stress parameters, as well as $\Delta\psi_{mit}$ with age at diagnosis, blasts in bone marrow, cytogenetic abnormalities, serum ferritin levels, and transfusion dependency. We noted a positive correlation between blasts superoxide/peroxides ratio and the percentage of blast in bone marrow (r=0.681; p<0.001). Serum ferritin levels were positively related with intracellular peroxides (r=0.576; p=0.002; Figure 28C), and negatively with GSH levels (r=–0.398; p=0.040). Moreover, transfusion dependent patients had lower GSH levels compared with independent ones (Figure 28D). GSH levels in erythroid precursors (p=0.041), granulocytes (p=0.033), and lymphocytes (p=0.001) were negatively correlated with transfusion dependency. We did not observe any

significant correlation between cytogenetic abnormalities or age at diagnosis with oxidative stress and $\Delta\psi_{mit}$ levels (data not shown).

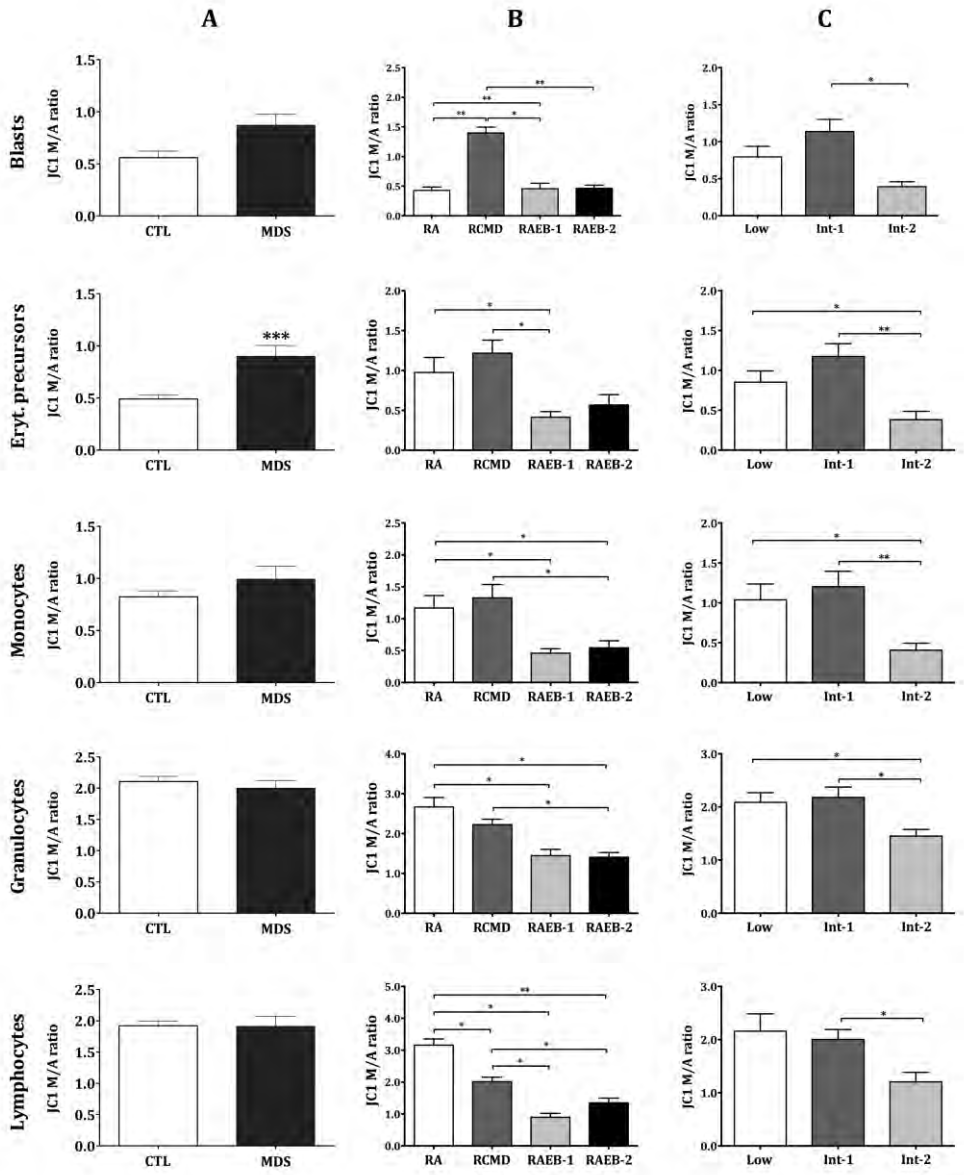


Figure 27. Analysis of mitochondrial dysfunction. Mitochondrial membrane potential ($\Delta\psi_{mit}$) was compared between MDS patients and controls (A), between MDS subtypes according WHO classification (B), and between MDS IPSS risk groups according to International Prognostic Scoring System. (C). $\Delta\psi_{mit}$ in bone marrow cell populations were analyzed by JC-1 monomer/aggregate ratio. The results are represented as mean ± SEM. MDS, myelodysplastic syndrome; CTL, control; Eryt. precursors, erythroid precursors; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess of blasts type 1; RAEB-2, refractory anemia with excess of blasts type 2; Int-1, intermediate-1; Int-2, intermediate-2; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

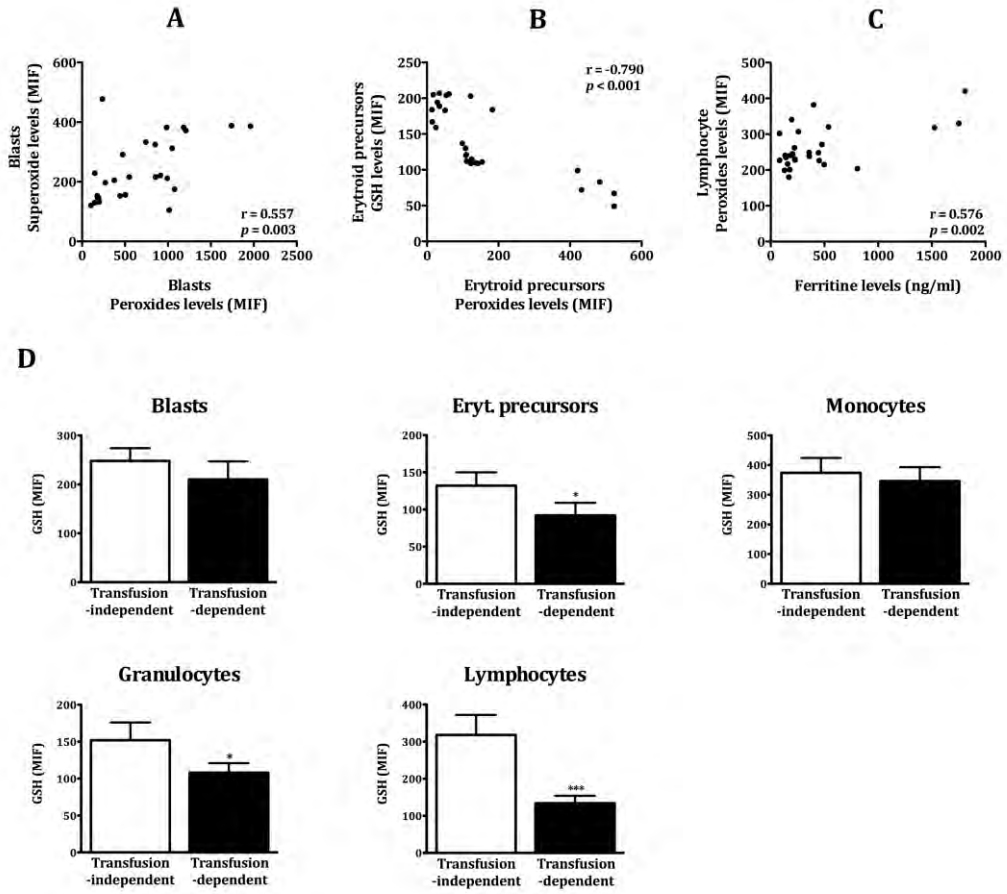


Figure 28. Relationship between oxidative stress parameters (A – B), and between oxidative stress and patient clinical characteristics (C – D). (A) represents the positive correlation of intracellular peroxides with superoxide levels in blast cells, and (B) the negative correlation of intracellular peroxides with GSH levels in erythroid precursors. (C) represents the positive correlation of intracellular peroxides levels from lymphocytes with serum ferritin levels, and (D) the relationship between transfusion dependency and GSH levels. Oxidative stress levels in bone marrow cell populations were analyzed as following: intracellular peroxides and superoxide levels using $DCFH_2$-DA and DHE dies, respectively; and GSH levels through MO dye. *r*, correlation coefficient; GSH, reduced glutathione; $DCFH_2$-DA, 2,7-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; MO, mercury orange; *, $p<0.05$; ***, $p<0.001$.

3.4.6. Oxidative stress levels could be use as predictive biomarker for MDS diagnosis

To evaluate the diagnostic value of oxidative stress parameters and $\Delta\psi_{mit}$, we constructed ROC curves. According to these curves (Table 11), the GSH levels in

blast cells were the most accurate biomarker for MDS diagnosis, with an area under the curve (AUC) of 1.000 (95% CI 1.000–1.000; $p<0.001$). GSH levels lower than 651 MIF were defined as the optimal cut-off value for differentiation of MDS patients from controls. This cut-off presented the maximal sensitivity (100%) and specificity (100%), as well as a strong power to rule in [positive likelihood ratio (PLR): undefined] and to rule out [negative likelihood ratio (NLR): 0.0]. Low GSH levels in erythroid precursors (AUC=0.886; 95% CI 0.784–0.988; $p<0.001$), monocytes (AUC=0.852; 95% CI 0.818–0.986; $p=0.001$), and granulocytes (AUC=0.841; 95% CI 0.712–0.970; $p=0.001$) were good biomarkers for MDS diagnosis. The best GSH cut-off values were 187 MIF (sensitivity: 92%; specificity: 74%; PLR: 3.5; NLR: 0.1), 496 MIF (sensitivity: 85%; specificity: 100%; PLR: 85.2; NLR: 0.1), and 139 MIF (sensitivity: 67%; specificity: 100%; PLR: 66.7; NLR: 0.3), respectively.

Furthermore, intracellular superoxide levels higher than 315 MIF in erythroid precursors (AUC=0.963; 95% CI 0.911–1.000; $p<0.001$) and 195 MIF in granulocytes (0.792; 95% CI 0.651–0.932; $p=0.004$) were also accurate biomarkers to discriminate MDS patients from controls. These cut-off values achieved good sensitivity (100% and 67%, respectively for erythroid precursors and granulocytes), specificity (89% and 92%), and likelihood ratios (PLR: 9.0 and 8.0; NLR: 0.0 and 0.4). Intracellular peroxides levels higher than 106 MIF in erythroid precursors (AUC=0.704; 95% CI 0.543–0.864; $p=0.045$), 243 MIF in granulocytes (AUC=0.832; 95% CI 0.700–0.963; $p=0.001$), as well as 211 MIF in lymphocytes (AUC=0.861; 95% CI 0.715–1.000; $p<0.001$) were similarly significant biomarkers. Using these cut-off values, the differentiation between MDS patients and controls presented good sensitivity (59%, 74%, and 85%, respectively for erythroid precursors, granulocytes, and lymphocytes), specificity (100%, 83%, and 83%), and likelihood ratios (PLR: 59.3, 4.4 and 5.1; NLR: 0.4, 0.3 and 0.2). Finally, low superoxide/peroxides ratio in blasts (AUC=0.818; 95% CI 0.673–0.963; $p=0.002$), erythroid precursors (AUC=0.824; 95% CI 0.696–0.952; $p=0.001$), and granulocytes (AUC=0.840; 95% CI 0.713–0.966; $p=0.001$) were also accurate biomarkers to discriminate MDS patients from controls. The best

superoxide/peroxides ratio cut-off values were 0.68 for blasts (sensitivity: 70%; specificity: 92%; PLR: 8.8; NLR: 0.3), 3.72 for erythroid precursors (sensitivity: 70%; specificity: 100%; PLR: undefined; NLR: 0.3), and 0.71 for granulocytes (sensitivity: 67%; specificity: 92%; PLR: 10.3; NLR: 0.2).

Table 11

Performance of oxidative stress and mitochondrion markers to discriminate myelodysplastic syndrome patients from controls

				Cut-off				
		AUC		value	Sensitivity	Specificity	Likelihood ratio	
Cells	Biomarkers	value (95% CI)	*p*-value	(MIF)	(%)	(%)	Pos	Neg
Blasts	Peroxides	0.667 (0.467 – 0.838)	0.100	nc	nc	nc	nc	nc
	Superoxide	0.657 (0.484 – 0.831)	0.121	nc	nc	nc	nc	nc
	$O_2^{\bullet-}/H_2O_2$	0.818 (0.673 – 0.963)	0.002	0.68	70	92	8.8	0.3
	GSH	1.000 (1.000 – 1.000)	<0.001	651	100	100	und	0.0
	$\Delta\psi_{mit}$	0.653 (0.480 – 0.826)	0.132	nc	nc	nc	nc	nc
Erythroid precursors	Peroxides	0.704 (0.543 – 0.864)	0.045	106	59	100	und	0.4
	Superoxide	0.963 (0.911 – 1.000)	<0.001	315	100	89	9.0	0.0
	$O_2^{\bullet-}/H_2O_2$	0.824 (0.696 – 0.952)	0.001	3.72	70	100	und	0.3
	GSH	0.880 (0.784 – 0.988)	<0.001	187	92	74	3.5	0.1
	$\Delta\psi_{mit}$	0.604 (0.442 – 0.865)	0.055	nc	nc	nc	nc	nc
Monocytes	Peroxides	0.580 (0.398 – 0.768)	0.411	nc	nc	nc	nc	nc
	Superoxide	0.546 (0.369 – 0.723)	0.648	nc	nc	nc	nc	nc
	$O_2^{\bullet-}/H_2O_2$	0.620 (0.439 – 0.801)	0.235	nc	nc	nc	nc	nc
	GSH	0.852 (0.718 – 0.986)	0.001	496	85	100	und	0.2
	$\Delta\psi_{mit}$	0.512 (0.332 – 0.695)	0.903	nc	nc	nc	nc	nc
Granulocytes	Peroxides	0.832 (0.700 – 0.963)	0.001	243	74	83	4.4	0.3
	Superoxide	0.792 (0.651 – 0.932)	0.004	195	67	92	8.0	0.4
	$O_2^{\bullet-}/H_2O_2$	0.840 (0.713 – 0.966)	0.001	0.71	82	92	10.3	0.2
	GSH	0.841 (0.712 – 0.970)	0.001	139	67	100	und	0.3
	$\Delta\psi_{mit}$	0.579 (0.400 – 0.758)	0.091	nc	nc	nc	nc	nc
Lymphocytes	Peroxides	0.861 (0.715 – 1.000)	<0.001	211	85	83	5.1	0.2
	Superoxide	0.506 (0.327 – 0.685)	0.951	nc	nc	nc	nc	nc
	$O_2^{\bullet-}/H_2O_2$	0.608 (0.434 – 0.782)	0.287	nc	nc	nc	nc	nc
	GSH	0.670 (0.505 – 0.834)	0.094	nc	nc	nc	nc	nc
	$\Delta\psi_{mit}$	0.542 (0.304 – 0.719)	0.681	nc	nc	nc	nc	nc

GSH, reduced glutathione; $\Delta\psi_{mit}$, mitochondrial membrane potential; AUC, area under the curve; 95% CI, 95%confidence interval; MIF, mean intensity of fluorescence; LR, Likelihood ratio; Pos, positive; Neg, negative; nc, not calculated; und, undefined; $O_2^{\bullet-}/H_2O_2$, superoxide/peroxides ratio.

3.4.7. Oxidative stress levels could be prognostic factors in MDS

To determine if oxidative stress and $\Delta\psi_{mit}$ could influence survival of MDS patients, we estimate the cut-off points that allowed us to predict death and used them to stratify patients. The ROC analysis allowed us to determine optimal cut-off values for intracellular peroxides, superoxide, and GSH levels from erythroid precursors and granulocytes, as well as for superoxide/peroxides ratio from blasts and granulocytes. We could not obtain valid cut-off values or significant differences in Kaplan-Meier analysis for any other tested parameter (data not shown).

As observed in Figure 29, patients with high intracellular peroxides levels in erythroid precursors and granulocytes, as well as those with high intracellular superoxide levels had worse overall survival than those with low levels. The mean survival time of patients with intracellular peroxides levels higher than 358 MIF in erythroid precursors (68.1±5.3 months) was significantly longer than those with lower levels (32.3±4.6 months). Similarly, patients with intracellular peroxides levels higher than 241 MIF in granulocytes (58.7±5.8 months) showed significantly longer mean survival than those with lower levels (29.1±5.1 months). Moreover, the mean survival time of patients with intracellular superoxide levels higher than 292 MIF in erythroid precursors (65.5±7.6 months), as well as 175 MIF in granulocytes (72.3±5.4 months) was significantly longer than those with lower levels (29.4±4.1 and 29.4±4.1 months, respectively). Furthermore, the mean survival time of patients with superoxide/peroxides ratio lower than 0.8 in blasts (44.2±5.4 months) and 0.9 in granulocytes (62.5±6.9 months) was longer than those with higher ratios (21.1±6.9 and 30.7±4.7 months, respectively). Finally, the mean survival time of patients with GSH levels lower than 110 MIF in erythroid precursors (64.8±8.3 months) and 141 MIF in granulocytes (62.2±10.4 months) was longer than those with higher levels (29.9±4.2 and 30.8±4.0 months, respectively).

MDS patients had an increased propensity to acute leukemia transformation, and this fact may also be related to oxidative stress and/or $\Delta\psi_{mit}$. However, in the present study we were not able to evaluate the role of these parameters in acute

leukemia transformation, since in our sample only one patient had transformed to acute myeloid leukemia.

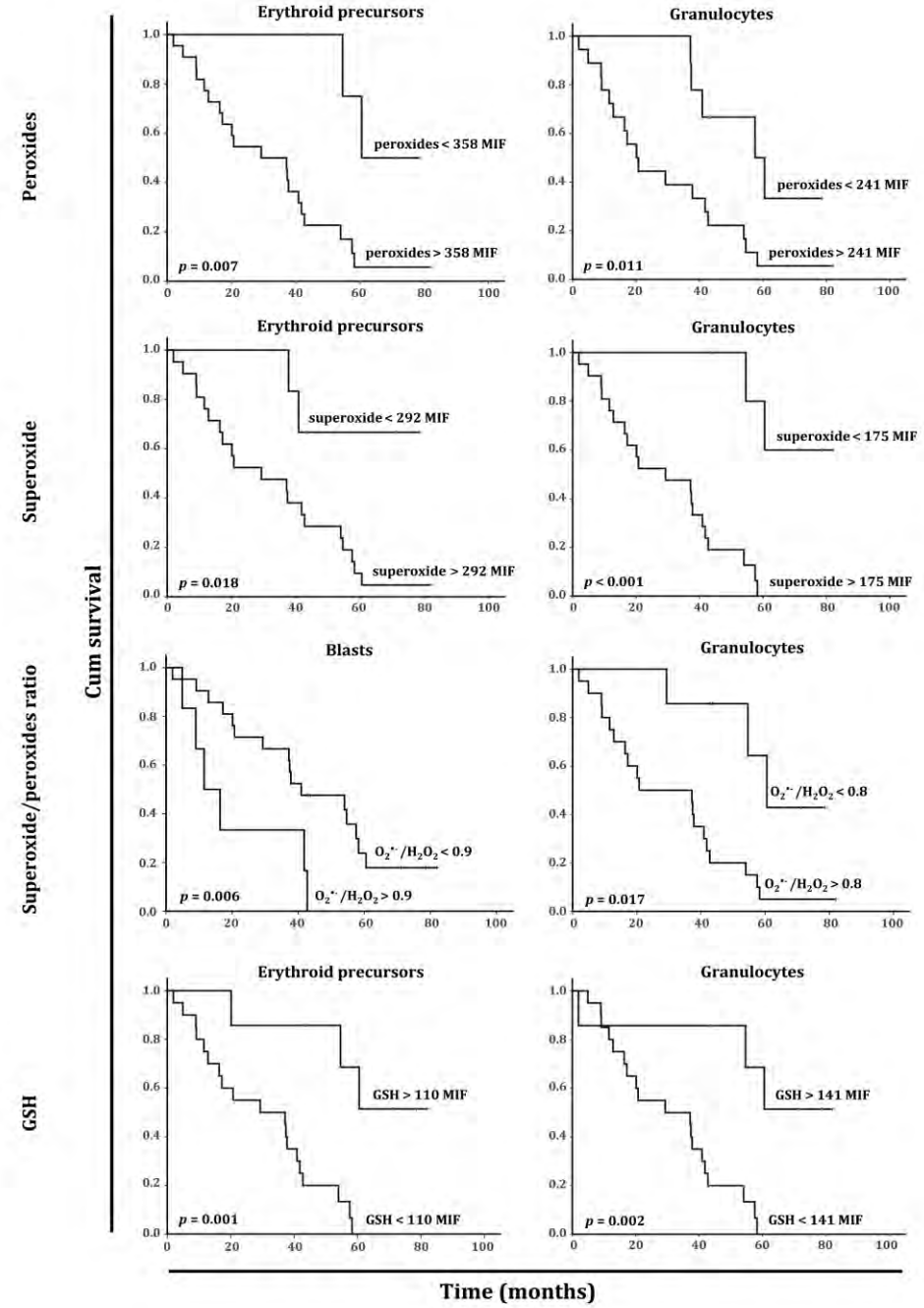


Figure 29. Overall survival curves of MDS patients, according to oxidative stress levels. Survival analysis was performed by Kaplan-Meier method. MDS patients were stratified through the cut-off points obtained from the ROC curves using intracellular peroxides, superoxide and GSH from erythroid precursors, as well as from granulocytes. MDS, myelodysplastic syndrome; GSH, reduced glutathione.

3.5. Discussion

Although several studies have shown that MDS patients had elevated ROS levels in peripheral blood and oxidative damage markers in peripheral blood and bone marrow [Ghoti *et al*., 2007; Ghoti *et al*., 2010; Honda *et al.,* 2000; Novotna *et al.*, 2009; Peddie *et al*., 1997; Saigo *et al.,* 2011; Voukelatou *et al.*, 2009], the analysis of oxidative stress levels, as well as the $\Delta\psi_{mit}$ in the different bone marrow cell types from MDS patients was not completely clear. Furthermore, the involvement of these parameters in diagnosis and prognosis of MDS patients are not yet studied.

In the present study, we described that bone marrow cells from MDS patients had increased intracellular peroxides levels and decreased GSH content, when compared with their normal counterpart cells. Blasts, erythroid precursors, and granulocytes had the highest ROS levels. These cells were the most affected by oxidative stress. In general, blast cells appear to be the ones that contribute more to MDS development and progression; however, all blood cell types were affected in a subtype dependent manner. The aberrant levels of oxidative stress parameters and mitochondrial dysfunction present in blasts may pass into mature cells and may be responsible for their abnormal phenotype. The oxidative stress changes observed in blasts and erythroid precursors suggest that these cells are the most relevant contributors to RA patients, while in RCMD, RAEB-1, and RAEB-2 patients blasts appear to be the most important. Moreover, patients stratified according to IPSS risk groups showed that only blasts, erythroid precursors, and monocytes had significant differences in oxidative stress parameters. RA patients, as well as RCMD, had the highest oxidative stress levels, which could contribute to the higher apoptotic rates translated into cytopenias observed in these patients. On the other hand, RAEB-1 and -2 patients were the MDS subtypes less affected by oxidative stress. Moreover, low risk patients, including low- and int-1-risk groups, showed higher intracellular ROS levels than high-risk patients (int-2-risk group), in agreement with MDS subtypes.

The increased ROS levels together with the decrease GSH in cells from MDS patients suggest that these cells are under oxidative stress. Oxidative stress in red

blood cells, platelets, and polymorphonuclear leukocytes of low risk MDS patients were already describe by Ghoti *et al.* [2007], while increased serum ROS levels were reported by Saigo *et al*. [2011]. Elevated ROS levels activate cellular signaling pathways that can induce proliferation or apoptosis depending on the stress levels [Circu & Aw, 2010; Valko *et al.,* 2007]. For example, ROS induce extrinsic and intrinsic apoptosis, through JNK activation or by decreasing cellular GSH levels that conduce to redox imbalance [Circu & Aw, 2010]. Increased ROS and low GSH content noted in MDS blasts, erythroid precursors, and granulocytes might be responsible for the higher susceptibility to apoptosis in RA and RCMD subtypes, as referred. This increase of apoptosis susceptibility in RA and RCMD subtypes, as well as in low-risk patients (low- and intermediate-1-risk), was previous reported by us [Cortesão *et al.,* 2008] and by others [Parker *et al.,* 2000; Parker & Mufti, 2001]. Furthermore, they may explain the increased cell death in low-risk patients. In myeloid cells, increased ROS may alter ROS-regulated pathways, particularly proliferation and survival pathways, conferring proliferative advantages to malignant cell population [Hole *et al.,* 2011]. In RAEB-1 and -2, we detected a decrease in ROS and GSH levels, especially in blasts. This fact may induces blasts to establish a new homeostatic redox balance responsible for the activation of proliferative signaling pathways, which could lead these cells to malignant clonal expansion and contribute to the proliferation potential reported previously [Cortesão *et al.,* 2008; Parker *et al.,* 2000; Parker & Mufti, 2001]. The highest oxidant status observed in precursors cells, such as blasts and erythroid precursors, might be related with the increased oxidative DNA damage in MDS $CD34^+$ cells, as described by Peddie *et al.* [1997]. Moreover, this oxidative state is possible associated with the significant increase of oxidative DNA damage in bone marrow cells from RA and RARS patients, as reported by Novotna *et al.* [2009]. The 8-OHdG is a marker of oxidative DNA damage and was found to be increased in MDS patients [Honda *et al.,* 2000; Jankowska *et al.,* 2008]. This fact could be a consequence of increased ROS production or decreased antioxidant defenses, namely GSH, in MDS patients. Persistent ROS together with a decrease in the antioxidant defenses and DNA repair systems could contribute to oxidative DNA damage and, consequently, to the accumulation of mutations and/or chromosomal

abnormalities [Sedelnikova *et al*., 2011], genetic changes observed in MDS patients. Furthermore, changes in superoxide/peroxides ratio observed in precursor cells from MDS patients (blasts and erythroid precursors), relatively to controls, could shift their fate between cell proliferation and apoptosis. As suggested by Pervaiz and Clement [2007], when superoxide/peroxides ratio favor peroxides, an apoptotic permissive intracellular environment is established facilitating apoptosis promotion and execution. On the other hand, in cells where superoxide is predominant, survival pathways are normally activated by direct or indirect mechanisms, such as PI3K-AKT or H^+ efflux pumps activation [Pervaiz & Clement, 2007]. Precursor cells from RAEB-1, RAEB-2, and int-2-risk patients had an increased superoxide/peroxides ratio, which may explain their cellular proliferative potential. Moreover, the positive correlation noted between superoxide/peroxides ratio in blasts and the percentage of blasts in bone marrow might reflect the proliferative advantage of these cells.

Mitochondria are the intracellular organelles responsible for energy formation, and may influence differentiation, proliferation, and apoptosis not only through ROS generation, but also by responding to changes in ROS-induced redox status [Honda *et al*., 2000; Orrenius *et al.,* 2007]. Cancer cells generally have increased $\Delta\psi_{mit}$ due to high glycolytic rates, compared with their normal counterparts. Moreover, transformed cells display this same characteristic [Gough *et al.,* 2009]. The increased glycolysis makes cancer cells more susceptible to apoptotic induction in the presence of apoptosis inductors, such as ROS. Since cell fate can be regulated by the superoxide/peroxide ratio, the changes in $\Delta\psi_{mit}$ could lead to survival signaling or trigger apoptosis [Greenberg *et al*., 2002; Kang & Pervaiz, 2012; Pervaiz & Clement, 2007]. In the present study, we report that erythroid precursors from MDS patients had a decrease in $\Delta\psi_{mit}$; however, when we stratified the patient sample, we noted an increase in $\Delta\psi_{mit}$ in all cell populations from RAEB-1 and -2, as well as from int-2-risk patients. Moreover, it was observed that blasts from RCMD patients had the lower $\Delta\psi_{mit}$ simultaneously with a low superoxide/peroxides ratio, which may also contribute to the peripheral cytopenias observed in this MDS subtype. The decrease in $\Delta\psi_{mit}$ observed in MDS

patients may be associated with the increase mitochondrial ROS production, since it was observed that patients with low $\Delta\psi_{mit}$ had high peroxides levels. Mitochondrial defects, namely mutations in mitochondrial DNA (mtDNA) and impairment mitochondrial gene transcription, were described in MDS patients by several authors [Fontenay *et al*., 2006; Greenberg *et al*., 2002; Schildgen *et al*., 2011; Wulfert *et al*., 2008]. The main differences between mtDNA and genomic DNA are the maternal inheritance, absence of introns and histones, and limited efficacy of repair of mtDNA. The proximity to the inner mitochondrial membrane, site of ROS formation, together with limited protection and repair, are responsible for the increased mutation rate in mtDNA, 10- to 20-fold higher than genomic DNA [Fontenay *et al*., 2006; Greenberg *et al.*, 2002]. Furthermore, these mutations change mitochondrial respiratory chain, that in turn decreases the $\Delta\psi_{mit}$ and activate apoptosis [Greenberg *et al*., 2002; Kang & Pervaiz, 2012]. The bone marrow erythroblasts from sideroblastic anemia patients showed a decrease in $\Delta\psi_{mit}$ [Fontenay *et al.,* 2006; Kang & Pervaiz, 2012], in accordance with our observation on bone marrow cells from RA and RCMD patients. The reported decrease in $\Delta\psi_{mit}$ can be a cause and a consequence of increased ROS production, since mitochondria play a major role as regulator and effector of apoptosis. In fact, high ROS production could be the most significant mechanism contributing to ineffective hematopoiesis in these MDS subtypes. On the other hand, the increase of $\Delta\psi_{mit}$ detected in RAEB-1 and -2, as well as in int-2-risk patients, may reflect their proliferative advantage. This evidence could be correlated with malignant evolution and, consequently, to a higher risk of leukemia progression.

In the present work, transfusion dependency was negatively correlated with GSH content, and intracellular peroxides levels were positively correlated with serum ferritin. In order to ameliorate the anemia, maintain quality of life, as well as prevent anemia-related morbidity and mortality, many patients repeated erythrocyte transfusion that can eventually result in iron overload [Adès *et al.*, 2014; Fenaux & Rose, 2009]. Transferrin is the protein responsible for the transport of iron trough the body. The transfusion dependency leads to transferrin saturation and, consequently, to excess of non-transferrin-bound iron (NTBI)

[Fenaux & Rose, 2009]. In the presence of peroxide and superoxide, NTBI may participate in Fenton and Harber-Weiss reactions and generate the highly reactive hydroxyl radical [Ghaffari, 2008; Ghoti *et al.,* 2007; Ghoti *et al.,* 2010; Invernizzi, 2010; Saigo *et al.,* 2011; Valko *et al.,* 2007], increasing the pro-oxidant status. The decrease in GSH levels observed in transfusion dependent patients could be associated with the increased in ROS production induced by free iron and suggest that patients with low GSH levels in erythroid precursors, granulocytes, and lymphocytes at diagnosis will probably be blood transfusion-dependents. However, GSH levels could not be correlated with blood transfusion volume, since same patients were treated with erythropoietin or azacytidine, drugs that could influence blood transfusion volume. GSH interacts directly with ROS, eliminating it, and operate as a cofactor for various antioxidant enzymes, namely glutathione peroxidase. As a consequence of these reactions, GSH is converted into oxidized glutathione (GSSG), and the excess of GSSG formation can contribute to mitochondrial dysfunction [Ribas *et al*., 2014]. Since GSH is converted in GSSG during ROS elimination, it might be responsible for the negative correlation observed between intracellular peroxides and GSH levels in erythroid precursors from MDS patients. Moreover, the relation between serum ferritin and ROS levels in MDS patients were also reported in other studies [Ghoti *et al.,* 2007; Ghoti *et al.,* 2010; Saigo *et al*., 2011]. The authors of these studies suggest that iron overload may be, at least in part, responsible for the increased apoptosis and ineffective hematopoiesis observed in low risk MDS patients. This hypothesis is based on the fact that treatment with iron chelators, like deferrioxamine and deferiprone, induces a reduction in transfusion requirement, as well as increase platelet and polymorphonuclear cells [Ghoti *et al.,* 2007; Ghoti *et al.,* 2010].

The possible role of oxidative stress and $\Delta\psi_{mit}$ as diagnostic biomarkers and survival predictors for MDS were also investigated. We found that GSH levels provide the most accurate and reliable indicator of MDS diagnosis, principally the GSH levels in blast cells. The association of oxidative stress levels with MDS diagnosis had not been done so far. The GSH accuracy as a diagnostic biomarker is one more indication that oxidative stress could be a major event in MDS

pathogenesis, despite our limited sampling. Moreover, oxidative stress also influences MDS prognosis. We observed that patients with high intracellular peroxides and superoxide levels in erythroid precursors or granulocytes had a shorter overall survival. The same pattern was detected in patients with low GSH levels, as well as in patients with high superoxide/peroxides ratio. Many prognostic factors have been identified in MDS patients. The identification of a few number of features with independent prognostic value, routinely available in all centers, have been assembled in the IPSS [Adès *et al.*, 2014]. However, additional prognostic systems have been suggested. These score systems provided other parameters that display meaningful differences in clinical outcomes. In this context, new prognostic systems are arising, namely WHO Prognostic Scoring System and Revised IPSS [Adès *et al.*, 2014; Greenberg, 2013; Tefferi & Vardiman, 2009]. Other MDS prognostic factors were found in several studies, such as age, bone marrow fibrosis, *TP53, RUNX1* or *ASXL1* mutations, as well as high serum ferritin and lactate dehydrogenase; however, these factors are not included in any prognostic systems [Adès *et al.*, 2014; Greenberg, 2013]. Marrow cells features evaluated by flow cytometry have also shown diagnostic and prognostic value [Adès *et al.*, 2014; Greenberg, 2013]. The present findings indicate that the evaluation of oxidative stress levels could increase the discriminative power of prognostic scoring systems to detect high risk features, and could be a prognostic tool to refine the current score systems.

The number of patients enrolled in the present study unable us to analyze oxidative stress parameters and $\Delta\psi_{mit}$ in all MDS subtypes; none RARS or MDS associated with isolated del(5q) patients as well as none high risk patient was studied. Our sample is composed predominantly of RCMD and low risk patients. However, previous reports already indicated that oxidative stress was more common event in low risk patients. Furthermore, control samples were obtained from patients undergoing scheduled bone marrow aspirates to exclude hematologic disease; although these individuals do not have any known oxidative stress-related disease. In this context, prospective trails and multicenter studies enrolling a significant number of patients will be needed to confirm our results.

Here we suggest the involvement of oxidative stress and mitochondrial dysfunction in MDS development and prognosis. Oxidative stress was present in bone marrow cells from MDS patients, and was dependent on subtype. Moreover, intracellular peroxides, superoxide, GSH levels, and superoxide/peroxides ratio may constitute novel biomarkers with diagnosis and/or prognosis value for these diseases. The present study contributes to a better understanding of molecular basis of MDS, a multifactorial and heterogeneous disease.

Chapter 4

Oxidative stress levels are correlated with P15 and P16 gene promoter methylation in myelodysplastic syndrome patients

Gonçalves, A.C., Cortesão, E., Oliveiros, B., Alves, V., Espadana, A.I., Rito, L., Magalhães, E., Pereira, S., Pereira, A., Nascimento Costa, J.M., Mota-Vieira, L. & Sarmento-Ribeiro, A.B. (2015) *Clin Exp Med* [Epub ahead of print, 2015 May 17]

4.1. Abstract

Oxidative stress and abnormal DNA methylation have been implicated in some types of cancer, namely in myelodysplastic syndromes (MDS). Since both mechanisms are observed in MDS patients, we analyzed the correlation of intracellular levels of peroxides, superoxide anion, and GSH, as well as ratios of peroxides/GSH and superoxide/GSH with the methylation status of *P15* and *P16* gene promoters in bone marrow leukocytes from MDS patients. Comparatively to controls, these patients had lower GSH content, higher peroxides levels, peroxides/GSH and superoxide/GSH ratios, as well as higher methylation frequency of *P15* and *P16* gene promoters. Moreover, patients with methylated *P15* gene had higher oxidative stress levels than patients without methylation (peroxides: 460±42 MIF *vs.* 229±25 MIF, p=0.001; superoxide: 383±48 MIF *vs.* 243±17 MIF, p=0.022; peroxides/GSH: 2.50±0.08 *vs.* 1.04±0.34, p<0.001; superoxide/GSH: 1.76±0.21 *vs.* 1.31±0.10, p=0.007). Patients with methylated *P16* and at least one methylated gene had higher peroxide levels as well as peroxides/GSH ratio than patients without methylation. Interestingly, oxidative stress levels allow the discrimination of patients without methylation from ones with methylated *P15*, methylated *P16*, or at least one methylated (*P15* or *P16*) gene promoter. Taken together, these findings support the hypothesis that oxidative stress is correlated with *P15* and *P16* hypermethylation.

4.2. Introdution

Reactive oxygen species (ROS) are a group of small, diffusible radical and non-radical molecules with one or more unpaired electrons [Farquhar & Bowen, 2003; Hole *et al*., 2011; Jones, 2008; Klaunig *et al*., 2010; Valko *et al.,* 2007]. These molecules might be originated not only from exogenous sources, such as ionizing radiation, ozone exposure, and hyperoxia, but also from endogenous ones, like mitochondria, NADPH oxidase enzymes and, cytochrome P450 [Birben *et al*., 2012; Hole *et al*., 2011; Klaunig *et al.,* 2010]. The superoxide anion ($O_2^{\bullet -}$) is considered the primary ROS, since it interact with other molecules inducing the formation of different free radicals, namely hydrogen peroxide (H_2O_2), which can be converted into hydroxyl radical ($^{\bullet}OH$) or hypochlorous acid [Ghaffari, 2008; Hole *et al*., 2011; Valko *et al.,* 2007]. Under normal physiological conditions, cells defend itself from ROS through enzymatic, as well as non-enzymatic defense mechanisms. The antioxidant molecules include cellular enzymatic defenses, for instance superoxide dismutase, glutathione peroxidase, and catalase; and non-enzymatic defenses, such as reduced glutathione (GSH), α-tocopherol, and vitamin C [Birben *et al*., 2012; Jones, 2008; Valko *et al*., 2007]. However, in pathological conditions, particularly in cancer, increased ROS production may overwhelm the cellular antioxidant defenses, leading to an oxidative stress state [Birben *et al*., 2012; Farquhar & Bowen, 2003; Hole *et al*., 2011; Jones, 2008; Klaunig *et al*., 2010; Valko *et al*., 2007]. Excess of ROS induces several DNA modifications, for example single- or double-stranded breaks, mutations, and cross-linking with proteins [Birben *et al.,* 2012; Klaunig *et al.,* 2010]. DNA modifications that result from persistent oxidative stress represent the first step of carcinogenesis. In addition to DNA lesions, free radicals can also cause oxidative damage to lipids, proteins, and non-coding RNAs [Klaunig *et al.,* 2010]. Moreover, ROS play an important role in intracellular signaling by changing the expression of growth factors and/or proto-oncogenes. These changes will lead to deregulation of cell proliferation and apoptosis [Klaunig *et al*., 2010; Klaunig & Kamendulis, 2004; Valko *et al*., 2007].

Epigenetic is a mechanism of gene expression regulation that does not alter gene sequence [Das & Singal, 2004; Taby & Issa, 2010]. The most commonly occurring

epigenetic event is DNA methylation at cytosines that precede guanine in the DNA sequence, the CpG dinucleotides. The addiction of a methyl group at the carbon 5 position of the cytosine ring is catalyzed by DNA methyltransferases (DNMTs) [Das & Singal, 2004; Galm *et al*., 2006; Taby & Issa, 2010]. Due to the high mutagenic potential of 5-methylcytosine, CpG dinucleotides are irregularly distributed along human genome [Galm *et al.,* 2006]. There are CpG-rich regions – the CpG islands – in the regulatory region of many genes, namely tumor suppressor genes. In general, CpG islands are normally unmethylated, but repetitive genomic sequences and introns are hypermethylated [Esteller, 2008; Galm *et al.,* 2006]. Contrarily, the genome of cancer cells is characterized by global hypomethylation and localized hypermethylation. *CDKN2B (P15)* and *CDKN2A (P16)* are examples of localized hypermethylation that had been described in hematological neoplasms [Esteller, 2008; Galm *et al*., 2006; Karlic *et al.,* 2014; Taby & Issa, 2010].

Oxidative DNA damage can modify DNA methylation patterns by several mechanisms. The 8-hydroxydeoxyguanosine (8-OHdG), the most frequent oxidative DNA lesion, is able to decrease the capacity of DNMTs to interact with DNA, leading to hypomethylation and, consequently, to genomic instability [Das & Singal, 2004; Donkena *et al*., 2010; Klaunig & Kamendulis, 2004; Ziech *et al*., 2011]. Additionally, 5-methylcytosine is susceptible to oxidation, generating 5-hydroxymethylcytosine that interferes with the subsequent epigenetic steps. Moreover, ROS may contribute to gene silencing by hypermethylation of tumor suppressor genes [Donkena *et al*., 2010; Ziech *et al*., 2011]. In this context, oxidative stress could be a key contributor to carcinogenesis, since it alters global and gene specific-methylation patterns [Cerda & Weitzman, 1997; Donkena *et al*., 2010; Franco *et al*., 2008; Ziech *et al.,* 2011].

Myelodysplastic syndromes (MDS) are a group of diseases characterized by inefficient hematopoiesis associated with an increased propensity to acute myeloid leukemia transformation [Adès *et al.,* 2014; Farquhar & Bowen, 2003; Karlic *et al.,* 2014; Shih & Levine, 2011]. MDS patients have normal or hypercellular bone marrow with morphological dysplastic cells and peripheral cytopenias [Karlic *et al.,* 2014]. The pathogenesis of MDS is not well understood. The molecular

mechanisms contributing to the pathogenesis of these diseases include, among others: 1. mutations in genes that encode transcription factors, such as *RUNX1*, as well as proteins involved in cell proliferation pathways, like *NRAS* or *JAK2* [Adès *et al.,* 2014; Shih & Levine, 2011]; 2. epigenetic modifications, including hypermethylation of *P15* and *P16* genes [Adès *et al*., 2014; Karlic *et al.,* 2014; Shih & Levine, 2011]; 3. oxidative stress, namely increased ROS levels and oxidative DNA damage [Farquhar & Bowen, 2003; Ghoti *et al.,* 2007; Novotna *et al.,* 2009].

Oxidative stress and abnormal DNA methylation are observed in MDS patients. Therefore, we hypothesize that oxidative stress may contribute to MDS development and progression, which may be correlated with DNA methylation of tumor suppressor genes. In the present work, we analyzed the levels of intracellular peroxides, superoxide anion, and GSH, the ratios of peroxides/GSH and superoxide/GSH, as well as the methylation pattern of *P15* and *P16* gene promoters in MDS patients, in order to investigate the potential relation between these two tumorigenic mechanisms.

4.3. Methods

4.3.1. Ethical statement

The present study was conducted in accordance with the Helsinki declaration. The Ethics Committee of Faculty of Medicine of University of Coimbra (Coimbra, Portugal) approved all research procedures. All participants provided their informed consent for participation prior to enrollment.

4.3.2. Study population

We enrolled 27 patients with de novo MDS at diagnosis with a median age of 78 years, ranging from 33 to 85 years, with 51.8% females (*n*=14) and 48.2% males (*n*=13), as well as 12 individuals without neoplastic malignancies (controls), with

six females (50.0%) and six males (50.0%) with a median age of 72 years, ranging from 38 to 89 years. Patients and controls do not have any known oxidative stress-related disease. Demographic and clinical characteristics of MDS patients are shown in Table 12. Patients were grouped according to the World Health Organization classification (2008) [Brunning *et al*., 2008]. Five patients were diagnosed with refractory anemia (18.5%), 12 with refractory cytopenia with multilineage dysplasia (44.4%), four with refractory anemia with excess blasts type 1 (14.8%), and six with refractory anemia with excess blasts type 2 (22.3%). According with the International Prognostic Scoring System (IPSS) [Brunning *et al*., 2008], the prognostic score were low in 10 patients (37.0%), intermediate 1 (int-1) in 12 (44.4%), and intermediate 2 (int-2) in five (18.6%). The serum ferritin at diagnosis presented an average value of 444±93 ng/ml, and three patients (11.1%) had ferritin levels higher than 1000 ng/ml.

4.3.3. Assessment of ROS and GSH in leukocytes

Bone marrow samples were obtained from MDS patients, at time of diagnosis and prior to any treatment, and from controls, by aspiration. Initially, bone marrow samples were stained with PerCP-Cy5.5 conjugate anti-CD45 monoclonal antibodies (BD Biosystems, San Diego, CA, USA) for 15 min at room temperature (RT), in the dark. Then, cells were incubated with FACS lysing solution (BD Biosystems) for 15 min (RT in the dark), washed twice with phosphate buffered saline (PBS), by centrifugation at 300g for 5 min. Intracellular peroxides and superoxide anion levels were measured using 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Molecular Probes, Life Technologies Corporation, Carlsbad, CA, USA) and dihydroethidium (DHE; Molecular Probes) dies, respectively, as described by others [Almeida *et al*., 2008; Zielonka *et al.,* 2008]. Briefly, 1×10^6 previously stained cells were incubated with 5 μM of $DCFH_2$-DA for 45 min at 37°C in a humidified atmosphere of 5% CO_2 or with 5 μM of DHE for 15 min at RT in the dark. Next, cells were washed twice with cold PBS by centrifugation at 300g for 5 min, resuspended in the same buffer, and kept on ice for an immediate detection.

The GSH content was measured using mercury orange (MO) dye (Sigma-Aldrich, Sintra, Portugal), by incubating 1×10^6 previously stained cells with 40 μM of MO for 15 min at RT, in the dark [O'Connor *et al*., 1988]. Cells were then washed twice with cold PBS by centrifugation at 300g for 5 min, resuspended in the same buffer, and kept on ice for an immediate detection by flow cytometry. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosystems), and at least 50 000 events were collected using CellQuest software (BD Biosystems). Results were analyzed using Paint-a-Gate software (BD Biosystems).

4.3.4. Methylation pattern of P15 and P16 tumor suppressor genes

DNA from bone marrow samples was extracted according to standard procedures. One μg of genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Methylation specific PCRs of *P15* and *P16* gene promoters were carried out as previously described [Yeh *et al*., 2003]. PCR products were resolved on 4% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

4.3.5. Statistical analysis

Statistical analysis was performed using SPSS version 21.0, and graphics were generated using GraphPad Prism version 5.0. Data are expressed as mean ± standard error of mean (SEM), unless otherwise specified. Normality was assessed by Kolmogorov Smirnov analysis. Student's t-test and ANOVA with Bonferroni Post Hoc test were used to assess the statistical significance of the difference between means of two groups (patients vs controls; patients with methylation vs patients without methylation) and more than two groups (IPSS risk groups: low *vs.* int-1 *vs.* int-2), respectively. The methylation frequency between groups (patients vs controls, IPSS risk groups) was compared using chi-square test. The receiver operating characteristic (ROC) curves analysis was performed to

analyze variables accuracy as methylation predictor. All statistical analyses were two sided, and a $p<0.05$ was considered statistically significant.

Table 12
Demographic and clinical characteristics of MDS patients

Demographic data			Clinical features							
Patient (No)	Age (years)	Sex (M/F)	MDS subtypes (WHO)	Hb (g/L)	ANC (10^9/L)	PTLs (10^9/L)	Blasts (%)[a]	Cytogenetics (FISH)	Risk groups (IPSS)	Ferritin (ng/ml)
1	68	F	RA	9.3	3.0	225	1	N	Low	225
2	82	M	RA	5.9	4.3	277	1	N	Low	221
3	61	M	RA	9.4	2.6	276	0	N	Low	195
4	84	F	RA	8.3	4.3	342	1	N	Low	476
5	85	F	RA	9.2	2.1	160	0	+8	Int-1	257
6	72	M	RCMD	7.4	1.9	12	1	N	Int-1	1809
7	72	M	RCMD	15.5	2.7	89	1	N	Low	183
8	82	M	RCMD	10.5	0.4	39	2	+8	Int-1	224
9	80	F	RCMD	12.7	0.4	140	2	Del(20q)	Low	177
10	56	M	RCMD	5.6	2.4	253	4	N	Low	497
11	81	F	RCMD	7.9	0.8	118	1	N	Int-1	128
12	78	F	RCMD	5.8	2.1	233	2	N	Low	168
13	65	F	RCMD	9.4	1.4	13	2	N	Int-1	357
14	33	F	RCMD	12.3	4.0	29	4	N	Low	141
15	81	F	RCMD	9.1	2.8	215	1	N	Low	82
16	81	M	RCMD	6.0	1.9	324	1	+8	Int-1	1750
17	79	F	RCMD	13.1	0.9	107	6	Del(7q)	Int-1	398
18	81	M	RAEB-1	10.4	1.3	170	6	N	Int-1	451
19	74	M	RAEB-1	6.4	1.2	39	5	N	Int-1	444
20	73	F	RAEB-1	9.7	0.9	76	9	N	Int-2	808
21	84	F	RAEB-1	7.9	2.4	367	5	Del(20q)	Int-1	134
22	84	M	RAEB-2	7.6	2.4	26	18	N	Int-2	358
23	76	M	RAEB-2	5.7	1.0	61	10	N	Int-1	83
24	78	F	RAEB-2	4.6	0.8	65	12	N	Int-2	1525
25	71	M	RAEB-2	5.4	2.7	19	16	N	Int-2	538
26	77	M	RAEB-2	9.8	5.1	49	11	N	Int-2	203
27	77	F	RAEB-2	9.0	0.1	29	10	N	Int-1	157

[a], bone marrow; No, sample number; M, male; F, female; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, RA with excess of blasts type 1; RAEB-2, RA with excess of blasts type 2; Hb, hemoglobin; ANC, absolute neutrophil count; PTLs, platelets; IPSS, International Prognostic Scoring System; Low, low-risk; Int-1, intermediate-risk-1; Int-2, intermediate-risk-2; N, normal; +8, trisomy 8; Del(7q), long arm deletion of chromosome 7; Del(20q), long arm deletion of chromosome 20.

4.4. Results

4.4.1. MDS patients have increased ROS levels

In order to verify if MDS cells were under oxidative stress, we initially investigated ROS levels in leukocytes (CD45 positive cells) from bone marrow patient and control samples. As shown in Figure 30A, intracellular peroxides levels were 1.5-fold higher in MDS patients than in controls (p=0.002). However, intracellular superoxide anion levels were similar between MDS patients [300±26 mean intensity of fluorescence (MIF)] and controls (274±8 MIF). The intracellular levels of GSH were significantly decreased in MDS patients (0.4-fold) comparatively with controls. Moreover, intracellular peroxides levels were significantly higher in low-risk patients (1.2-fold relatively to int-1- and 2.3-fold comparatively to int-2-risk patients), and inversely correlated with IPSS risk (Figure 30B). Oxidative stress levels were measured by determining the ratios of peroxides/GSH and superoxide/GSH. As observed in Figure 30, peroxides/GSH and superoxide/GSH ratios were, respectively, 3.8- and 2.4-fold higher in MDS patients than in controls (p<0.001). Furthermore, peroxides/GSH ratio was inversely correlated with IPSS risk, being higher in low-risk patients relatively to int-1- and int-2-risk patients [1.3-fold comparatively to int-1-risk patients (p=0.104) and 2.1-fold comparatively with int-2-risk patients (p=0.037)].

4.4.2. P15 and P16 promoters are frequently methylated in MDS patients

To assess the methylation status of *P15* and *P16* gene promoters, methylation specific PCRs were performed. We observed that MDS patients had a *P15* methylation frequency of 40.7% (11/27) and a *P16* methylation frequency of 33.3% (9/27) (Figure 31A). Moreover, 55.6% (15/27) of these patients had at least one methylated gene (*P15* or *P16*), and 18.5% (5/27) had both methylated genes. In control group, we only detected one individual with methylated P15 (8.3%; 1/12). The methylation frequency was higher in low-risk patients relatively with int-1 or int-2 ones (Figure 31B).

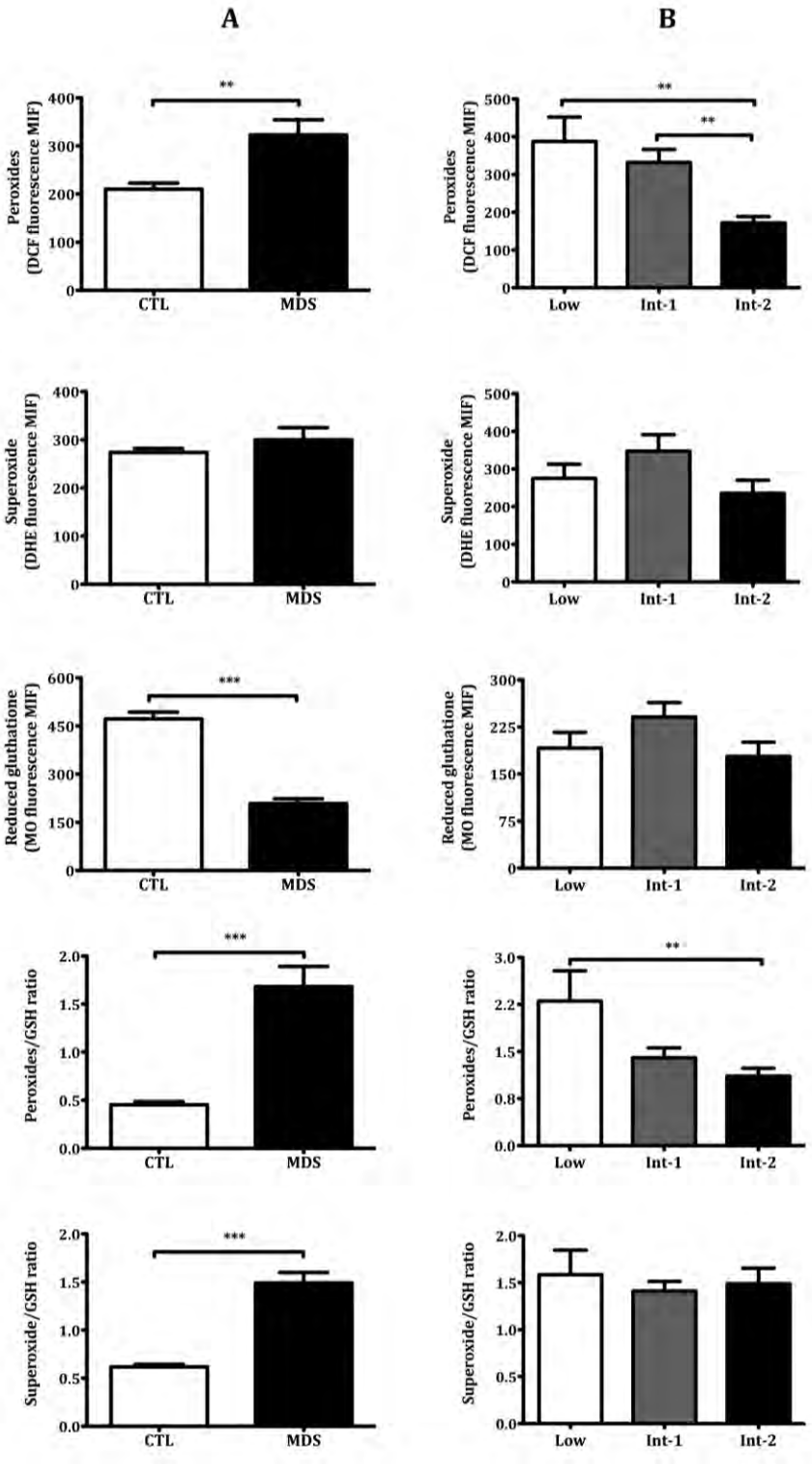


Figure 30. Analysis of oxidative stress parameters. Intracellular peroxides, superoxide anion, and GSH levels, as well as peroxides/GSH and superoxide/GSH ratios were compared between MDS patients and controls (A), and between MDS IPSS risk groups (B). The results are represented as mean ± SEM. MDS, myelodysplastic syndrome; CTL, control; low, low-risk; Int-1, intermediate-1-risk; Int-2, intermediate-2-risk; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

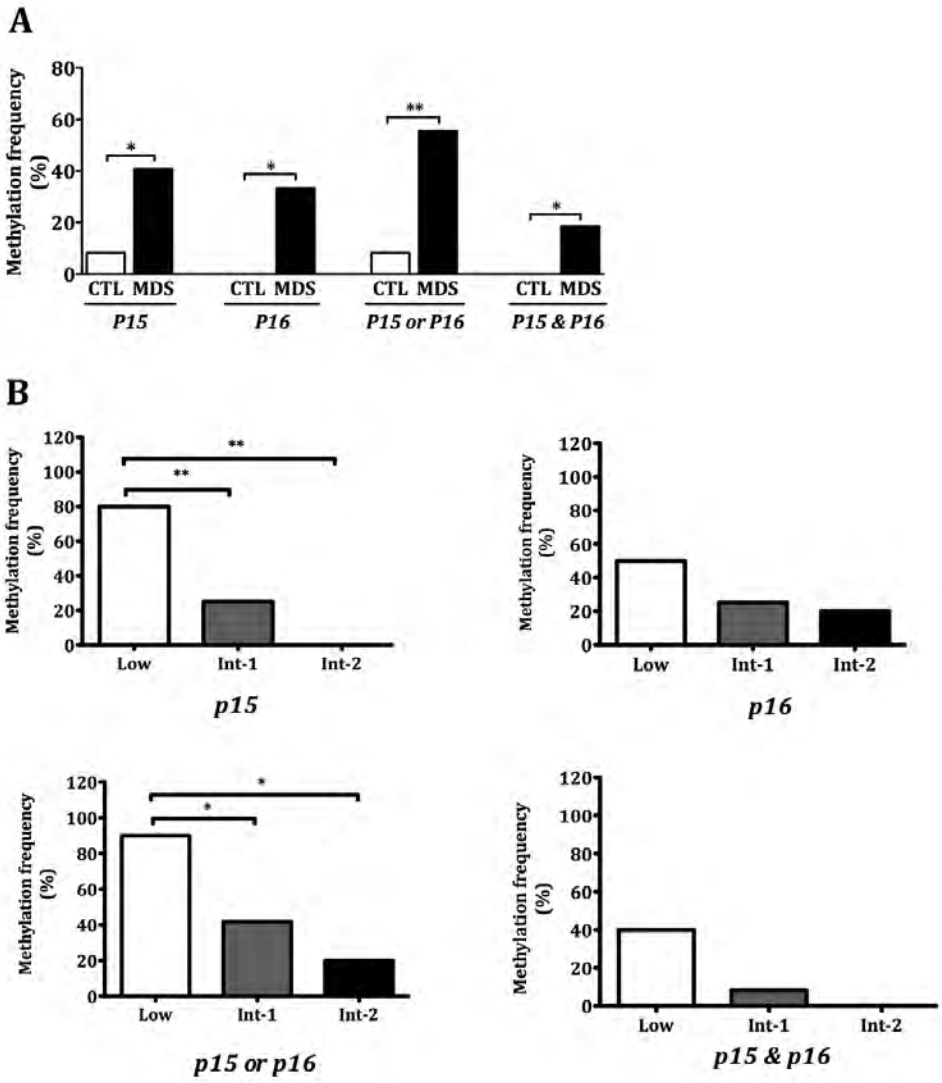


Figure 31. *P15* and *P16* genes promoter methylation status in MDS patients and controls. Methylation frequency of *P15, P16,* at least one gene (*P15* or *P16*), and both genes (*P15* & *P16)* were compared between MDS patients and controls (A), and between MDS IPSS risk groups (B). CTL, control; MDS, myelodysplastic syndrome; low, low-risk; Int-1, intermediate-1-risk; Int-2, intermediate-2-risk; *, $p<0.05$; **, $p<0.01$.

Furthermore, *P15* gene promoter were methylated in 80.0% (8/10) of low-risk patients and in 25.0% (3/12) of int-1-risk ones ($p=0.004$), while *P15* methylation were not observed in int-2-risk patients. Moreover, 90.0% (9/10) of low-risk patients, 41.7% (5/12) of int-1, and 20.0% (1/5) of int-2 had methylated *P15* or *P16* ($p=0.016$).

4.4.3. Oxidative stress is correlated with P15 and P16 gene promoter methylation in MDS patients

In order to correlate the methylation status with oxidative stress parameters, we analyzed the intracellular peroxides, superoxide anion and GSH levels, as well as peroxides/GSH and superoxide/GSH ratios in MDS patients, according to methylation profile. These parameters were not correlated in controls, since we only observed one control with methylated *P15.*

We found that patients with methylated genes had highest intracellular ROS levels, as well as peroxides/GSH and superoxide/GSH ratios than patients without methylation (Figure 32). In patients with methylated *P15*, intracellular peroxide levels were 2.0-fold higher (methylated: 460±42 MIF, unmethylated: 229±25 MIF; $p<0.001$), intracellular superoxide anion was 1.6-fold higher (methylated: 383±48 MIF, unmethylated: 243±17 MIF; $p=0.022$), peroxides/GSH ratio was 2.5-fold higher (methylated: 2.61±0.34, unmethylated: 1.04±0,08; $p<0.001$), and superoxide/GSH ratio was 1.3-fold higher (methylated: 1.76±0.21, unmethylated: 1.31±0.10; $p=0.007$), comparatively with patients without methylation. Moreover, patients with methylated *P16* had significant highest intracellular peroxides levels and peroxides/GSH ratio than those without methylation [intracellular peroxides: 460±42 MIF (methylated) *vs*. 229±25 MIF (unmethylated), $p=0.003$; peroxides/GSH ratio: 2.55±0.48 (methylated) *vs*. 1.25±0.12 (unmethylated), $p=0.005$]. We also observed that intracellular peroxides levels and peroxides/GSH ratio were, respectively, 1.8- and 2.3-fold higher in patients with at least one methylated gene [intracellular peroxides: 405±42 MIF (methylated) *vs*. 221±27 MIF (unmethylated), $p=0.001$; peroxides/GSH ratio: 2.24±0.31 (methylated) *vs*. 0.99±0.08 (unmethylated), $p < 0.001$], independently of the gene, and in patients with both methylated genes (methylated: 516±88 MIF, unmethylated: 279±26; $p=0.020$), compared with patients without methylation. Although patients with gene promoters methylated had higher intracellular superoxide anion levels, compared with unmethylated ones, the differences were only statistically significant for methylated *P15* ($p=0.022$).

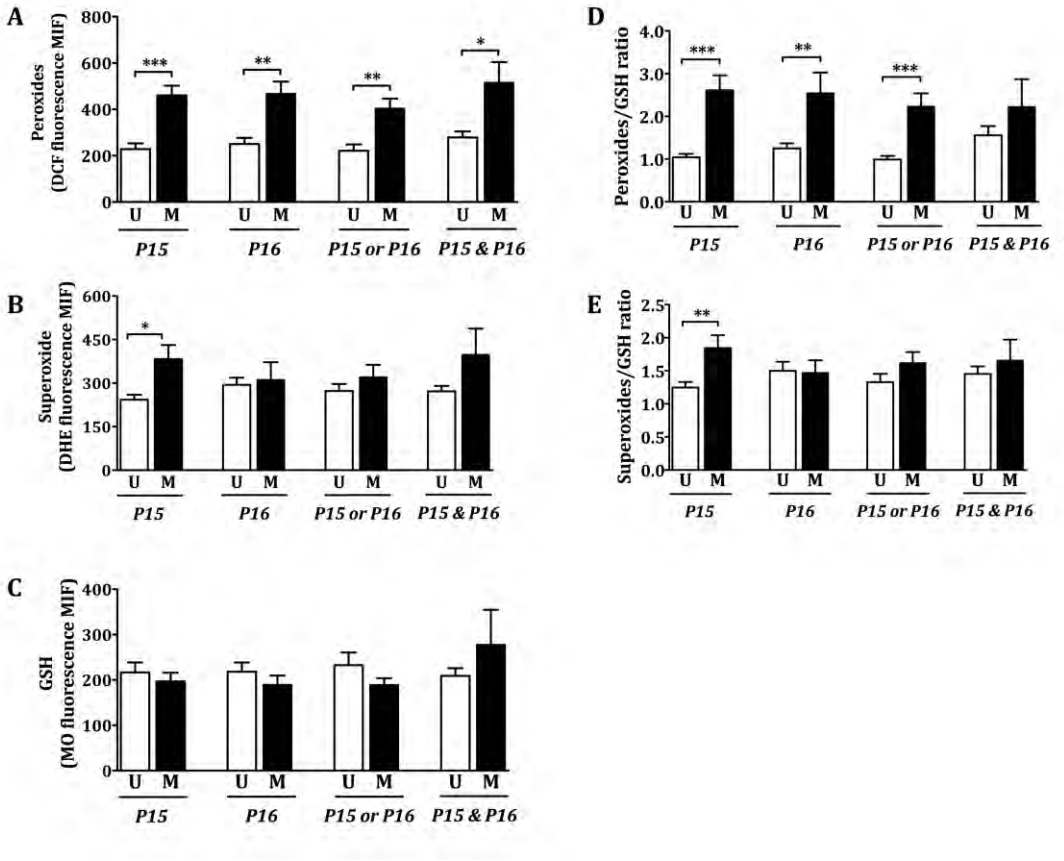


Figure 32. Analysis of oxidative stress parameters in MDS patients, according to *P15* and *P16* methylation status. Patients were stratified, according to methylation status, in *P15, P16*, at least one gene (*P15* or *P16*), and both genes (*P15* & *P16*). The results are represented as mean ± SEM. U, unmethylated; M, methylated; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

To explore the possible predictive value of oxidative stress parameters in gene methylation status, we performed ROC curves analysis and summarize significant results in Table 13. According to this analysis, intracellular peroxides levels as well as peroxides/GSH and superoxide/GSH ratios were able to discriminate patients without methylation from ones with methylated *P15*, being the peroxides/GSH ratio the more accurate parameter for methylation assessment with an area under the curve (AUC) of 0.994 (95% CI 0.976–1.000; $p<0.001$). Peroxides/GSH ratio higher than 1.631 was defined as the optimal cut off value for differentiation of MDS patients with methylation from those without. This cut-off presented high sensitivity (100%) and specificity (94%), as well as a strong power to rule in

[positive predictive value (PPV): 92%] and to rule out [negative predictive value (NPV): 100%].

Table 13

Significant performance of oxidative stress biomarkers to discriminate *P15* and *P16* promoter methylation status in myelodysplastic syndrome patients

Gene Biomarkers	AUC value (95% CI)	*p*-value	Cut-off value	SEN (%)	SPE (%)	PPV	NPV
P15							
Peroxides (MIF)	0.756 (0.543 – 0.968)	0.026	404	65	94	100	80
Peroxides/GSH	0.994 (0.976 – 1.000)	< 0.001	1.631	100	94	92	100
Superoxide/GSH	0.813 (0.647 – 0.977)	0.007	1.459	73	75	67	80
P16							
Peroxides/GSH	0.840 (0.683 – 0.996)	0.005	1.631	78	72	63	87
P15* or *P16							
Peroxides/GSH	0.861 (0.715 – 1.000)	0.002	1.631	80	83	100	80

GSH, reduced glutathione; AUC, area under the curve; CI, confidence interval; SEN, sensitivity; SPE, specificity; MIF, mean intensity of fluorescence; PPV, positive predictive value; NPV, negative predictive value.

Superoxide/GSH ratio (AUC=0.813; 95% CI 0.647–0.977; p=0.007) and intracellular peroxides levels (AUC=0.756; 95% CI 0.543–0.968; p=0.026) were also good biomarkers of *P15* methylation. The best superoxide/GSH ratio and intracellular peroxides cut-off values were respectively 1.459 (sensitivity: 73%; specificity: 75%; PPV: 67%; NPV: 80%) and 404 MIF (sensitivity: 65%; specificity: 94%; PPV: 100%; NPV: 80%). Moreover, peroxides/GSH ratio higher than 1.631 was also able to discriminate *P16* methylation (AUC=0.840; 95% CI 0.683–0.996; p=0.005) and at least one methylated gene (AUC=0.861; 95% CI 0.715–1.000; p=0.002). This cut-off achieved good sensitivity (78% and 80%, respectively, for *P16* methylation and *P15* or *P16* methylation), specificity (72% and 83%), and predictive values (PPV: 63% and 100%; NPV: 87% and 80%). Intracellular superoxide anion and GSH levels were unable to discriminate any methylation pattern (data not shown).

4.5. Discussion

The present work demonstrated a correlation of *P15* and *P16* gene promoters hypermethylation with intracellular levels of ROS, as well as with ratios of peroxides/GSH and superoxide/GSH in MDS patients. Moreover, *P15* hypermethylation was associated with the increased intracellular superoxide anion levels. To our knowledge, this is the first report that associates, *in vivo*, intracellular ROS levels, namely peroxides and superoxide anion levels, as well as peroxides/GSH and superoxide/GSH ratios with methylation status of tumor suppressor genes, such as the cell cycle inhibitors *P15* and *P16*. Only two reports correlate *in vivo* oxidative stress with methylation pattern. One of these studies [Nishida *et al*., 2013] showed that 8-OHdG, a marker of oxidative DNA damage, were associated with an increased number of methylated tumor suppressor genes in liver samples from chronic hepatitis C; and the other one [Patchsung *et al*., 2012] positively related the decrease in total antioxidant status with hypomethylation of long interspersed nuclear element-1 (LINE-1) in bladder cancer patients and normal individuals. *In vitro*, there are a few studies linking oxidative stress levels with methylation status, but none of them were carried out in hematological neoplasms [Lim *et al*., 2008; Min *et al*., 2010; Quan *et al.,* 2011].

Abnormal DNA methylation and oxidative stress, established either by increased ROS production or decrease antioxidant levels, have been recognized as key players in the carcinogenesis process [Donkena *et al.,* 2010; Klaunig *et al*., 2010; Klaunig & Kamendulis, 2004; Valko *et al.,* 2007; Wongpaiboonwattana *et al.,* 2013; Ziech *et al*., 2011]. Here, we demonstrated that MDS patients had higher *P15* and *P16* methylation frequencies compared with controls. Several authors reported similar results, showing that DNA methylation is a common event in MDS [Claus & Lübbert, 2003; Galm *et al*., 2006; Karlic *et al*., 2014; Solomon *et al.,* 2008]. Moreover, the present investigation indicates that abnormal methylation were more frequent in low-risk patients. This data is not in agreement with the majority of others studies that shown that *P15* and *P16* hypermethylation is more frequently in high-risk patients [Aggerholm *et al.,* 2006; Quesnel *et al.,* 1998; Tien *et al.,* 2011]. The divergence observed between our methylation results and

published ones may be due to sampling. Here, the majority of subjects were low-risk patients. On the other hand, comparatively to controls, we reported that MDS patients have increased levels of peroxides and decreased GSH content as well as increased ratios of peroxides/GSH and superoxide/GSH. Furthermore, intracellular peroxides levels and peroxides/GSH ratio were inversely correlated with IPSS prognostic risk. Ghoti *et al.* [2007] described that red blood cells, platelets, and polymorphonuclear leukocytes of low risk MDS patients were under oxidative stress, while Saigo *et al.* [2011] reported increased serum ROS levels. Furthermore, indirect evidences of oxidative stress in MDS patients, such as oxidative DNA damage, were also observed [Honda *et al*., 2000; Novotna *et al.,* 2009; Peddie *et al.,* 1997]. To the best of our knowledge, the peroxides/GSH and superoxide/GSH ratios were not yet studied in MDS. Together, these data confirmed that MDS cells were under oxidative stress.

Despite the fact that abnormal DNA methylation and oxidative stress are mechanisms underlying MDS pathogenesis, little is known about the relation between them. As referred above, several mechanisms has been proposed to explain how oxidative stress modifies DNA methylation patterns, most of all justifying the hypomethylation effect of oxidative stress. Here, we showed a correlation of oxidative stress parameters with hypermethylation of *P15* and *P16* tumor suppressor genes. Interestingly, intracellular peroxides levels, as well as peroxides/GSH and superoxide/GSH ratios, allowed the discrimination of patients without methylation from ones with methylated *P15*, methylated *P16*, or at least one methylated (*P15* or *P16*) gene promoter. These facts support the idea that methylation status is correlated with oxidative stress.

Other studies had demonstrated that ROS induced methylation of tumor suppressors genes. For example, *E-cadherin, catalase*, and *POU class 2 homeobox 1* (alias *OCT-1*) gene promoters were hypermethylated when exposed to hydrogen peroxide in hepatocellular carcinoma cell lines [Lim *et al*., 2008; Min *et al*., 2010; Quan *et al*., 2011]. Similarly, in bladder cancer cells, peroxide hydrogen treatment induced *RUNX3* promoter hypermethylation [Wongpaiboonwattana *et al.,* 2013]. Furthermore, Soberanes and collaborators [2012] reported that in mice lung

oxidative stress, induced by particulate matter air pollution, results in increased DNMT1 protein expression and *P16* hypermethylation. Our group had exposed acutely and chronically hematological cell lines (normal and neoplastic) to hydrogen peroxide and menadione (a superoxide donor). Under these oxidative stress conditions, we observed an increase in 5-methylcytosine levels, a decrease in LINE-1 methylation, as well as the induction of hypermethylation of *P15*, *P16*, and *KEAP1* gene promoters in a cell type and exposure dependent manner [Gonçalves *et al*., 2015]. However, it is not precisely known how peroxides and superoxide anion levels induce hypermethylation. The hydrogen peroxide can modulate DNA methylation through the formation and relocalization of a silencing complex, composed by DNMT1, DNMT3B, SIRT1, and members of polycomb repressive complex 4, which stimulates cancer specific hypermethylation [O'Hagan *et al.,* 2011]. In agreement with this study, we observed that hematological cell lines chronically exposed to oxidative stress inducers had an increase in *DNMT1* and *DNMT3A* gene expression [Gonçalves *et al*., 2015]. Beside that, according to Afanas'ev [2014], superoxide anion may directly deprotonate cytosine at carbon 5 position allowing the nucleophilic attack of S-adenosylmethionine on this cytosine, and inducing the formation of 5-methylcytosine through a DNMT-independent manner [Afanas'ev, 2014; Rang & Boonstra, 2014]. Another mechanism that could be associated with the hypermethylation induced by ROS is single stranded DNA breaks (SSBs). The formation of SSBs by oxidative stress will signal for *de novo* methylation, contributing to the increase of methylation in tumor suppressor genes [Franco *et al.,* 2008]. Furthermore, the incomplete repair of SSBs by base excision repair enzymes can results in it conversion to double strand break, leading to unprogrammed methylation [Jankowska *et al.,* 2008; Peddie *et al.,* 1997]. In this context, any or even all of these mechanisms could participate in *P15* and *P16* abnormal methylation observed in MDS patients.

Many MDS patients receive erythrocyte transfusion in order to ameliorate anemia, as well as to prevent anemia-related morbidity and mortality, and/or maintain quality of life. Repeated blood transfusions can eventually result in iron overload [Adès *et al.,* 2014; Fenaux & Rose, 2009]; however, some MDS patients develop

iron overload before blood transfusion in part as consequence of ineffective erythropoiesis that increased dietary iron absorption due hepcidin suppression [Bystrom & Rivella, 2015; Kikuchi *et al.,* 2012; Steensma & Gattermann, 2013]. Iron overload occurs when transferrin becomes saturated and/or ferritin, the main intracellular iron storage protein, become deregulated. Under this condition, the concentration of non-transferrin-bound iron (NTBI) increases [Fenaux & Rose, 2009]. In the presence of ROS, such as peroxide and superoxide, NTBI may participates in Fenton and Harber-Weiss reactions and generates the highly reactive hydroxyl radical [Ghoti *et al*., 2007; Ghoti *et al*., 2010; Hole *et al.,* 2011; Valko *et al.,* 2007], increasing the pro-oxidant status. In the present study, three MDS patients presented ferritin levels higher than 1000 ng/ml, which can indicate that these patients had iron overload before erythrocyte transfusion treatments. Moreover, serum ferritin levels were positively correlated with intracellular peroxides and negatively with GSH levels (data not shown), suggesting that ferritin levels may reflect a cellular pro-oxidant status. Some studies demonstrated that treatment with iron chelators (such as deferrioxamine, deferiprone, and deferasirox), as well as with antioxidant molecules [like *N*-acetylcysteine (NAC)] decreased ROS levels and increased GSH content, suggesting that iron chelation therapy may improve hemoglobin levels and reduced transfusion requirements through it antioxidant features [Ghoti *et al*., 2007; Ghoti *et al*., 2010]. On the other hand, it has been demonstrated that NAC inhibited the ROS-induced methylation of *E-cadherin, catalase*, and *POU class 2 homeobox 1* [Lim *et al.,* 2008; Min *et al.,* 2010; Patchsung *et al.,* 2012]. Taken together, these findings raise the possibility that iron-chelation therapy may ameliorate hematological parameters and prolong survival time of MDS patients, by an indirect action in DNA methylation. However, this hypothesis was not yet investigated.

As mentioned above, the hypermethylation of *P15* and *P16* tumor suppresser genes occurs frequently in MDS patients, and these epigenetic abnormalities had been associated with disease progression and transformation to AML [Galm *et al.,* 2006; Karlic *et al.,* 2014; Mufti, 2004]. Here, we found a correlation of oxidative stress with methylation of *P15* and, to a lesser extent, *P16* promoters. In this

context, a treatment that effectively reduces the oxidative stress, namely iron chelators and/or antioxidants, may reverse *P15* and *P16* hypermethylation, contributing, consequently, to reduce disease progression and AML transformation. The present study presented some limitations. A reduced sample size was analyzed that unable MDS subtype analysis. Moreover, *in vitro* and *in vivo* mechanistic studies were needed to understand how oxidative stress influences methylation in these tumor suppressor genes, since we could only speculate the possible mechanisms. In future studies, it will be necessary to evaluate gene promoter methylation profiles and gene expression levels of the two tumor suppressor genes – *P15* and *P16* –, in order to confirm that oxidative stress induces transcriptional silencing by DNA methylation.

In summary, we firstly demonstrated that MDS patients with high intracellular peroxides and superoxide anion levels, as well as those with high peroxides/GSH and superoxide/GSH ratios had increased methylation frequency of *P15* and *P16* gene promoters. Moreover, oxidative stress levels (intracellular peroxides levels, as well as peroxides/GSH and superoxide/GSH ratios) were able to discriminate MDS patients with methylation from those without, being the peroxides/GSH ratio the more accurate methylation biomarker. Together, these finding supports the hypothesis that oxidative stress is correlated with tumor suppressor genes methylation.

Chapter 5

Localized DNA hypermethylation and global DNA hypomethylation are correlated with oxidative stress levels in myeloid neoplasms

Gonçalves, A.C., *Alves, R., Baldeiras, I., Cortesão, E., Carda, J.P., Oliveiros, B., Brás, M., Pereira, A., Nascimento Costa, J.M., Mota-Vieira, L. & Sarmento-Ribeiro, A. B.*

(Under review, Sept 2015)

5.1. Abstract

Oxidative stress and abnormal DNA methylation have been implicated in some types of cancer, such as myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN). This fact leads us to investigate whether oxidative stress parameters were correlated with localized and global DNA methylation in peripheral blood of patients with MDS and MPN. Localized DNA methylation was assessed by the methylation status of *P15*, *P16*, *TP53*, *MGMT*, *DAPK,* and *KEAP1* genes, whereas global DNA methylation was measured by the levels of 5-methylcytosine (5-mC), 5-hydroxymetylcytosine (5-hmC), and by the levels of long interspersed nucleotide elements 1 (LINE-1) methylation. When compared to controls, MDS patients had lower levels of reduced glutathione (GSH) and total antioxidant status (TAS), as well as higher levels of peroxide, peroxide/GSH, and peroxide/TAS. Moreover, MDS and MPN patients had higher 5-mC levels and a lower 5-hmC/5-mC ratio, as well as increased methylation of at least one methylated gene (*P15*, *P16*, *DAPK,* or *KEAP1*). The hypermethylation of *TP53* and *MGMT* gene promoters was absent in MDS and MPN patients. Peroxide levels were 1.5-, 1.8-, and 1.6-fold higher in patients with methylated *P15*, *DAPK*, and *KEAP1* promoters, respectively, than those without methylation. Similarly, peroxide/GSH ratio was 1.7-, 1.5-, and 1.4-fold higher in patients with those methylated gene promoters, respectively, in comparison to those with methylation. We also found a negative correlation of LINE-1 methylation with peroxide levels (r=–0.620, p<0.001) and peroxide/GSH ratio (r=–0.539, p<0.001); and, inversely, a positive correlation of 5-mC with peroxide levels (r=0.571, p<0.001) and peroxide/GSH ratio (r=0.502, p<0.001). Overall, this study points to a possible relationship between oxidative stress and DNA methylation, two common pathogenic mechanisms involved in MDS and MPN.

5.2. Introdution

Myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPN) are hematological malignancies with high propensity to develop acute myeloid leukemia [Nimer, 2008]. MDSs are a heterogeneous group of clonal stem cell disorders characterized by dysplasia, impaired differentiation, and inefficient hematopoiesis, which leads to peripheral cytopenias [Adés *et al.*, 2014; Issa, 2013; Nimer, 2008]. Likewise, MPNs are a group of neoplasms that arise from genetically altered myeloid stem or progenitor cells [Klco *et al.*, 2010; Tefferi *et al.*, 2009; Tefferi & Vainchenker, 2011]. These malignancies are characterized by the expansion of one or more hematopoietic cell lineages, with a hypercellular bone marrow due to the overproduction of myeloid cells [Klco *et al.*, 2010]. It is recognized that multiple genetic and epigenetic modifications, which change gene expression, are required for the development of MDS and MPN [Kitamura *et al.*, 2014].

Reactive oxygen species (ROS) are considered an important player in the initiation and progression of hematological malignancies [Sardina *et al.*, 2012]. They can have both beneficial and deleterious effects [Ghaffarri, 2008; Imbesi *et al.*, 2013]. In the case of imbalance in redox homeostasis, ROS levels overwhelm cellular antioxidant defenses, and oxidative stress is established [Ghaffarri, 2008; Sardina *et al.*, 2012]. Several biological processes – namely those involved in the activation of signaling pathways such as proliferation, differentiation, and cell death are dependent upon appropriate intracellular ROS levels [Hasselbalch *et al.*, 2014; Imbesi *et al.*, 2013]. An increase in ROS levels with a decrease in GSH content was already observed in blood cells from MDS patients [Ghoti *et al.*, 2007; Gonçalves *et al.*, 2015b]. Similar findings were observed in MPN patients [Vener *et al.* 2010]. High levels of ROS may contribute to cancer development through both genetic and epigenetic mechanisms [Wu & Ni, 2015]. At an epigenetic level, both DNA hypermethylation and hypomethylation can be induced by ROS [Afanas'ev, 2014; Campos *et al.*, 2007; Weitzman *et al.*, 1994; Wu & Ni, 2015].

In MDS and MPN neoplasms, two different categories of epigenetic alterations occur: aberrant DNA methylation and mutations in epigenetic regulator genes [Mascarenhas *et al*., 2011; Patchsung *et al*., 2012; Santini *et al*., 2013; Woods & Levine, 2015]. The hypermethylation of genes crucial to cell survival, differentiation, and proliferation, such as *CDKN2B* (*P15*), *CDKN2A* (*P16*), *DAPK*, and *MGMT* genes, are observed in these myeloid malignancies [Bodoor *et al*., 2014; Mascarenhas *et al*., 2011; Medeiros *et al*., 2012; Santini *et al*., 2013]. Furthermore, *TP53* hypermethylation has been associated with acute lymphoblastic leukemia [Agirre *et al*., 2003] and lung cancer [Muscarella *et al*., 2011a]. Moreover, DNA hypomethylation of the cancer genome occurs in repetitive sequences, namely in long interspersed nuclear element-1 (LINE-1) [Patchsung *et al*., 2012]. On the other hand, several genes involved in the regulation of DNA methylation, such as *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *EZH2*, and *ASXL1*, are mutated in MDS patients [Santini *et al*., 2013]. Similarly, most of the mutated genes in MPNs are those involved in the regulation of epigenetic mechanisms, and these genetic events affect a high percentage of patients with myeloid malignancies [Mascarenhas *et al*., 2011; Woods & Levine, 2015].

Despite the advances in the understanding of myeloid malignancies pathogenesis, the link between these two common molecular mechanisms – oxidative stress and abnormal methylation – remain poorly understood. In a previous pilot study involving 27 MDS patients, we firstly demonstrated that bone marrow (BM) cells from MDS patients with methylated *P15* and *P16* gene promoters had high levels of intracellular peroxides and superoxide anion, as well as those with high ratios of peroxides/reduced glutathione (GSH) and superoxide/GSH [Gonçalves *et al*., 2015c]. However, peripheral blood (PB) collection is more convenient for patients than BM aspiration due to it less invasive nature. In this context, we first analyzed the concordance between oxidative stress parameters (intracellular levels of peroxides, GSH, and peroxides/GSH ratio) as well as *P15* and *P16* methylation status detected in PB and BM samples from MDS patients (n=10) and controls (n=8), previously enrolled in our pilot study [Gonçalves *et al*., 2015c]. Additionally, we also evaluated in these subjects the concordance of plasmatic peroxide levels.

Subsequently and in order to improve the knowledge on the cross talk between these two molecular mechanisms, we expanded the evaluation of oxidative stress and DNA methylation parameters, as well as the studied pathologies. Therefore, we analyzed localized and global DNA methylation, as well as a broad spectrum of non-enzymatic and enzymatic antioxidants, free radicals, and oxidative damage parameters, in peripheral blood samples from myeloid neoplasm patients (MDS and MPN) and controls. Moreover, we also investigated the association of oxidative stress with DNA methylation, and the usefulness of these parameters as biomarkers for the diagnosis of MDS and MPN.

5.3. Methods

5.3.1. Ethical statement

The Ethics Committee of Faculty of Medicine of University of Coimbra (Coimbra, Portugal) approved the research procedures, and the study was conducted in accordance with the Declaration of Helsinki. Prior to enrollment, participants provided their informed consent for participation. The international ethical guidelines of confidentiality, anonymity of personal data, and abandonment option in case of expressed will were followed.

5.3.2. Study population

In the present study, 91 patients with myeloid malignancies at diagnosis (66 MDS patients and 25 MPN) and 26 controls, without hematological malignancies, were enrolled from October 2012 to March 2014. MDS patients were diagnosed according to World Health Organization 2008 classification of myeloid neoplasms [Brunning *et al*., 2008] in the following subtypes: refractory cytopenia with unilineage dysplasia (RCUD), refractory anemia with ring sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess of blasts (RAEB) type 1 (RAEB-1), and RAEB type 2 (RAEB-2). The MPN patients were diagnosed according to the same classification in polycythemia vera

(PV) and essential thrombocytosis (ET) [Tefferi & Vardiman, 2008]. The prognostic score for MDS patients was calculated using the International Prognostic Scoring System (IPSS), and patients were stratified according to their risk score in low-risk, intermediate-1 (int-1), and high-risk (including intermediate-2 and high-risk) patients [Brunning *et al*., 2008]. Biodemographic (age and gender) and clinical data (hematological features, karyotype, as well as the mutation status for *FLT3*, *JAK2*, and *TET2* genes), when available, were obtained from medical records. The methylation status and the levels of peroxide and 8-hydroxy-2-deoxyguanosine (8-OHdG) were evaluated in all participants (*n*=117); however, due to several constraints and operational reasons, the other oxidative stress parameters were only assessed in 69 individuals (57 patients and 12 controls). To examine the concordance between oxidative stress parameters and DNA methylation status detected in PB and BM samples, we analyzed ten MDS patients and eight controls, previously enrolled in our pilot study [Gonçalves *et al*., 2015c].

5.3.3. Sample preparation

At diagnosis, peripheral blood samples were collected, after fasting, into sodium heparin (oxidative stress studies) and EDTA tubes (methylation studies). Samples for oxidative stress evaluation were immediately centrifuged; plasma and red blood cells (with a concentration of hemoglobin adjusted at 100 g/l) were stored frozen at -20°C until analysis, as previously described [Baldeiras *et al*., 2010]. For normalization of some oxidative stress parameters, total plasma protein and cholesterol were measured.

5.3.4. Assessment of intracellular levels of peroxides and GSH in leukocytes

Peripheral blood and bone marrow samples from MDS patients and controls, previously enrolled in our pilot study [Gonçalves *et al*., 2015c], were stained with PerCP-Cy5.5 conjugate anti-CD45 monoclonal antibodies (BD Biosystems, San Diego, CA, USA). Intracellular peroxides levels were measured using

2',7'-dichlorodihydrofluorescein diacetate dye ($DCFH_2$-DA; Molecular Probes, Life Technologies Corporation, Carlsbad, CA, USA), as described previously by us [Gonçalves *et al*., 2015c]. Peripheral blood and bone marrow leucocytes were identified by the positive expression of CD45.

5.3.5. Uric acid determinations

Plasmatic levels of uric acid were determined by a colorimetric method [Barham & Trinder, 1972]. This method was based on the reduction of uric acid by the enzyme uricase, which releases hydrogen peroxide and forms a chromogenic compound that was then spectrophotometrically evaluated at 550 nm.

5.3.6. Vitamin A and E measurements

The assessment of plasmatic levels of vitamins A (vit A) and E (vit E) were initiated by lipid extraction from plasma samples. Next, vitamins were quantified by high performance liquid chromatography (HPLC), using an analytic column spherisorb ODS1-5 µm (250×4.6 mm), eluted at 2.5 ml/min with a water solution of methanol (90%), at 45°C, with spectrophotometric detection (Gilson) at 340 nm (for vit A) or 295 nm (for vit E).

The levels of vitamin E in red blood cells were extracted in n-hexane and quantified by reverse-phase HPLC [De Leenheer *et al*., 1979; Vatassery *et al*., 1978], using an analytic column spherisorb S10w (250×4.6 mm), eluted at 1.5 ml/min with n-hexane modified with 0.9% of methanol, and detected by spectrophotometry at 287 nm (Gilson).

5.3.7. Oxidized and reduced glutathione quantification

Reduced (GSH) and oxidized glutathione (GSSG) in red blood cells were also evaluated by HPLC with fluorimetric detection (excitation at 385 nm, and emission at 515 nm), using the Immunodiagnostik kit (Immunodiagnostik AG, Bensheim,

Germany), as described by the manufacturer. Total glutathione (GS) was calculated according to the following equation: [total GS] = [GSH] + 2×[GSSG].

5.3.8. Total antioxidant status evaluation

Total antioxidant status (TAS) was evaluated by a chromogenic method (Randox Laboratories), based on the plasma capacity to inhibit the formation of the $ABTS^{+}$ radical cation (2,2'-azino-di-[3-etilbenzotiazolin sulfonate]), and detected at 600 nm, as described by the manufacturer.

5.3.9. Antioxidant enzymes activity determination

Erythrocyte glutathione peroxidase (GPX) was evaluated by spectrophotometry using an indirect determination method and *tert*-butyl hydroperoxide as substrate [Paglia & Valentine, 1967]. The oxidized glutathione formation was monitored through the quantification of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm in a thermostatized spectrophotometer UVIKON 933 UV/Visible. Erythrocyte glutathione reductase (GR) was evaluated by spectrophotometry at 340 nm [Goldberg & Spooner, 1983], using GSSG as a substrate, and monitoring its reduction to GSH through the quantification of NADPH oxidation at 37°C in a spectrophotometer UVIKON 933 UV/Visible.

5.3.10. Lipid peroxidation measurements

Levels of lipid peroxidation in plasma and red blood cells were assessed by the formation of thiobarbituric acid (TBA) adducts of malondialdehyde (MDA), separated by HPLC (Gilson), and quantified fluorimetrically using the ClinRep complete kit (RECIPE), as described by the manufacturer. Briefly, 100 µl blank, standard, controls, and patients samples were first derivatized at 100°C for 60 min in glass light-protected vial. After cooling, samples were neutralized, precipitated, and centrifuged at 10 000g for 5 min. Finally, 20 µl of the supernatants were injected into the HPLC and the MDA adducts were determined fluorimetrically

(excitation at 515 nm, and emission at 553 nm; FP-2020/2025, Jasco, Tokyo, Japan).

5.3.11. Plasmatic nitric oxide quantification

The plasmatic levels of nitric oxide (NO) were determined by a photometric method (Roche Diagnostics GmbH) via its oxidation products, nitrite and nitrate [Titheradge, 1998]. First, the nitrate present in the ultrafiltrated plasma was reduced to nitrite, which then reacted with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye, and detected by spectrophotometry at 550 nm.

5.3.12. Plasmatic peroxide quantification

The plasmatic levels of peroxide were measured by a colorimetric method (Thermo Scientific Pierce Quantitative Peroxide Assay Kit – lipid-compatible formulation, Life Technologies), based on the oxidation of ferrous to ferric ion in the presence of xylenol orange. Plasmatic peroxide were detected by spectrophotometry at 595 nm, and determined by comparison with a hydrogen peroxide standard curve, as described by the manufacturer, in a Synergy™ multi-mode microplate reader (BioTek Instruments).

5.3.13. Plasmatic 8-hydroxy-2-deoxyguanosine quantification

The plasmatic 8-OHdG levels were measured using a competitive quantitative ELISA Kit (8-hydroxy-2-deoxyguanosine ELISA Kit, Abcam), according to manufacturer instructions. The assay is based on the competition between 8-OHdG and an 8-OHdG-acetylcholinesterase conjugate for a limited amount of 8-OHdG monoclonal antibody. The colorimetric intensity was determined spectrophotometrically in a Synergy™ multi-mode microplate reader, and its value was inversely proportional to the amount of free 8-OHdG in plasma.

5.3.14. Global DNA methylation analysis

Global methylation and hydroxymethylation were determined in DNA samples by specific ELISA assays (5-methylcytosine DNA ELISA kit and 5-hydroxymethylcytosine DNA ELISA Kit; Enzo), according to manufacturer's protocol. Genomic DNA was extracted from whole blood, as previously described by Bartlett & White [2003]. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). These assays use monoclonal antibodies against 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) to obtain the percentage of 5-mC and 5-hmC in total DNA. Global methylation was also assessed by methylation analysis of LINE-1 repetitive elements, using combined bisulfite restriction analysis (COBRA) as previously described [Yang *et al*., 2004]. Briefly, the genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen). PCR was performed on bisulfite treated DNA using specific primers for LINE-1 repetitive sequences (5'-GATCTTTTTATTAAAAATATAAAAATTAGT-3' and 5'-GATCCCAAACTAAAATAC-AATAA-3'). The final PCR product was digested with the *HinfI* restriction enzyme. The digested PCR products were resolved by electrophoresis on 10% polyacrylamide gels, and stained with ethidium bromide. Gel images were acquired using a Gel Doc XR (Bio-Rad), and band intensity was measured by densitometry through QuantityOne software (Bio-Rad). The ratio of intensity of the digested band to the sum of digested and undigested indicated the percent of LINE-1 methylation.

5.3.15. Methylation pattern of tumor suppressor genes

The methylation status of tumor suppressor genes *P15*, *P16*, *TP53*, *MGMT*, *DAPK*, and *KEAP1* were carried out by methylation-specific PCR (MSP), as previously described by others [Esteller *et al*., 2000; Gonzalez-Gomez *et al*., 2003; Hanada *et al*., 2012; Yeh *et al*., 2003]. Each assay was validated by the amplification of unmethylated and methylated universal DNA controls (EpiTect PCR Control DNA Set, Qiagen) and a no template control (NTC). PCR products were resolved on 3% agarose gel stained with ethidium bromide, and visualized under UV illumination.

5.3.16. Statistical analysis

Statistical analysis was performed using SPSS version 22.0, and graphics were constructed through GraphPad Prism version 5.0. Continuous variables were expressed as mean ± SEM, unless otherwise specified, and categorical variables as numbers and percentages. To account for changes in plasma lipid content, vitamin A and E were expressed in relation to cholesterol, because lipids affect the concentration of these vitamins. Normality was assessed by the Kolmogorov-Smirnov test. For normally distributed continuous variables, the Student's t-test or ANOVA with Bonferroni post-hoc test were performed to assess the statistical significance of the difference between means of two groups (for example: patients *vs.* controls; patients with methylation *vs.* patients without methylation) and more than two groups (subtype groups: RA *vs.* RCMD *vs.* RAEB-1 *vs.* RAEB-2; IPSS risk groups: low *vs.* int-1 *vs.* high), respectively. When continuous variables did not show normal distribution, the Mann Whitney U or Kruskal Wallis tests were used. The association between continuous variables was determined by Spearman or Pearson correlation coefficients. The methylation frequency between groups (patients *vs.* controls, IPSS risk groups) was compared using the chi-square test. Logistic regression, adjusted to age and gender, was performed to establish the factors that were associated with MDS or MPN. Moreover, the same analysis was performed to establish the oxidative stress factors that were correlated with DNA methylation.

Factors that showed a significant association in the univariate analysis were included in the multivariate logistic regression to determine the independent associated variables. The calibration of logistic models was assessed by the Homer-Lemeshow goodness-of-the-fit test. Results from logistic analysis were expressed as adjusted odds ratios (OR) with the corresponding 95% confidence interval (CI). In this analysis, the OR corresponds to a 1-unit increase in the explanatory variable. Finally, receiver operating characteristic (ROC) curves were performed to evaluate the accuracy of significant parameters as diagnostic biomarkers of MDS and MPN. The area under the curve (AUC) was calculated as measurement of the accuracy of the test, and an optimal cut-off point was

determined as the value of the parameter that maximized the sum of specificity and sensitivity (Youden's J Index). All statistical analyses were two-sided, and a $p<0.05$ was considered statistically significant.

5.4. Results

5.4.1. Biodemographic and clinical characteristics of MDS and MPN patients

The present study enrolled 91 patients, of which 66 (72.5%) were diagnosed with MDS [median age of 72 years (range 22–89), 60.1% (*n*=40) females and 39.9% (*n*=26) males], and 25 (27.5%) were diagnosed with MPN [median age of 68 years (range 46–77), 52.0% (*n*=13) females and 48.0% (*n*=12) males]. The control group consisted of 26 subjects without hematological malignancies or oxidative stress-related disorders [median age of 67 years (range 32–79), 53.8% females (*n*=14) and 46.2% males (*n*=12)]. Biodemographical and clinical characteristics of all participants are shown in Table 14. There were no statistical differences in biodemographic characteristics between the three groups: MDS, MPN and control.

MDS and MPN patients were diagnosed according to WHO classification (2008). Regarding MDS patients, nine (13.6%) were diagnosed with RCUD, 10 (15.2%) with RARS, 40 (60.6%) with RCMD, four (6.1%) with RAEB-1, and three (4.5%) with RAEB-2. The IPSS prognostic score was low in 25 (49.0%) patients, intermediate-1 (int-1) in 17 (33.3%), and intermediate-2/high (high) in nine (17.7%). Fifteen MDS patients had cytogenetic abnormalities, and one presented a *FLT3* internal tandem duplication (ITD) mutation. In the MPN group, 12 (48.0%) patients were diagnosed with polycythemia vera (PV) and 13 (52.0%) with essential thrombocytosis (ET). Fifteen MPN patients had gene mutations distributed as followed: 11 on *JAK2* c.1849G>T (p.Val617Phe) [PV: *n*=8 (66.7%); ET: *n*=3 (23.0%)], one had *FLT3* ITD [PV: *n*=1 (4.0%)], and three had *TET2* exon 4 [PV: *n*=3 (15.8%)].

Table 14
Biodemographic and clinical characteristics of patients and controls

Characteristics	Patients		Controls (n=26)	
	MDS (*n*=66)		MPN (*n*=25)	
Demographic features				
Gender (%)				
Male	26	(39.9)	12	(48.0)	12	(46.2)
Female	40	(60.1)	13	(52.0)	14	(53.8)
Age (years)				
Median	74		68		67	
Range	22 – 89		46 – 77		32 – 79	
Clinical features				
Hematological parameters (median, range)				
WBC (x10^9/l)	3.5	(1.3–13.0)	9.9	(7.6–14.4)		
Hb (g/l)	10.6	(5.4–16.0)	16.3	(14.3–17.3)		
Platelets (x10^9/l)	98	(12–324)	726	(382–1453)		
WHO 2008 classification				
Myelodysplastic syndrome				
RCUD (%)	9	(13.6)	–			
RARS (%)	10	(15.2)	–			
RCMD (%)	40	(60.6)	–			
RAEB-1 (%)	4	(6.1)	–			
RAEB-2 (%)	3	(4.5)	–			
Myeloproliferative neoplasms				
Polycythemia vera	–		12	(48.0)		
Essential thrombocytosis	–		13	(52.0)		
IPSS risk groups	*n*=51					
Low	25		–			
Int-1	17		–			
High (Int-2 + High)	9		–			
Karyotype (conventional or FISH)	*n*=51		*n*=25			
Normal	36		–			
Deletion 20q	2		–			
Trisomy 8	11		–			
Monosomy 7/deletion 7q	1		–			
Complex (≥ 3 chromosomal abnormalities)	1		–			
Philadelphia chromosome translocation: t(9;22)(q34;q11)	–		0			
Gene mutations				
JAK2 c.1849G>T (p.Val617Phe)	ND		11:25			
FLT3 ITD	1:66		1:25			
TET2 exon 4 mutated	0:16 (ND = 50)		3:19 (ND = 6)			

MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; WHO, World Health Organization; WBC, white blood cells; Hb, hemoglobin; RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RAEB-1, refractory anemia with excess blasts type 1; RAEB-2, refractory anemia with excess blasts type 2; IPSS, international prognostic scoring system; Int-1, intermediate-1; Int-2, intermediate-2; FISH, fluorescence *in situ* hybridization; ITD, internal tandem duplications; ND, not determined.

5.4.2. Concordance between oxidative stress parameters and DNA methylation status in peripheral blood and bone marrow samples

In order to investigate the concordance between PB and BM regarding intracellular and plasmatic levels of oxidative stress in MDS patients and controls, we correlate the leucocytes intracellular levels of peroxides, GSH, and peroxides/GSH ratio, as well as the peroxide plasmatic levels (Figure 33A). We observed that peroxides intracellular levels were correlated in PB and BM with a correlation coefficient of 0.738 (p=0.046) in MDS patients and 0.898 (p=0.005) in controls. Moreover, GSH intracellular levels were highly significant correlated in MDS patients 0.969 (p=0.003) and controls 0.976 (p<0.001). Furthermore, the peroxides/GSH ratio was highly correlated in MDS patients (r=0.927, p=0.002) and significantly correlated in controls (r=0.738, p=0.046). Similarly, plasmatic levels of peroxide were highly correlated in MDS patients (r=0.952, p=0.001) and significantly correlated in controls (r=0.755, p=0.037). Finally, plasmatic and intracellular peroxides were correlated in PB (r=0.905, p=0.005) and BM (r = 0.810, p = 0.022) of MDS patients, as well as in controls (PB: r=0.952, p=0.001; BM: r=0.970, p<0.001).

The analysis of DNA methylation concordance between PB and BM were performed using the methylation status of *P15* and *P16* gene promoters (Figure 33B). The detection of *P15* methylated promoters was concordant in 8 out of 10 (80%) MDS patients. As observed in Figure 33B, in patient number 7 (MDS 7) the methylation of *P15* gene was only detected in the BM sample, while in patient number 10 (MDS 10) the methylation of this gene was only in PB. On the other hand, a completely concordance between PB and BM was observed in the detection of *P16* gene promoter methylation. Regarding unmethylated *P15* and *P16* gene promoters, the results from MSP were completely concordant in both MDS patients and controls.

A

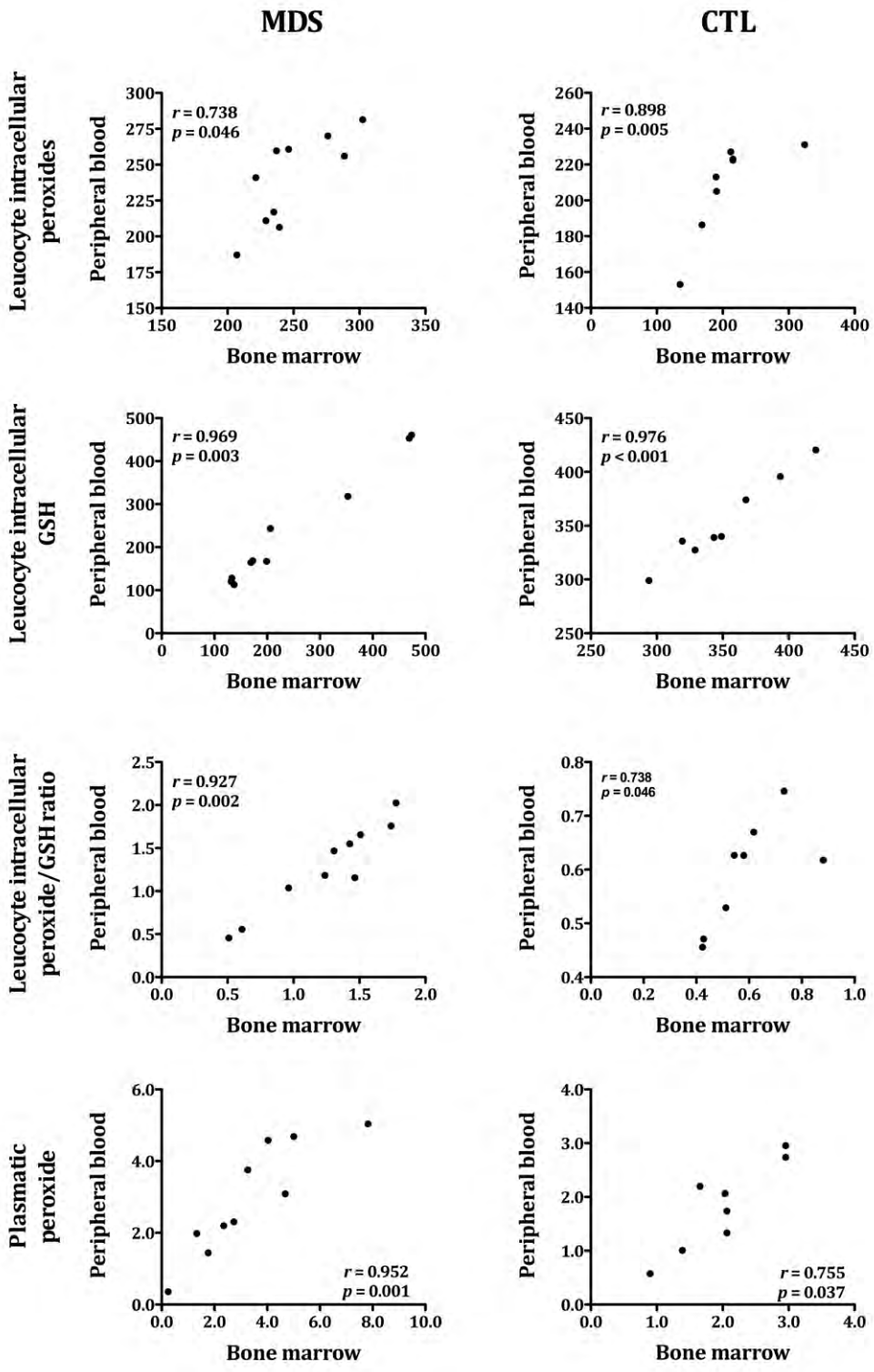


Figure 33. Concordance analysis of oxidative stress parameters (A) and DNA methylation status (B) in peripheral blood and bone marrow samples of myelodysplastic syndrome patients and controls.

B

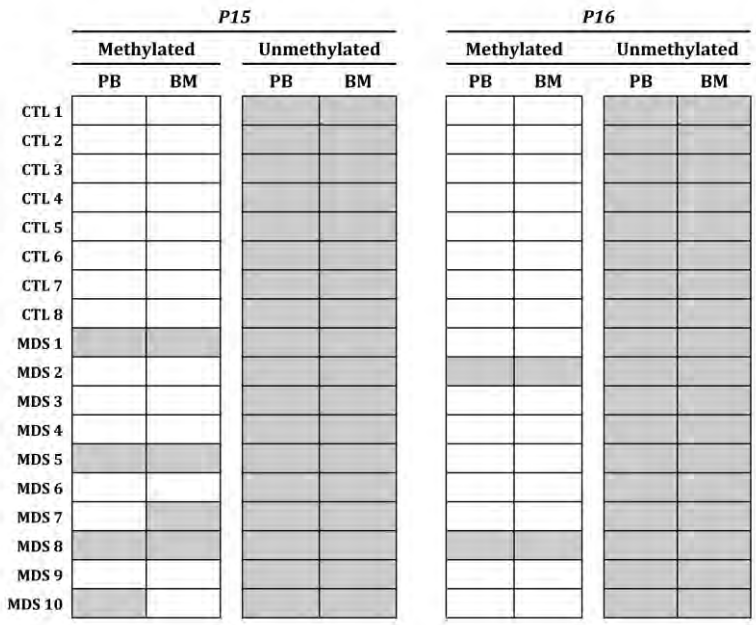


Figure 33. Concordance analysis of oxidative stress parameters (A) and DNA methylation status (B) in peripheral blood and bone marrow samples of myelodysplastic syndrome patients and controls (*continued*). The dot plots (A) represent the following correlations: leucocyte intracellular peroxides (peripheral blood *vs.* bone marrow), leucocyte intracellular reduced glutathione (peripheral blood *vs.* bone marrow), leucocyte intracellular peroxides/reduced glutathione ratio (peripheral blood *vs.* bone marrow), and plasmatic peroxide (peripheral blood *vs.* bone marrow). The concordance analysis between peripheral blood and bone marrow performed for *P15* and *P16* methylation status is represented in (B). PB, peripheral blood; BM, bone marrow; MDS, myelodysplastic syndrome; CTL, control.

5.4.3. Oxidative stress levels in myeloid malignancies

In order to analyze the participation of oxidative stress in myeloid malignancies development, we examined the systemic levels of reactive species (peroxide and NO), antioxidant defenses [uric acid, vitamin E (plasmatic and erythrocytic), vitamin A, GSH, GSSG, TAS, erythrocyte GPX and GR activities], and oxidative damage [8-OHdG and MDA (plasmatic and erythrocytic)] between patients and controls. Moreover, since oxidative stress results from the imbalance between free radicals and antioxidants, we also calculated the following ratios: peroxide/GSH, peroxide/TAS, NO/GSH, and NO/TAS.

As shown in Figure 34A–H, the results from plasmatic oxidative stress indicated that peroxide levels (Figure 34A) were increased in myeloid malignancies patients (MDS: 4.33±0.22 μM, $p<0.001$; MPN: 6.47±0.54 μM, $p<0.001$) in comparison to controls (1.98±0.23 μM), as well as in MPN patients in relation to MDS ones ($p=0.001$). The NO levels (Figure 34B) were increased in MDS patients (11.37±0.79 μM, $p=0.002$) compared to those with MPN (5.83±0.70 μM, $p=0.044$). Moreover, the TAS levels (Figure 34C) were decreased in MDS patients (0.81±0.03 mM, $p=0.003$) in comparison with controls (0.96±0.05 mM). The DNA damage (8-OHdG levels; Figure 34G) was increased in MDS patients (MDS: 37.49±0.76 ng/ml) in comparison to those with MPN (32.52±0.81 ng/ml; $p=0.002$) and to controls (29.15±0.94 ng/ml; $p<0.001$), whereas lipid peroxidation levels (MDA; Figure 34H) were increased in MPN patients (1.08±0.08 μM, $p=0.017$) in relation to controls (0.85±0.04 μM). We did not find significant differences in plasmatic levels of uric acid, vitamin A, and vitamin E between patients and controls (MDS *vs.* CTL and MPN *vs.* CTL) nor between MDS and MPN patients (Figure 34D–F). Additionally, we observed that erythrocyte levels of GSH levels (Figure 34M) were decreased in MDS patients (7.35±0.45 μmol/gHb), in comparison to MPN ones (8.32±0.35 μmol/gHb, $p=0.09$) and to controls (9.90±0.31 μmol/gHb, $p=0.027$). However, we did not observed significant differences between patients and controls or MDS and MPN patients in erythrocyte levels of GSSG and total GS, in GPX and GR activities, as well as in MDA (Figure 34N–S).

Next, we calculated oxidative stress indexes as the ratios of peroxide/TAS, NO/GSH, and NO/TAS (Figure 34I–L). The peroxide/GSH ratio was increased in myeloid malignancies patients (MDS: 0.72±0.06, $p<0.001$; MPN: 0.79±0.21, $p=0.011$) in comparison to controls (0.25±0.02). The peroxide/TAS ratio was increased in patients with MDS (4.78±0.34, $p<0.001$) and MPN (6.02±0.59, $p<0.001$), in relation with controls (2.09±0.21), as well as in MPN patients when compared with MDS ones ($p=0.048$). Furthermore, the NO/GSH ratio was increased in MDS patients (1.94±0.18) in relation to those with MPN (0.87±0.08, $p=0.06$) and to controls (0.94±0.08, $p=0.09$). The NO/TAS was increased in MDS

patients (12.66±1.09), in comparison to those with MPN (8.34±0.65, $p<0.001$) and to controls (8.09±0.77, $p=0.027$).

Finally, we analyzed the differences in oxidative stress parameters between patients grouped according to WHO classification and to IPSS risk groups, respectively. As shown in Table 15, in MDS subtypes GSH levels were 1.6- and 1.7-fold higher in patients with RAEB-1 and RAEB-2 in comparison to those with RCDU and RARS ($p<0.05$). The 8-OHdG levels were 1.2-fold higher in RAEB-2 patients in relation to those with RCDU ($p=0.035$).

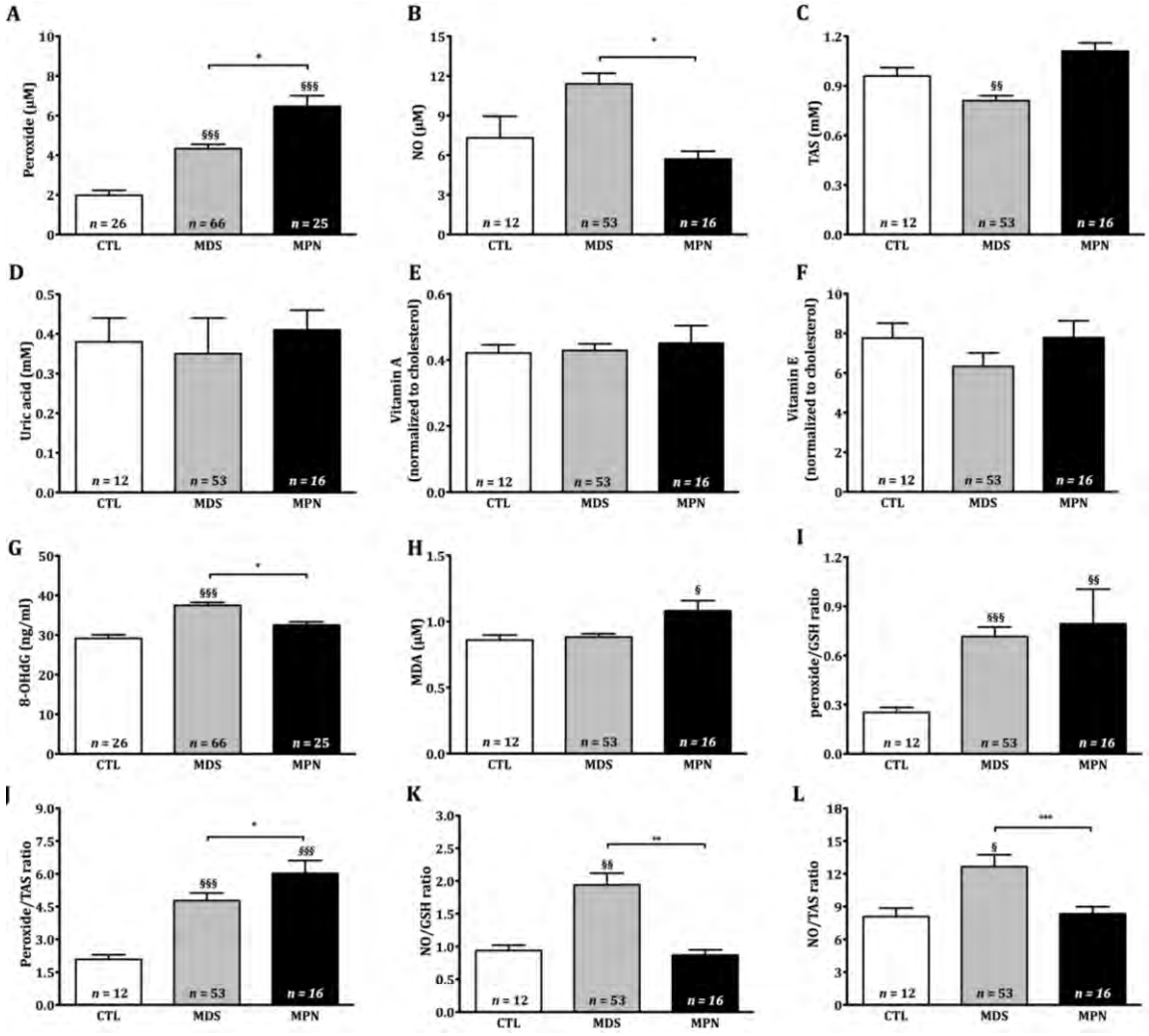


Figure 34. Analysis of plasmatic (A – L) and erythrocyte (M – S) oxidative stress parameters in patients with myelodysplastic syndrome and myeloproliferative neoplasm, as well as in controls.

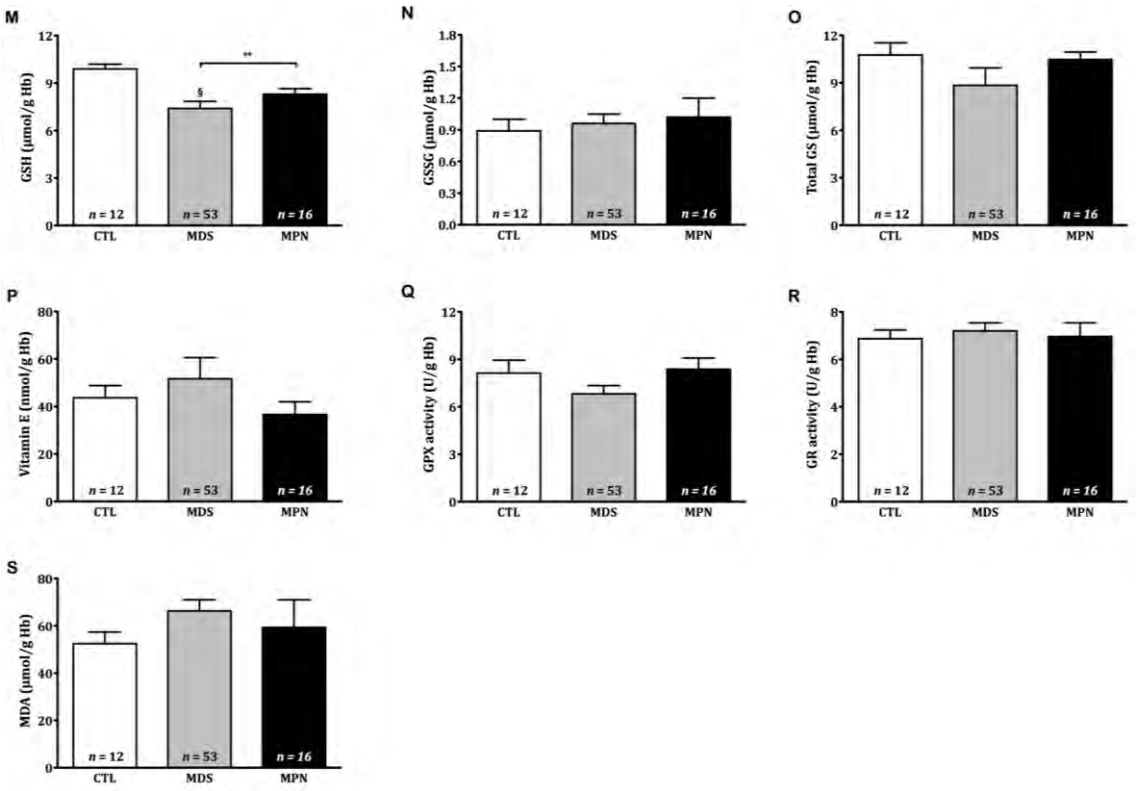


Figure 34. Analysis of plasmatic (A – L) and erythrocyte (M – S) oxidative stress parameters in patients with myelodysplastic syndrome and myeloproliferative neoplasm, as well as in controls *(continued)***.** The following plasmatic oxidative stress levels are represented: (A) peroxide, (B) nitric oxide (NO), (C) total antioxidant status (TAS), (D) uric acid, (E) vitamin A, (F) vitamin E, (G) 8-hydroxy-2-deoxyguanosine (8-OHdG), (H) malondialdehyde (MDA), (I) peroxide/GSH ratio, (J) peroxide/TAS ratio, (K) NO/GSH ratio, and (L) NO/TAS ratio. Additionally, the following erythrocyte oxidative stress levels are represented: (M) reduced glutathione (GSH), (N) oxidized glutathione (GSSG), (O) total glutathione (total GS), (P) vitamin E, (Q) glutathione peroxidase (GPX) enzymatic activity, (R) glutathione reductase (GR) enzymatic activity, and (S) MDA. The results are represented as mean ± SEM. MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; §, $p<0.05$ (*vs*. control); §§, $p<0.01$ (*vs*. control); §§§, $p<0.001$ (*vs*. control); *, $p<0.05$ (MDS *vs*. MPN); **, $p<0.01$ (MDS *vs*. MPN); ***, $p<0.001$ (MDS *vs*. MPN).

We did not observed significant differences between MDS IPSS risk groups (data not shown). In MPN subtypes, peroxide levels were 1.5-fold higher in PV patients in relation to ET ones (p=0.035), and GSH levels were 1.1-fold higher in ET patients when compared with PV ones (p=0.034).

Table 15
Plasma levels of peroxide, glutathione, 8-hydroxy-2'-deoxyguanosine, and 5-hydroxymethylcytosine on myelodysplastic syndrome and myeloproliferative neoplasm subtypes

Parameter	MDS						MPN		
	RCDU	RARS	RCDM	RAEB-1	RAEB-2	p^a	PV	TE	p^b
Peroxide (μM)	4.8±0.5	4.6±0.5	4.7±0.3	3.6±0.9	3.8±0.8	NS	7.6±0.7	5.1±0.6	0.035
GSH (μmol/gHb)	5.2±0.7	5.1±0.9	7.4±0.7	8.2±0.4†	8.6±2.6†	0.022	8.0±0.5	8.9±0.5	0.034
8-OH-dG (ng/ml)	35.8±1.4	35.3±2.3	38.3±1.0	38.4±2.6	42.3±1.1*	0.009	32.2±0.8	33.0±1.6	NS
5-hmC (%)	0.3±0.1‡	0.4±0.1‡	0.4±0.1‡	0.5±0.1	0.8±0.2	0.044	0.3±0.1	0.4±0.1	NS

p, *p*-value; [a]Kruskal-Wallis test; [b]Mann-Whitney U test. *, $p<0.05$ (*vs.* RCDU); †, $p<0.05$ (*vs.* RCDU and *vs.* RARS); ‡, $p<0.05$ (*vs.* RAEB-2). RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RAEB-1, refractory anemia with excess blasts type 1; RAEB-2, refractory anemia with excess blasts type 2; GSH, reduced glutathione; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 5hmC, 5-hydroxymethylcytosine; NS, non significant.

5.4.4. DNA methylation status in myeloid malignancies

The DNA methylation status in myeloid malignancies was measured through global DNA methylation (5-mC, 5-mC/5-hmC, and LINE-1 methylation levels; Figure 35). Additionally, we examined the localized DNA methylation in gene promoters of *P15*, *P16*, *TP53*, *DAPK*, *MGMT*, and *KEAP1* (Table 16 and Figure 36).

As observed in Figure 35A, 5-mC levels were increased in patients with MDS (0.91±0.06%, $p=0.001$) and MPN (0.91±0.11%, $p=0.013$), when compared to controls (0.26±0.03%). In accordance we these results, 5-hmC/5-mC levels (Figure 35B) were decreased in patients with MDS (0.61±0.15, $p<0.001$) and MPN (0.48±0.15, $p<0.001$), in relation to controls (1.26±0.25). The LINE-1 methylation levels (Figure 35C) were also decreased in patients with MDS (69.5±1.0%, $p<0.001$) and MPN (67.8±1.9%, $p<0.001$), in relation to controls (77.8±0.8%, $p=0.013$). Additionally, we found that RAEB-2 subtype (Table 15) had increased 5-hmC levels (0.76±0.20%), in comparison with RCDU (0.30±0.07%, $p=0.033$) and RCDM subtypes (0.36±0.39, $p=0.038$). The 5-hmC levels were increased in RAEB-2 patients (0.8±0.2%) in relation to those with RCDU (0.3±0.1%), RARS (0.4±0.1%), and RCDM (0.4±0.1%).

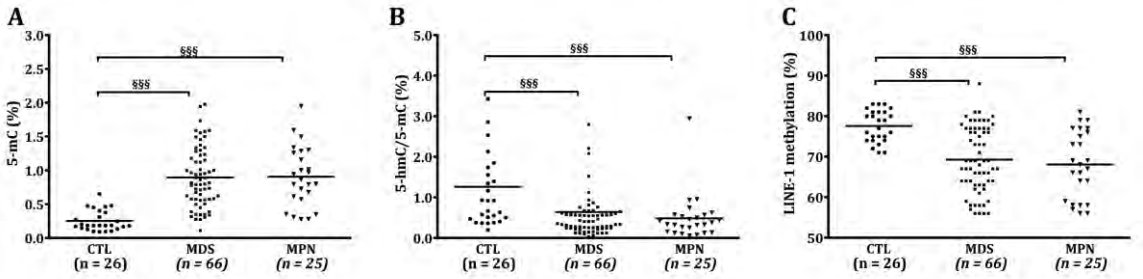


Figure 35. Global and localized DNA methylation status in patients with myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN), as well as in controls (CTL). Global methylation were determined by the quantification of: (A) 5-methylcytosine (5-mC), (B) 5-hydroxymethylcytosine (5-hmC)/5-mC ratio, and (C) long interspersed nucleotide elements 1 (LINE-1) methylation. §, $p<0.05$ (*vs*. control); §§, $p<0.01$ (*vs*. control); §§§, $p<0.001$ (*vs*. control).

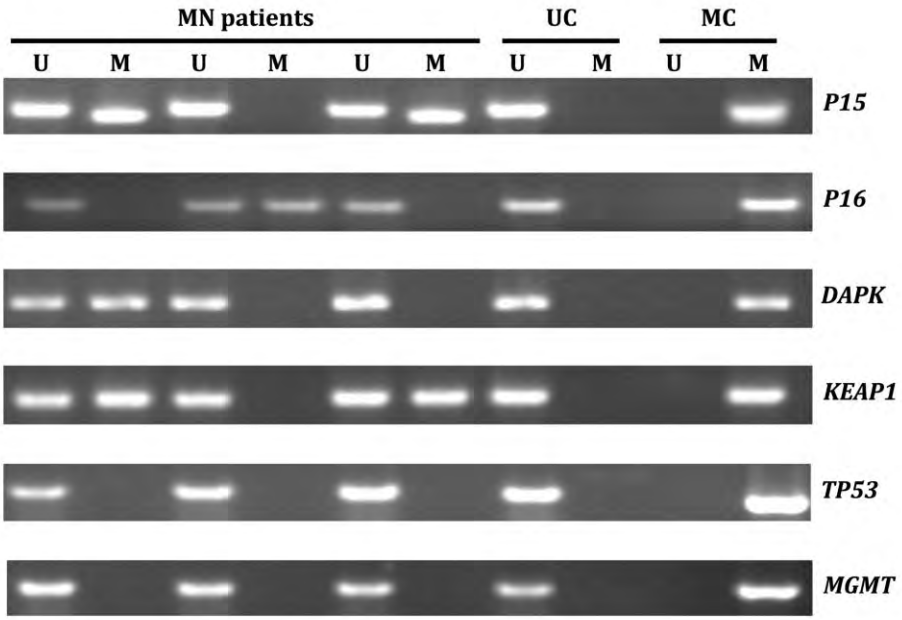


Figure 36. Representative methylation-specific polymerase chain reaction (MSP) results of *P15*, *P16*, *DAPK*, *KEAP1*, *TP53*, and *MGMT* aberrant methylation in myeloid neoplams. U, results obtained using unmethylated primers; M, results obtained using methylated primers; UC, total unmethylated control; MC, total methylated control.

In regard to localized DNA methylation (Table 16), MDS patients had a significant higher methylation frequency of *P15* (48.5%, 32/66), *DAPK* (42.4%, 28/66), and

KEAP1 (33.3%, 22/66) gene promoters, when compared with controls (*P15*: 11.5%, 3/26; *DAPK*: 0.0%, 0/26; *KEAP1*: 11.5%, 3/26). Moreover, MDS patients had a significant increase in *DAPK* methylation frequency when compared to MPN ones (4.0%, 1/25; $p<0.001$). Additionally, MPN patients had hypermethylation of *P16* (24.0%, 6/25) and *KEAP1* (28.0%, 7/25) gene promoters, however without statistical significance.

Table 16
Methylation frequency of studied genes on myelodysplastic syndrome and myeloproliferative neoplasm patients, as well as on their subtypes

Disease	Methylation frequency							
	P15		*P16*		*DAPK*		*KEAP1*	
	n	%	*n*	%	*n*	%	*n*	%
MDS	32/66	48.5**	13/66	19.7	28/66	42.4***	26/66	33.3*
RCDU	3/9	33.3	3/9	11.1	3/9	33.3	1/9	11.1
RARS	4/10	40.0	4/10	10.0	7/10	70.0	2/10	20.0
RCDM	20/40	50.0	20/40	20.0	14/40	35.0	17/40	42.5
RAEB-1	2/4	50.0	2/4	25.0	2/4	50.0	1/4	25.0
RAEB-2	3/3	100.0	3/3	50.0	2/3	66.7	1/3	33.3
MPN	8/25	32.0	8/25	32.0	1/25	4.0†††	8/25	32.0
PV	6/12	50.0	6/12	33.3	0/12	0.0	2/12	16.7
ET	2/13	15.4	2/13	30.8	1/13	7.7	6/13	46.2
Controls	3/26	11.5	1/26	3.8	0/26	0.0	3/26	11.5

Disease	Methylation frequency							
	TP53		*MGMT*		1 ≥ methylated genes		2 ≥ methylated genes	
	n	%	*n*	%	*n*	%	*n*	%
MDS	0/66	0.0	0/66	0.0	43/66	65.2***	32/66	48.5***
RCDU	0/9	0.0	0/9	0.0	4/9	44.4	1/9	11.1
RARS	0/10	0.0	0/10	0.0	5/10	70.0	3/10	30.0
RCDM	0/40	0.0	0/40	0.0	29/40	72.5	23/40	57.5
RAEB-1	0/4	0.0	0/4	0.0	2/4	50.0	2/4	50.0
RAEB-2	0/3	0.0	0/3	0.0	3/3	100.0	3/3	100.0
MPN	0/25	0.0	0/25	0.0	10/25	40.0*	6/25	24.0*
PV	0/12	0.0	0/12	0.0	5/12	41.6	3/12	25.0
ET	0/13	0.0	0/13	0.0	5/13	38.5	3/13	23.0
Controls	0/26	0.0	0/26	0.0	4/26	15.4	0/26	0.0

*, $p<0.050$ (*vs*. controls); **, $p<0.010$ (*vs.* controls); ***, $p<0.010$ (*vs.* controls); †††, $p<0.001$ (*vs.* MDS). RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RAEB-1, refractory anemia with excess blasts type 1; RAEB-2, refractory anemia with excess blasts type 2.

Finally, the majority of MDS patients had at least one methylated gene (65.5%, 43/66) and, to a lesser extent, two or more methylated genes (48.5%, 32/66). Similarly, 40.0% (10/25) of MPN patients had at least one methylated gene and 24.0% (6/25) of these patients had two or more methylated genes. Contrarily, *TP53* and *MGMT* gene promoters were unmethylation in all subjects. In control group, none subject had two or more methylated genes. The *P15* gene promoter methylation (Table 16) was higher in RAEB-2 subtype (100.0%, 3/3) in comparison with RCDU ones (33.3%, 3/9).

5.4.5. Association of oxidative stress and DNA methylation with myeloid malignancies

Logistic regression was performed to assess the oxidative stress and DNA methylation parameters that were associated with myeloid malignancies (Table 17). The levels of peroxide and 8-OHdG were significantly associated with MDS in the univariate analysis; and a similar association was found with the ratios of peroxide/GSH, peroxide/TAS, NO/GSH, and NO/TAS. In addition to these oxidative stress biomarkers, we found an association between DNA methylation features (5-mC, 5-mC/5-hmC, LINE-1 methylation, methylated *P15*, methylated *KEAP1*, two or more methylated genes) and MDS. The univariate analysis indicated that MPN was associated with high levels of peroxide, plasmatic MDA, and peroxide/GSH ratio, as well as with high 5-mC levels. Moreover, low levels of LINE-1 methylation and 5-hmC/5-mC ratio were associated with MPN.

The multivariate logistic regression indicated that levels of peroxide (OR=1.12, 95% CI 1.03–1.22, p=0.011), 8-OHdG (OR=1.23, 95% CI 1.01–1.59, p=0.041), and 5-mC (OR=2.13, 95% CI 1.30–3.48, p=0.003), as well as the presence of two or more methylated genes (OR=4.52, 95% CI 3.39–8.80, p<0.001) were independent predictors of MDS development. Furthermore, the MPN development was associated with high levels of peroxide (OR=1.18, 95% CI 1.07–1.31, p<0.001) and plasmatic MDA (OR=4.24, 95% CI 2.93–5.00, p=0.001).

To analyze the diagnostic value of the independent predictors found in the multivariate logistic regression, we constructed ROC curves (Table 18). Regarding MDS patients, the levels of peroxide (AUC=0.877; 95% CI 0.800–0.955; p<0.001) and 8-OHdG (AUC=0.863; 95% CI 0.790–0.937; p<0.001) were accurate biomarkers to discriminate MDS patients from controls. The cut-off values were projected to be 3.28 μM and 34.7 ng/ml, respectively for peroxide and 8-OHdG levels, which achieved good sensitivity (73% and 72%), specificity (92% and 92%), and predictive value (PPV: 96% and 96%; NPV: 77% and 80%). However, the 5-mC had the greatest diagnostic value with the highest AUC (AUC=0.936, 95% CI 0.887–0.984, p<0.001). The optimal cut-off value of 5-mC was 0.48%, and this cut-off value achieved good sensitivity (82%), specificity (96%), and predictive value [positive predictive value (PPV): 93%; negative predictive value (NPV): 78%].

Table 17
Univariate and multivariate logistic regression analysis in patients with myelodysplastic syndrome and myeloproliferative neoplasms.

Disease	Biomarkers	Univariate analysis			Multivariate analysis		
		OR	95% CI	*p*-value	OR	95% CI	*p*-value
MDS	Peroxide	1.13	1.07 – 1.91	<0.001	1.12	1.03 – 1.22	0.011
	8-OH-dG	1.27	1.03 – 1.41	<0.001	1.23	1.01 – 1.59	0.041
	Peroxide/GSH	1.77	1.30 – 2.42	<0.001	–		
	Peroxide/TAS	1.09	1.04 – 1.14	<0.001	–		
	NO/GSH	2.03	1.45 – 6.34	0.003	–		
	NO/TAS	1.11	1.02 – 1.21	0.017	–		
	5-mC	2.43	1.62 – 3.64	<0.001	2.13	1.30 – 3.48	0.003
	5-hmC/5-mC	0.94	0.88 – 1.31	0.005	–		
	LINE-1	0.83	0.75 – 0.91	<0.001	–		
	Methylated *P15*	8.42	3.85 – 9.40	<0.001	–		
	Methylated *KEAP1*	3.83	1.04 – 7.18	0.044	–		
	2 ≥ methylated genes	7.51	4.59 – 9.34	<0.001	4.52	3.39 – 8.80	<0.001
MPN	Peroxide	1.18	1.08 – 1.30	<0.001	1.18	1.07 – 1.31	<0.001
	MDA (plasma)	6.28	5.05 – 8.33	0.002	4.24	2.93 – 5.00	0.001
	Peroxide/GSH	1.93	1.31 – 2.85	0.001	–		
	5-mC	2.22	1.43 – 3.47	<0.001	–		
	5-hmC/5-mC	0.21	0.15 – 0.79	0.021	–		
	LINE-1	0.78	0.68 – 0.90	0.001	–		

MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; OR, odds ratio; CI, confidence interval; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; LINE-1, long interspersed nuclear element-1; 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; NO, nitric oxide; GSH, reduced glutathione; TAS, total antioxidant status; MDA, malondialdehyde.

For MPN, the MDA levels were the most accurate diagnostic biomarker (AUC=0.981, 95% CI 0.944–1.000, p<0.001). The optimal cut-off value of MDA was 0.81 μM, and this cut-off value achieved good sensitivity (100%), specificity (96%), and predictive value (PPV: 92%, NPV: 96%). Peroxide levels were good MPN diagnostic biomarker (AUC=0.811; 95% CI 0.694–0.927; p<0.001), with a cut-off value at 3.56 μM, ROS levels achieved good sensitivity (88%), specificity (82%), and predictive value (PPV: 88%, NPV: 85%).

Table 18

Significant oxidative stress and DNA methylation parameters as diagnostic biomarker of myelodysplastic syndrome and myeloproliferative neoplasms

Disease	Biomarkers	AUC		Cut-off				
		value (95% CI)	*p*-value	value	SEN (%)	SPE (%)	PPV (%)	NPV (%)
MDS	Peroxide (μM)	0.877 (0.800 – 0.955)	< 0.001	32.8	73	92	96	77
	8-OH-dG (ng/ml)	0.863 (0.790 – 0.937)	< 0.001	34.7	72	92	96	80
	5-mC (%)	0.936 (0.887 –0.984)	< 0.001	0.48	82	96	93	78
MPN	Peroxide (μM)	0.981 (0.944 – 1.000)	< 0.001	35.6	100	96	92	96
	MDA (μM)	0.811 (0.694 – 0.927)	< 0.001	0.81	88	82	88	85

MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; OR, odds ratio; CI, confidence interval; 5-mC, 5-methylcytosine; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; AUC, area under the curve; CI, confidence interval; SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value.

5.4.6. Correlation between oxidative stress and DNA methylation in myeloid malignancies

To test the hypothesis that oxidative stress levels can be associated with gene promoter methylation, we analyzed oxidative stress parameters according to *P15*, *P16*, *DAPK*, and *KEAP1* gene promoter methylation profile. In addition, we correlated the levels of LINE-1, 5-mC and 5-hmC/5-mC with the oxidative stress parameters. We found that patients with methylated genes had higher peroxide levels and peroxide/GSH ratio than patients without methylation (Figure 37A–D). In patients with methylated *P15*, peroxide levels were 1.5-fold higher (4.58±0.32 μM, p<0.001) and peroxide/GSH ratio was 1.7-fold higher (0.74±0.06, p<0.001), when compared with patients without methylation (peroxide: 3.01±0.07 μM; peroxide/GSH: 0.43±0.06).

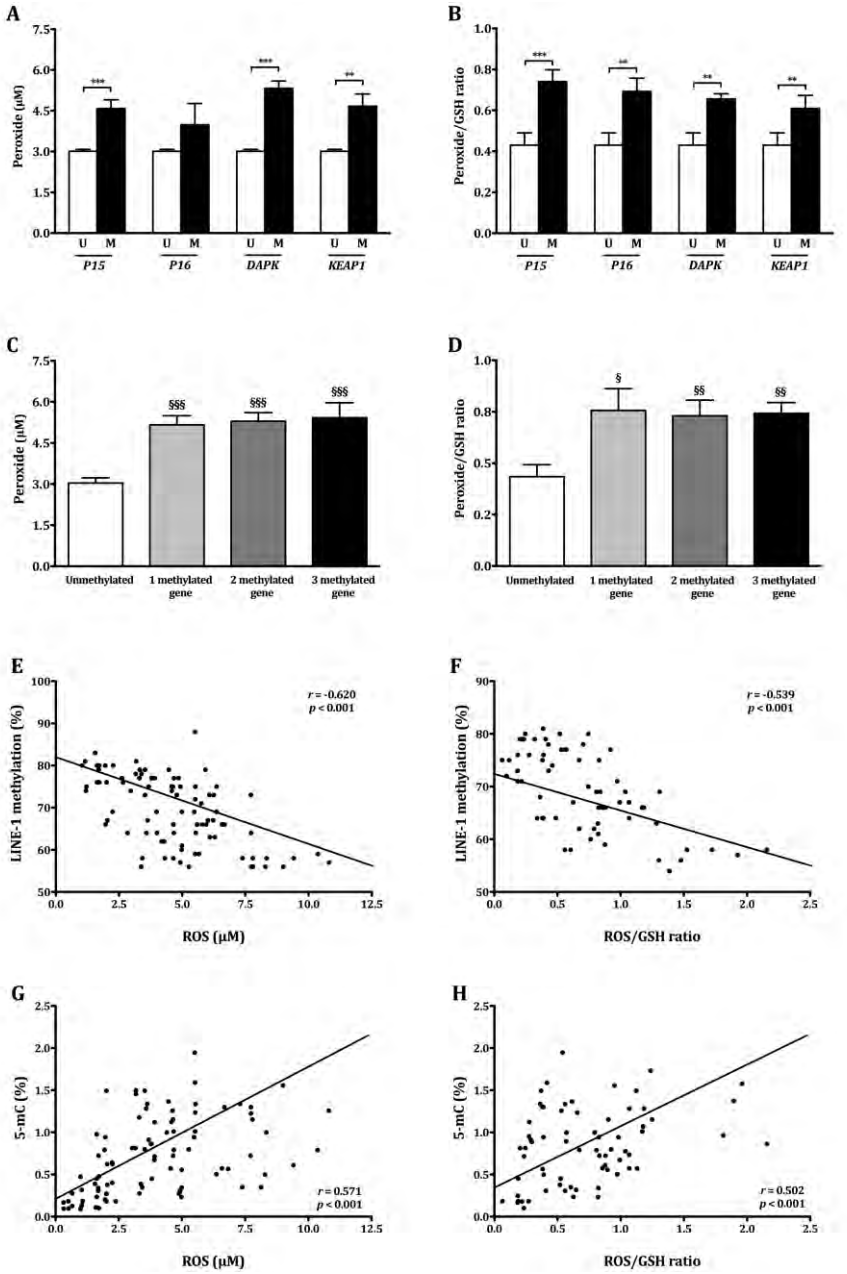


Figure 37. Analysis of oxidative stress parameters in myeloid neoplasm patients according to localized DNA methylation status (A – D), and the correlation of oxidative stress parameters with global DNA methylation (E-G). The levels of peroxide (A) and peroxide/ GSH (B) were analyzed in patients stratified according to their methylation status of *P15*, *P16*, *DAPK*, and *KEAP1* gene. Next, levels of peroxide (C) and peroxide/GSH (D) were examined in patients stratified according to the number of methylated genes. The following significant correlations between oxidative stress and DNA methylation were observed: long interspersed nucleotide elements 1 (LINE-1) methylation with peroxide levels (E); LINE-1 with peroxide/GSH ratio (F), 5-methylcytosine (5-mC) with peroxide levels (G), and 5-mC with peroxide/GSH ratio (H). Graphic A, C, E, and G: *n*=91; Graphic B, D, F, and H: *n*=69. GSH, reduced glutathione; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Moreover, patients with methylated *P16* had significantly higher peroxide/GSH ratio (0.69±0.07, p=0.006) than those without methylation (0.43±0.06). Likewise, peroxide levels and peroxide/GSH ratio were, respectively, 1.8- and 1.5-fold higher in patients with methylated *DAPK* (peroxide: 5.33±0.26 μM, p<0.001; peroxide/GSH ratio: 0.66±0.03, p=0.007) and 1.6- and 1.4-fold higher in patients with methylated *KEAP1* (peroxide: 4.67±0.45 μM, p=0.005; peroxide/GSH ratio: 0.61±0.06, p=0.009), compared with patients without methylation. Moreover, we observed that peroxide levels (Figure 37C) and peroxide/GSH ratio (Figure 37D) were significantly increased in patients with one (peroxide: 5.16± 0.34 μM, p<0.001; peroxide/GSH ratio: 0.76±0.11, p=0.007), two (peroxide: 5.29±0.31 μM, p<0.001; peroxide/GSH ratio: 0.73±0.08, p=0.001), or three methylated genes (peroxide: 5.42±0.55 μM, p<0.001; peroxide/GSH ratio: 0.74±0.05, p=0.001), independent of the gene, than those without methylation.

In myeloid malignancy patients, we also found that LINE-1 methylation was negatively correlated with peroxide levels (r=–0.620, p<0.001; Figure 37E) and with peroxide/GSH ratio (r=–0.539, p<0.001; Figure 37F). Furthermore, 5-mC levels were positively correlated with peroxide levels (r=0.571, p<0.001; Figure 37G) and with peroxide/GSH ratio (r=0.502, p<0.001; Figure 37H). We observed similar correlations in patients grouped according to WHO classification (MDS and MPN) and in controls (data not shown).

In multivariate logistic analysis (Table 19), we found that the peroxide/GSH ratio was associated with high levels of 5-mC (OR=1.30, CI 1.13–1.50, p<0.001) and with low levels of LINE-1 methylation (OR=0.85, 95% CI 0.77– 0.95, p=0.003). Our results also indicated that *P15* methylation was associated with high ROS/GSH ratio (OR=1.42, 95% CI 1.01–1.99, p=0.045) and with high peroxide levels (OR=1.05, 95% CI 1.00–1.09, p=0.047). Finally, the methylation of two or more tumor suppressor genes was associated with high peroxide levels (OR=1.02, 95% CI 1.00–1.05, p=0.049) and with high peroxide/GSH ratio (OR=1.39, 95% CI 1.15–2.12, p=0.006).

Table 19
Association of oxidative stress with DNA methylation in patients with myeloid neoplasms

Oxidative stress	DNA methylation	Multivariate analysis		
		OR	95% CI	*p*-value
Peroxide				
	Methylated *P15*	1.05	1.00 – 1.09	0.047
	2 ≥ methylated genes	1.02	1.00 – 1.05	0.049
Peroxide/GSH ratio				
	LINE-1	0.85	0.77 – 0.95	0.003
	5-mC	1.30	1.13 – 1.50	<0.001
	Methylated *P15*	1.42	1.01 – 1.99	0.045
	2 ≥ methylated genes	1.39	1.15 – 2.12	0.006

OR, odds ratio; CI, confidence interval; LINE-1, long interspersed nuclear element-1; 5-mC, 5-methylcytosine.

5.5. Discussion

Different cancer models suggest that high ROS levels contribute to cancer development and progression through genetic and epigenetic mechanisms. In regard of epigenetic events, ROS can induce both tumor suppressor hypermethylation and global DNA hypomethylation [Wu & Ni, 2015]. In the present study, we demonstrated a correlation between DNA methylation and oxidative stress levels in myeloid neoplasm patients. Firstly, we demonstrated that oxidative stress (intracellular levels of peroxides, GSH, and peroxides/GSH; plasmatic peroxide) as well as *P15* and *P16* methylation status were correlated in PB and BM samples from MDS patients and controls. Secondly, we confirmed our previous results showing that hypermethylation of *P15* and, to a lesser extent, *P16* gene promoters were correlated with ROS levels, as well as with peroxide/GSH ratio. Thirdly, we showed, for the first time, that hypermethylation of *DAPK* and *KEAP1* gene promoters, 5-mC levels, and LINE-1 methylation were associated with oxidative stress levels (peroxide and peroxide/GSH ratio) in these myeloid neoplasm patients.

Oxidative stress has been implicated in the pathogenesis of myelodysplastic syndrome and myeloproliferative neoplasms [De Souza *et al*., 2015; Durmus *et al*., 2013; Durmus *et al*., 2014; Gonçalves *et al*., 2015b; Hasselbalch *et al*., 2014; Vener *et al*., 2010]. Here, we found that patients with MDS and MPN had increased levels of peroxide; and MDS patients also had decreased levels of GSH and TAS. Several authors reported similar results, indicating that MDS patients were under oxidative stress [De Souza *et al*., 2015; Ghoti *et al*., 2007; Saigo *et al*., 2011]. Moreover, a significant disturbance in the free radical-antioxidant balance, in favor of the former, was observed in these patients, as previously observed by us [Gonçalves *et al*., 2015c]. Vener *et al*. [2010] and Durmus *et al*. [2013] observed that patients with PV and ET had increased levels of pro-oxidants and decreased levels of antioxidants. In comparison with control subjects, patients with MDS and MPN show oxidative stress damage. In fact, MDS patients showed increased levels of DNA damage (8-OHdG) and MPN patients had increased levels of MDA. These findings were also observed in other studies [Durmus *et al*., 2014; Honda *et al*., 2000; Jankowska *et al*. 2008; Novotna *et al*., 2009]. Furthermore, we found that patients on more advanced stages of MDS (RAEB-2 subtype) showed higher levels of DNA damage and GSH, in accordance with our previous report [Gonçalves *et al*., 2015b]. This fact may contribute to the establishment of a new homeostatic redox balance responsible for the activation of proliferative signaling pathways, which could lead RAEB-2 neoplastic cells to clonal expansion and contribute to their proliferation potential [Gonçalves *et al*., 2015b]. Moreover, patients with PV had higher peroxide levels and lower GSH content than patients with TE. Importantly, we found that peroxide levels and DNA damage were independent risk factors for MDS development, while peroxide and lipid peroxidation (MDA) were independent risk factors for MPN, and that 8-OHdG and MDA levels were found to be accurate diagnostic biomarkers for MDS and MPN, respectively. The confirmation of these results in independent studies will support and highlight the clinical usefulness of these oxidative damage parameters as diagnostic biomarkers. Altogether, these data support that oxidative stress is involved in the pathogenesis of MDS and, possibly, of MPN.

Epigenetic abnormalities, such as changes in DNA methylation pattern, are other key players in the development of myeloid malignancies. In the present study, MDS patients had a significant hypermethylation of *P15* and *DAPK* tumor suppressor genes. These results are similar to those reported in the literature [Gonçalves *et al*., 2015c; Qian *et al*., 2010; Solomon *et al*., 2008]. We also observed a significant hypermethylation of *KEAP1* gene promoter in MDS patients; however, any other study investigated the methylation status of this gene in hematological malignancies. KEAP1, the major negative regulator of cellular defenses against ROS, binds to NRF2 transcription factor, targeting it to degradation. The NRF2 activated by *KEAP1* hypermethylation leads to antioxidant enzymes expression and promotes cancer cells growth [Hanada *et al*., 2012]. These mechanisms may contribute to the development and progression of myeloid neoplasms. Here, we found that patients with MDS and, to a lesser extent, with MPN had frequently one or more methylated genes. These results suggest that abnormal tumor suppressor gene hypermethylation is a common event in myeloid malignancies.

Additionally, we found that patients with MDS and MPN had increased levels of 5-mC and decreased 5-hmC/5-mC ratio, as well as decreased levels of LINE-1 methylation. As mentioned above, epigenetic abnormalities are common events in myeloid malignancies; however, only few studies investigated their global methylation status (5-mC and/or 5-hmC levels). Bujko *et al.* [2013], Figueroa *et al*. [2010], and Yamazaki *et al*. [2012] reported that myeloid neoplasm patients had global hypermethylation, whereas Ko *et al.* [2010] observed a decrease in 5-hmC levels. The significant downregulation of *TET2* expression observed by Scopim-Ribeiro *et al.* [2014] in total bone marrow cells from MDS patients, may contribute, at least in part, to the decreased ratio of 5-hmC/5-mC observed here. Since repeated DNA sequences, such as LINE-1, are enriched in CpG sites, it has been considered that global hypomethylation largely arise from demethylation of these sequences [Ross *et al.*, 2010]. Therefore, global hypomethylation may be present in MDS and MPN patients, since a decrease in LINE-1 methylation was observed in our cohort of patients. This methylation pattern has been associated with genome instability, changes in chromatin structure, and increased frequency of copy

number abnormalities [Walker *et al*., 2011], features also found in MDS patients. To the best of our knowledge, only one study investigated the LINE-1 methylation in MDS patients. In this study, Römermann *et al*. [2008] reported that LINE-1 was hypermethylated in these patients. However, Bolatti *et al*. [2009] found that LINE-1 was hypomethylated in multiple myeloma, and Fabris *et al*. [2011] observed similar results in chronic myelogenous leukemia, other MPN subtype. Despite controversial results in global DNA methylation, the high levels of 5-mC found here could be a molecular explanation for the clinical success of hypomethylating agents, such as decitabine and azacytidine, in these diseases. Moreover, the aberrant DNA methylation pattern observed in the present study reinforces the role of epigenetics in myeloid neoplasm development.

DNA hypomethylation and hypermethylation can be induced by ROS [Wu & Ni, 2015]. The positive correlation of LINE-1 hypomethylation and oxidative stress, observed in our cohort of myeloid neoplasm patients, already has been demonstrated in patients with bladder cancer [Patchsung *et al*., 2012]. Several molecular mechanisms could explain the correlation of LINE-1 hypomethylation with oxidative stress. Firstly, cells with increased production of ROS, such as neoplastic cells, required high GSH levels. In this context, cells under oxidative stress may redirect *S*-adenosylmethionine (SAM) – the universal endogenous donor of methyl groups – from the methionine cycle in one-carbon metabolism to the synthetize GSH, resulting in a decreased availability of SAM to DNA methylation processes [Hitchler & Domann, 2007]. Moreover, the increased levels of 8-OHdG may explain the hypomethylation of LINE-1 observed in patients with MDS and MPN. The formation of this oxidized DNA base in a CpG site may not only inhibit the methylation of the adjacent cytosine by DNA methyltransferases, but can also induce a guanine to thymine transversion that will result in the loss of a CpG site [Kuchino *et al*., 1987; Patchsung *et al*., 2012; Weitzman *et al*., 1994]. Additionally, we found a positive correlation of 5-mC and tumor suppressor gene hypermethylation with oxidative stress (peroxide levels and peroxide/GSH ratio). Interestingly, the peroxide levels and the peroxide/GSH ratio were similar between patients with one, two, or three methylated genes. This fact suggests that

hypermethylation of tumor suppressor genes is not proportional to oxidative stress levels. Additionally, single-stranded breaks (SSBs) induced by oxidative DNA damage could be responsible for the increased levels of 5-mC and the hypermethylation of tumor suppressor genes observed in the present study. The incomplete repair of SSBs by base excision repair enzymes result in their conversion in double-strand breaks, signaling for *de novo* methylation and, therefore, contributing to unprogrammed methylation [Christman *et al*., 1995; Franco *et at*., 2008]. Furthermore, ROS can induce hypermethylation of tumor suppressor genes by the upregulation of DNMT1 and histone deacetylase 1, enzymes involved in gene silencing through promoter methylation and histone deacetylation [Kang *et al*., 2012a], as well as by the formation and relocalization of a silencing complex, composed by DNMT1, DNMT3B, SIRT1, and members of polycomb repressive complex 4, stimulating cancer-specific hypermethylation [O'Hagan *et al*., 2011]. Finally, superoxide anion may directly deprotonate cytosine at the carbon 5 position, allowing the formation of methylated cytosine through the nucleophilic attack of SAM [Afanas'ev, 2014].

In the present study a number of issues must be taken into account. Firstly, although we had enrolled almost all newly diagnosed patients during recruitment period, this study analyzed a relatively small cohort of patients, especially those with myeloproliferative neoplasms. This limitation did not allow us to evaluate oxidative stress and methylation parameters in all MDS and MPN subtypes or risk groups. Secondly, and despite the correlations found between PB and BM in a small number of MDS patients and controls, the same studies must be replicated in isolated neoplastic cells from bone marrow. Finally, part of the methodology used was qualitative. For instance, the tumor suppressor methylation analysis will be more informative using quantitative methodologies, such as MethyLight PCR or pyrosequencing.

In the present report, we demonstrated a correlation between DNA methylation and oxidative stress levels in myeloid neoplasm patients. We found an association of peroxide levels and peroxide/GSH ratio not only with tumor suppressor gene hypermethylation, but also with the LINE-1 hypomethylation. Moreover, we

showed, for the first time, that hypermethylation of *KEAP1* gene promoter is a frequent event in MDS patients. Overall, this study reflects the complexity of myeloid malignancies and points to a possible link between oxidative stress and DNA methylation, two common pathogenic mechanisms of MDS and MPN.

Chapter 6

Genetic variants involved in oxidative stress, base excision repair, DNA methylation, and folate metabolism pathways influence myeloid neoplasms susceptibility and prognosis

Gonçalves, A.C., *Alves, R., Baldeiras, I., Cortesão, E., Carda, J.P., Branco, C.C., Oliveiros, B., Loureiro, L., Pereira, A., Nascimento Costa, J.M., Sarmento-Ribeiro, A. B. & Mota-Vieira, L.*

(Under review, Sept 2015)

6.1. Abstract

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) share common features: elevated oxidative stress, DNA repair deficiency, and aberrant DNA methylation. We performed a hospital-based case-control study to evaluate the association in variants of genes involved in oxidative stress, folate metabolism, DNA repair, and DNA methylation with susceptibility and prognosis of these malignancies. To that end, 16 SNPs (one *per* gene: *CAT*, *CYBA*, *DNMT1*, *DNMT3A*, *DNMT3B*, *GPX1*, *KEAP1*, *MPO*, *MTRR*, *NEIL1*, *NFE2F2*, *OGG1*, *SLC19A1*, *SOD1*, *SOD2*, and *XRCC1)* were genotyped in 191 patients (101 MDS and 90 AML) and 261 controls. We also measured oxidative stress (reactive oxygen species/total antioxidant status ratio), DNA damage (8-hydroxy-2'-deoxyguanosine), and DNA methylation (5-methylcytosine) in 50 subjects (40 MDS and 10 controls). Results showed that five genes were associated with MDS (*GPX1*, *NEIL1*, *NFE2L2*, *OGG1*, and *SOD2*), two with AML (*DNMT3B* and *SLC19A1*), and two with both diseases (*CYBA* and *DNMT1*). We observed a correlation of *CYBA* TT, *GPX1* TT, and *SOD2* CC genotypes with increased oxidative stress levels, as well as *NEIL1* TT and *OGG1* GG genotypes with higher DNA damage. The 5-methylcytosine levels were negatively associated with *DNMT1* CC, *DNMT3A* CC, and *MTRR* AA genotypes, and positively with *DNMT3B* CC genotype. Furthermore, *DNMT3A*, *MTRR*, *NEIL1*, and *OGG1* variants modulated AML transformation in MDS patients. Additionally, *DNMT3A, OGG1, GPX1*, and *KEAP1* variants influenced survival of MDS and AML patients. Altogether, data suggest that genetic variability influence predisposition and prognosis of MDS and AML patients, as well AML transformation rate in MDS patients.

6.2. Introdution

Myeloid neoplasms are defined as a group of clonal disorders of hematopoietic stem or progenitor cells that include three clinicopathologic categories: myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and myeloproliferative neoplasms. These malignancies are initiated by the accumulation of randomly acquired genetic and epigenetic modifications that lead to cellular deregulated self-renewal, proliferation, and differentiation [Fröhling *et al.,* 2005; Kim *et al.,* 2015; Murati *et al.,* 2012]. MDS is a multistep neoplasm characterized by inefficient hematopoiesis that leads to peripheral cytopenias, and by an increased propensity to acute leukemia transformation [Adès *et al.,* 2014]. AML is defined as a clonal disorder of hematopoietic progenitor cells with a severe arrest of myeloid differentiation that can arise from different chromosomal aberrations or gene mutations [Mehdipour *et al.,* 2014]. However, MDS and AML share some common features, such as: oxidative stress [Hole *et al.,* 2011; Sardina *et al.,* 2012; Zhou *et al.,* 2013], DNA repair deficiency [Economopoulou *et al.,* 2011; Esposito & So, 2014; Zhou *et al.,* 2015], and aberrant DNA methylation [Meldi & Figueroa, 2014; Schoofs *et al.,* 2014; Woods & Levine, 2015].

Oxidative stress is a major player in carcinogenesis [Sosa *et al.,* 2013; Valko *et al.,* 2007]. It is caused by an imbalance between reactive oxygen species (ROS) and antioxidant defenses, which neutralize the former molecules [Birben *et al.,* 2012; Klaunig *et al.,* 2010; Valko *et al.,* 2007]. A certain level of ROS is essential for cell signaling processes. Exceeding the optimal level of ROS may result in DNA damage, lipid peroxidation, and protein oxidation [Klaunig *et al.,* 2010; Valko *et al.,* 2007]. To prevent DNA damage and its potential deleterious effect, cells have several defense mechanisms [Birben *et al.,* 2012]. These include DNA damage sensing and repair systems, cell cycle arrest, apoptosis, and senescence [Zhou *et al.,* 2015]. DNA repair mechanisms are essential in maintaining genomic integrity in cells under oxidative stress, since defective DNA repair leads to genetic and epigenetic abnormalities [Chung *et al.,* 2014; Cuozzo *et al.,* 2007; Zhou *et al.,* 2015]. The 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most commonly oxidized bases in DNA. The accumulation of 8-OHdG within the genome is minimized by the

effectiveness of several DNA repairing mechanisms, such as: homologous recombination, non-homologous end joining, base excision repair (BER), nucleotide excision repair, and mismatch repair (MMR) [Economopoulou *et al.,* 2011].

DNA methylation is a reversible epigenetic mechanism that regulates gene expression without altering the gene sequence [Meldi & Figueroa, 2014; Woods & Levine, 2015]. Under normal physiological conditions, CpG islands are unmethylated, but repetitive genomic sequences and introns are hypermethylated [Akhavan-Niaki & Samadani, 2013; Esteller, 2008; Taby & Issa, 2010]. However, cancer cells are characterized by global hypomethylation and localized hypermethylation. In addition to oxidative stress and DNA repair, folate levels also influence DNA methylation. Folates are an important component in the synthesis of S-adenosylmethionine – the methyl group donor in DNA methylation reactions –, and its deficiency has been associated not only with global hypomethylation, but also with localized hypermethylation [Akhavan-Niaki & Samadani, 2013].

Inherited genomic variations, such as single nucleotide polymorphisms (SNPs), may influence genetic susceptibility to cancer. The identification of susceptibility genes can improve the understanding of leukemogenic mechanisms, offering the possibility to detect individuals at risk of developing hematological malignancies, as well as to identify potential biomarkers of disease progression and survival. Moreover, the association of oxidative stress and DNA damage repair with methylation is supported by the following observations: oxidative stress induces DNA damage, DNMTs form a complex with DNA replication and repair factors, DNMT1 is recruited to DNA repair sites, and global inhibition of methylation leads to genome instability [Chung *et al.,* 2014; Kitkumthorn & Mutirangura, 2011; O'Hagan *et al.,* 2011].

Since myeloid neoplasms are multifactorial disorders, the study of specific gene variants involved in different pathways may contribute to a deeper understanding of their molecular mechanisms. In this context, we investigated whether genetic

polymorphisms in oxidative stress, DNA damage repair, DNA methylation, and folate metabolism, individually or combined, could predispose individuals to MDS and AML development. In addition, we analyzed the possible association of these variants with disease prognosis (rate of MDS transformation into AML and survival).

6.3. Methods

6.3.1. Ethical Statement

The present study was conducted in accordance with the Helsinki declaration. The Ethics Committee of Faculty of Medicine of University of Coimbra (Coimbra, Portugal) approved all research procedures. Prior to enrollment, the participants provided their informed consent for participation. The international ethical guidelines of confidentiality, anonymity of personal data, and abandonment option in the case of expressed will were followed.

6.3.2. Study Population

We conducted a hospital-based case-control study that included 191 myeloid neoplasms cases (101 MDS patients and 90 AML patients), as well as 261 control individuals. All cases and controls were enrolled from two hospitals of the central region of Portugal – "Centro Hospitalar e Universitário de Coimbra, EPE (CHUC, EPE)" and "Hospital Distrital da Figueira da Foz, EPE (HDFF, EPE)" –, from June 2010 to March 2013. Patients were diagnosed according to the World Health Organization classification of myeloid neoplasms and acute leukemia [Brunning *et al.,* 2008; Döhner *et al.,* 2010]. Controls were selected among healthy blood donors and from individuals presenting no history of cancer in these two hospitals, during the same period of time. In addition, a sub-cohort of 50 subjects (40 MDS patients and 10 controls) was used to perform genotype-phenotype studies. In order to control the effects of confounders, cases and controls were matched based on

gender and age (± 5 years). Basic demographic characteristics of patients and controls are shown in Table 20.

Table 20
Basic demographic and clinical characteristics of myeloid neoplasm patients and controls

Characteristics	Cases (*n* = 191)		Controls (*n* = 261)	
	n	%	*n*	%
Demographic data				
Gender				
Male	97	50.8	135	51.7
Female	94	49.2	126	48.3
Age (years)				
Median age	70		68	
Range	16–90		19–85	
Clinical data				
Myelodysplastic syndrome (*n*=101)				
RCUD	17	16.8		
RCMD	51	50.5		
RARS	11	10.9		
RAEB-1	8	7.9		
RAEB-2	10	9.9		
del(5q)	4	4.0		
Acute myeloid leukemia (*n*=90)				
AML with minimal differentiation	7	7.8		
AML without maturation	7	7.8		
AML with maturation	16	17.8		
APL with t(15;17)(q22;q12); PML-RARA	13	14.4		
Acute myelomonocytic leukemia	12	13.3		
Acute monoblastic/monocytic leukemia	13	14.4		
Acute erythroid leukemia	2	2.2		
AML with myelodysplasia-related changes	20	22.2		

AML, acute myeloid leukemia; RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RAEB-1, refractory anemia with excess blasts type 1; RAEB-2, refractory anemia with excess blasts type 2; del(5q), myelodysplastic syndrome with isolated del(5q); APL, acute promyelocytic leukemia

6.3.3. Genes and SNPs Selection

The selection of candidate genes was based on their involvement in oxidative stress, DNA methylation, base excision repair, and folate metabolism pathways. Sixteen SNPs (one per gene) were chosen according to the following criteria: 1.

known or promising relevance to cancer development, 2. reported association to human cancer, 3. minor allele frequency (MAF) ≥5% in Caucasians in public databases or published literature, 4. validated allele substitutions, or 5. previously published functional changes associated to allele substitutions. For this purpose, we used the Human Genome Epidemiology (HuGE) Literature Finder (http://www.hugenavigator.net), a database that provides information on the prevalence of genetic variants populations, gene-disease associations, as well as interactions of gene-gene and gene-environment [Yu *et al.,* 2008]. Relevant information of the 16 selected SNPs is summarized in Table 21.

6.3.4. DNA extraction and SNP genotyping

Human genomic DNA from cases and controls was extracted from whole blood samples, collected into EDTA tubes, as previously describe by Bartlett & White [2003]. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), and 100 ng of DNA was used in each genotyping assay.

We genotyped eight SNPs using PCR-RFLP assays: *CAT* (rs1001179), *CYBA* (rs4673), *GPX1* (rs1050450), *MPO* (rs2333227), *OGG1* (rs1052134), *SLC19A1* (rs1051266), *SOD1* (rs2070424), and *SOD2* (rs4880). The PCR-RFLP primers and protocols were adapted from previously described methods [Dervieux *et al.,* 2004; Inoue *et al.,* 1998; Li *et al.,* 2005; London *et al.,* 1997; Sutton *et al.,* 2006; Tarng *et al.,* 2001; Zhang *et al.,* 2011]. The other eight SNPs [*DNMT1* (rs759920), *DNMT3A* (rs2289195), *DNMT3B* (rs2424908), *KEAP1* (rs11085735), *MTRR* (rs162036), *NEIL1* (rs4462560), *NFE2L2* (rs13001694), and *XRCC1* (rs1799782)] were genotyped through tetra-primer-ARMS-PCR assays, using primers designed with BatchPrimer3 1.0 software (http://probes.pw.usda.gov/batchprimer3/) [You *et al.,* 2005]. ARMS-PCR assays were performed as described in Supplementary Table S1). For both PCR techniques, results were first confirmed by direct sequencing. Samples previously sequenced and found to contain the three possible genotypes

were used as positive controls in each genotype assay. A negative control containing water instead of genomic DNA was also tested simultaneously. Genotyping was repeated in approximately 10% of total samples to check for genotyping accuracy.

Table 21
Relevant information of the 16 selected candidate SNPs

Gene symbol*	Pathway	dbSNP	Chr. position	Variants	Molecular consequence	Global MAF§
CAT	Oxidative stress	rs1001179	11:34438684	c.-330C>T	2KB Upstream	0.215 (T)
CYBA	Oxidative stress	rs4673	16:88646828	c.214T>C; p.Tyr72His	Missense	0.374 (T)
DNMT1	DNA methylation	rs759920	19:10174102	c.649-197T>C	Intron	0.472 (T)
DNMT3A	DNA methylation	rs2289195	2:25240614	c.2173+26C>T	Intron	0.402 (T)
DNMT3B	DNA methylation	rs2424908	20:32772577	c.-6-7741C>T	Intron	0.159 (T)
GPX1	Oxidative stress	rs1050450	3:49357401	c.599C>T; p.Pro200Leu	Missense	0.346 (T)
KEAP1	KEAP1–NRF2	rs11085735	19:10491504	c.1325+73T>G	Intron	0.051 (T)
MPO	Oxidative stress	rs2333227	17:58281401	c.-643G>A	2KB Upstream	0.299 (A)
MTRR	Folate metabolism	rs162036	5:7885846	c.1049A>G; p.Lys350Arg	Missense	0.136 (G)
NEIL1	Base excision repair	rs5745920	15:75353009	c.976+308T>C	Intron	0.351 (T)
NFE2L2	KEAP1–NRF2	rs13001694	2:177254262	c.-4+9141T>C	Intron	0.430 (C)
OGG1	Base excision repair	rs1052133	3:9757089	c.977C>G; p.Ser326Cys	Missense	0.192 (G)
SLC19A1	Folate metabolism	rs1051266	21:45537880	c.80A>G; p.His27Arg	Missense	0.486 (G)
SOD1	Oxidative stress	rs2070424	21:31667007	c.240-251A>G	Intron	0.070 (G)
SOD2	Oxidative stress	rs4880	6:159692840	c.47T>C; p.Val16Ala	Missense	0.444 (C)
XRCC1	Base excision repair	rs1799782	9:43553422	c.580C>T; p.Arg194Trp	Missense	0.092 (T)

*According to HUGO Gene Nomenclature Committee (HGNC). †According to Sequence Ontology. M/m, major/minor allele. *CAT*, Catalase; *CYBA*, Cytochrome b-245, alpha polypeptide; *DNMT1*, DNA (cytosine-5-)-methyltransferase 1; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3 alpha; DNMT3B, DNA (cytosine-5-)-methyltransferase 3 beta; GPX1, Glutathione peroxidase 1; *KEAP1*, Kelch-like ECH-associated protein 1; *MPO*, Myeloperoxidase; *MTRR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; *NEIL1*, Nei endonuclease VIII-like 1 (E. coli); *NFE2L2*, Nuclear factor, erythroid 2-like 2; *OGG1*, 8-oxoguanine DNA glycosylase; *SLC19A1*, Solute carrier family 19 (folate transporter), member 1; *SOD1*, Superoxide dismutase 1 (soluble); *SOD2*, Superoxide dismutase 2 (mitochondrial); *XRCC1*, X-ray repair complementing defective repair in Chinese hamster cells 1; Trans Reg, transcriptional regulation; Prot Coding, protein coding; Splicing Reg, splicing regulation.

Table 21
Relevant information of the 16 selected candidate SNPs (*Continued*)

Gene symbol*	SNP functional effect			Reported association		
	FS‡	Category	Pathogenicity	Cancer type	OR	95% CI
CAT	0.242	Trans Reg	Changed	CML	0.6	0.4–0.9
CYBA	0.908	Prot Coding Splicing Reg	Deleterious Changed	NHL	1.6	1.1–2.4
DNMT1	0.208	Trans Reg	Changed	Ovarian	1.9	1.2–3.2
DNMT3A	0.000	Trans Reg	Not changed	–	–	–
DNMT3B	–	–	–	AML	0.6	0.4–0.9
GPX1	0.540	Prot Coding Splicing Reg	Deleterious Changed	Breast	1.9	1.1–3.3
KEAP1	–	–	–	–	–	–
MPO	0.208	Trans Reg	Changed	Lung	0.6	0.4–0.9
MTRR	0.955	Prot Coding Splicing Reg	Neutral Changed	–	–	–
NEIL1	0.050	Trans Reg	Changed	–	–	–
NFE2L2	0.101	Trans Reg	Changed	–	–	–
OGG1	0.294	Prot Coding Splicing Reg	Neutral Changed	Colorectal	1.8	1.2–2.7
SLC19A1	0.500	Prot Coding Splicing Reg Trans Reg	Neutral Changed Changed	ALL	2.1	1.3–3.2
SOD1	0.208	Trans Reg	Changed	–	–	–
SOD2	0.330	Prot Coding Splicing Reg	Neutral Changed	Leukemia	1.9	1.1–3.4
XRCC1	0.842	Prot Coding Splicing Reg	Deleterious Changed	NHL	1.5	1.1–2.0

‡According to F-SNP. The F-SNP functional score (FS) incorporates functional effects of SNPs predicted at splicing, transcriptional, translational, and post-translational level. The functional effect from protein coding category was predicted using PredictSNP, and from transcriptional and splicing regulation categories were predicted by F-SNP. §MAF source: 1000 Genomes (Caucasians/European/Iberian population in Spain).
CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.
Association data are derived from the following references: Arslan *et al.* [2011], Bănescu *et al.* [2014a], Chen *et al.* [2003], De Jonge *et al.* [2009], Lan *et al.* [2007], Méplan *et al.* [2014], Mostowska *et al.* [2013], Przybylowska *et al.* [2013], Vineis *et al.* [2007], and Zheng *et al.* [2013].

6.3.4. Oxidative stress quantification

The plasmatic peroxide levels were measured by a colorimetric method (Thermo Scientific Pierce Quantitative Peroxide Assay Kit – lipid-compatible formulation, Life Technologies), based on the oxidation of ferrous to ferric ion in the presence of xylenol orange. Plasmatic peroxide were detected by spectrophotometry at

595 nm, and determined by comparison with a hydrogen peroxide standard curve, as described by the manufacturer, in a SynergyTM multi-mode microplate reader (BioTek Instruments, Winooski, USA). Total antioxidant status (TAS) was evaluated by a chromogenic method (Randox Laboratories), based on the plasma capacity to inhibit the formation of the $ABTS^{+}$ radical cation (2,2'-azino-di-[3-etilbenzotiazolin sulfonate]), and detected at 600 nm, as described by the manufacturer. Since oxidative stress results from the imbalance between free radicals and antioxidants, we calculated the peroxide/TAS ratio to evaluate the effect of genotypes in oxidative stress.

The plasmatic 8-OHdG levels (DNA damage levels) were measured using a competitive quantitative ELISA Kit (8-hydroxy-2-deoxyguanosine ELISA Kit, Abcam), according to manufacturer instructions. The assay is based on the competition between 8-OHdG and an 8-OHdG-acetylcholinesterase conjugate for a limited amount of 8-OHdG monoclonal antibody. The colorimetric intensity was determined spectrophotometrically in a Synergy™ multi-mode microplate reader (BioTek Instruments, Winooski, USA), and its value was inversely proportional to the amount of free 8-OHdG in plasma.

6.3.5. DNA methylation analysis

Methylation was determined in DNA samples by specific ELISA assays (5-methylcytosine DNA ELISA kit, Enzo), according to manufacturer's protocol. These assays use monoclonal antibodies against 5-methylcytosine (5-mC) to obtain the percentage of 5-mC in total DNA. Genomic DNA obtained from peripheral blood was used to quantify 5-mC levels.

6.3.6. Statistical analysis

Normality and differences of confounding variables (age and gender) between groups (myeloid neoplasms vs. controls, MDS *vs*. controls, and AML vs. controls)

were assessed by the Kolmogorov-Smirnov test and nonparametric Mann-Whitney U test, respectively. Allele and genotype frequencies were determined by direct counting. The Hardy-Weinberg equilibrium (HWE) in the study groups (MDS, AML, and controls) was determined by Arlequin software v.3.5.1.2 [Excoffier & Lischer, 2010]. Genotypic profile frequencies were inferred using the maximum likelihood method (expectation maximization – EM – algorithm). Furthermore, the Pearson's chi-square test was calculated in order to compare the allele, genotype, and genotypic profile frequencies among groups (MDS *vs*. controls, and AML *vs*. controls). The association between genotypes and MDS or AML was analyzed by calculating the odds ratio (OR) and its 95% confidence interval (CI) by applying the Fisher's exact test with GraphPad Prism version 5.0 or unconditioned logistic regression with SPSS version 22.0. The Kaplan-Meier method was performed to estimate time to AML transformation and overall survival of patients dichotomized according to their genotypes using SPSS. Differences in survival were tested through log rank statistic. The hazard ratio (HR) and its 95% CI were calculated using the Cox proportional hazard model. All statistical analyses were two-sided, and a $p<0.05$ was considered statistically significant.

6.4. Results

6.4.1. Characteristics of the study groups

The present study enrolled a myeloid neoplasms group (n=191) and healthy control group (n=261). The myeloid neoplasms group, with a median age of 70 years (range 16–90), was composed of 97 (50.8%) males and 94 (49.2%) females. It included a subgroup of MDS patients (n=101), with a median age of 74 years (range 22–89), of which 55 (54.5%) were males and 46 (45.5%) were females, and a subgroup of AML patients (n=90), with a median age of 65 years (range 16–90), of which 42 (46.7%) were males and 48 (53.3%) were females. The healthy control group consisted of 135 (51.7%) males and 126 (48.3%) females, and had a median age of 68 years (range 19–85). In order to avoid confounding bias and to confirm

adequate matching between groups, we assessed differences in the demographic features. There were no significant differences between cases (MDS and AML) and controls in terms of their age (p=0.108 and p=0.112, respectively) or gender (p=0.467 and p=0.292, respectively). These results indicated adequate group matching.

According to 2008 WHO classification, the MDS subgroup included patients with the following subtypes: refractory cytopenia with unilineage dysplasia (RCUD; n=17, 16.8%), refractory cytopenia with multilineage dysplasia (RCMD; n=51, 50.5%), refractory anemia with ring sideroblasts (RARS; n=11, 10.9%), refractory anemia with excess blasts type 1 (RAEB-1; n=8, 7.9%), refractory anemia with excess blasts type 2 (RAEB-2; n=10, 9.9%), and myelodysplastic syndrome with isolated del(5q) (n=4, 4.0%). The distribution of MDS cases according IPSS risk groups showed a predominance of low risk patients [low risk (low + int-1), n=76 (87%); high risk (int-2 + high), n=11 (13%)].The AML subgroup included patients with the following diagnosis: AML with minimal differentiation (n=7, 7.8%), AML without maturation (n=7, 7.8%), AML with maturation (n=16, 17.8%), acute promyelocytic leukemia with t(15;17)(q22;q12) (n=13, 14.4%), acute myelomonocytic leukemia (n=12, 13.3%), acute monoblastic/monocytic leukemia (n=13, 14.4%), acute erythroid leukemia (n=2, 2.2%), and AML with myelodysplasia-related changes (n=20, 22.2%).

6.4.2. Allele and genotype distribution

In order to evaluate the contribution of variants in genes involved in oxidative stress, DNA repair, DNA methylation, and folate metabolism to the risk of MDS and AML development, we investigated whether selected SNPs were associated with these specific malignancies. We explored disease association with alleles (minor allele was compared with major allele as reference) and genotypes. The genotype association analyses were performed according to three genetic models: 1. codominant model (each genotype was compared with homozygous of major

allele), 2. dominant model (minor allele carriers were compared with homozygous of major allele), and 3. recessive model (homozygous of minor allele were compared with major allele carriers). The allele distribution of selected SNPs in patients with MDS and AML, as well as in controls is shown in Table 22.

Table 22
Allele distribution of selected SNPs in myeloid neoplasms and controls, and their association with risk of myelodysplastic syndrome and acute myeloid leukemia

Gene	Minor allele‡	MDS			AML			Controls
		MAF	OR (95% CI)	*p-value*	MAF	OR (95% CI)	*p*-value	MAF
CAT	T	0.233	0.90 (0.61–1.31)	0.631	0.200	0.74 (0.49–1.12)	0.158	0.253
CYBA	T	0.277	1.40 (0.97–2.04)	0.078	0.339	**1.88 (1.29–2.72)***	**0.001**	0.215
DNMT1	T	0.510	**1.60 (1.15–2.21)***	**0.006**	0.439	1.20 (0.85–1.69)	0.333	0.395
DNMT3A	T	0.084	0.78 (0.44–1.38)	0.489	0.122	1.18 (0.70–2.00)	0.580	0.105
DNMT3B	T	0.163	0.98 (0.63–1.51)	1.000	0.100	**0.56 (0.32–0.95)†**	**0.030**	0.167
GPX1	T	0.490	1.31 (0.94–1.81)	0.113	0.367	0.79 (0.56–1.12)	0.188	0.423
KEAP1	T	0.069	1.14 (0.60–2.19)	0.734	0.830	1.39 (0.74–2.64)	0.303	0.061
MPO	A	0.302	1.53 (0.99–2.21)	0.056	0.272	1.32 (0.90–1.95)	0.184	0.220
MTRR	G	0.193	1.12 (0.74–1.70)	0.592	0.206	1.21 (0.79–1.85)	0.374	0.176
NEIL1	T	0.579	**1.52 (1.10–2.11)***	**0.013**	0.500	1.11 (0.79–1.55)	0.604	0.475
NFE2L2	C	0.515	**1.63 (1.17–2.26)***	**0.004**	0.467	1.34 (0.95–1.89)	0.096	0.395
OGG1	G	0.436	**1.58 (1.34–2.21)***	**0.007**	0.317	0.95 (0.66–1.37)	0.854	0.328
SLC19A1	G	0.490	1.24 (0.89–1.72)	0.212	0.622	**2.12 (1.50-3.00)***	**<0.001**	0.437
SOD1	G	0.054	0.54 (0.28–1.07)	0.075	0.067	0.67 (0.35–1.30)	0.287	0.096
SOD2	C	0.599	**1.61 (1.16–2.24)***	**0.005**	0.506	1.10 (0.79–1.55)	0.604	0.481
XRCC1	T	0.099	1.36 (0.77–2.40)	0.291	0.056	0.73 (0.36–1.49)	0.498	0.075

‡Minor allele of controls and database (Caucasians/European/Iberian population in Spain). Bold indicates statistically significant association (* susceptibility or † protection). MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia.

In the control group, the allele frequencies of selected SNPs were similar to those reported in other European populations, except for *DNMT3A.* For this SNP, we detected a lower T allele frequency in control group (0.105) in comparison to the one stated in 1000 Genomes database (Caucasians/European/Iberian population in Spain: 0.402; $p<0.001$). Moreover, we observed the lowest minor allele frequency (MAF) for the *KEAP1* gene (0.061) and the highest for *SOD2* (0.481).

Based on disease association analysis, the predisposition to MDS was increased in individuals carrying the following alleles: *DNMT1* T (OR=1.60, 95% CI 1.15–2.21, *p*=0.006), *NEIL1* T (OR=1.52, 95% CI 1.10–2.11, *p*=0.013), *NFE2L2* C (OR=1.63, 95% CI 1.17–2.26, *p*=0.004), *OGG1* G (OR=1.58, 95% CI 1.34–2.21, *p*=0.007), and *SOD2* C (OR=1.61, 95% CI 1.16–2.24, *p*=0.005). Furthermore, the minor allele of *CYBA* (T allele, OR=1.88, 95% CI 1.29–2.72, *p*=0.001) and *SLC19A1* (G allele, OR=2.12, 95% CI 1.50–3.00, *p*<0.001) genes were associated with an increased risk for the development of AML, while the minor allele of *DNMT3B* (T allele, OR=0.56, 95% CI 0.32–0.95, *p*=0.030) was found to have a protective effect against this malignancy.

The genotype frequencies of selected SNPs were also compared (significant associations are shown in Table 23). All genotypes in the study groups and subgroups were in HWE. The *XRCC1* CC genotype was only detected in the MDS group, and *SOD1* GG or *KEAP1* AA genotypes were absent in this group. The *CYBA* TT genotype was associated with the predisposition to MDS (codominant model: OR=3.19, 95% CI 1.23–8.27, *p*=0.017; recessive model: OR=3.08, 95% CI 1.21–7.81, *p*=0.018) and AML (codominant model: OR=5.72, 95% CI 2.32–14.10, *p*<0.001; recessive model: OR=5.16, 95% CI 2.15–12.38, *p*<0.001). The *DNMT1* TT genotype was also found to be a risk factor for MDS (codominant model: OR=2.49, 95% CI 1.30–4.79, *p*=0.006; recessive model: OR=1.90, 95% CI 1.10–3.30, *p*=0.022), and the CT genotype was associated with an increased risk of AML development (codominant model: OR=1.76, 95% CI 1.01–3.05, *p*=0.045).

Additionally, the predisposition to MDS was increased in carriers of the following genotypes: *GPX1* TT (recessive model: OR=2.02, 95% CI 1.16–3.51, *p*=0.013), *NEIL1* TT (codominant model: OR=2.14, 95% CI 1.13–4.03, *p*=0.019; recessive model: OR=2.26, 95% CI 1.37–3.73, *p*=0.001), *NFE2L2* CC (codominant model: OR=2.83, 95% CI 1.42–5.62, *p*=0.003; recessive model: OR=1.99, 95% CI 1.13–3.52, *p*=0.018), *NFE2L2* CT and CC (dominant model: OR=1.96, 95% CI 1.14–3.34, *p*=0.014), *OGG1* GG (codominant model: OR=2.32, 95% CI 1.15–4.70, *p*=0.019), *OGG1* GC and GG (codominant model: OR=1.81, 95% CI 1.08–3.03, *p*=0.023; dominant model: OR=1.92, 95% CI 1.18–3.13, *p*=0.009), *SOD2* CC (OR=2.55, 95% CI

1.30–4.98, *p*=0.006; recessive model: OR=1.98, 95% CI 1.20–3.28, *p*=0.007), and *SOD2* CT (codominant model: OR=1.78, 95% CI 1.05–3.01, *p*=0.033). Finally, *SLC19A1* AG (codominant model: OR=2.59, 95% CI 1.29–5.18, *p*=0.007), GG genotypes (codominant model: OR=4.60, 95% CI 2.19–9.65, *p*<0.001; recessive model: OR=2.38, 95% CI 1.41–4.02, *p*=0.001), and G carrier genotypes (dominant model: OR=3.19, 95% CI 1.65–6.18, *p*=0.001) were found to be risk factors for AML, while *DNMT3B* T carrier genotypes conferred a protective effect against AML development (dominant model: OR=0.55, 95% CI 0.30–0.99, *p*=0.045).

Table 23
Significant genotype distribution of selected SNPs in myeloid neoplasm patients and controls, and their association with risk of myelodysplastic syndrome and acute myeloid leukemia

Gene:	MDS				AML				Controls	
dbSNP	*n*	%	OR (95% CI)	*p*-value	*n*	%	OR (95% CI)	*p*-value	*n*	%
***CYBA*: rs4673**										
CC§	55	54.5	Ref.		43	47.8	Ref.		158	60.5
CT§	36	35.6	1.10 (0.67–1.80)	0.703	33	36.7	1.29 (0.77–2.17)	0.338	94	36.0
TT§	10	9.9	**3.19 (1.23–8.27)***	**0.017**	14	15.6	**5.72 (2.32–14.10)***	**<0.001**	9	3.4
CC + CT¶			1.28 (0.81–2.04)	0.292			1.78 (0.99–2.72)	0.053		
TT‡			**3.08 (1.21–7.81)***	**0.018**			**5.16 (2.15–12.38)***	**<0.001**		
***DNMT1*: rs759920**										
CC§	25	24.8	Ref.		24	26.7	Ref.		97	37.2
CT§	49	48.5	1.56 (0.90–2.70)	0.114	53	58.9	**1.76 (1.01–3.05)***	**0.045**	122	46.7
TT§	27	26.7	**2.49 (1.30–4.79)***	**0.006**	13	14.4	1.25 (0.58–2.69)	0.567	42	16.1
CC + CT¶			1.80 (0.97–3.02)	0.052			1.63 (0.96–2.76)	0.072		
TT‡			**1.90 (1.10–3.30)***	**0.022**			0.88 (0.45–1.73)	0.711		
***DNMT3B*: rs2424908**										
CC§	73	82.2	Ref.		73	81.1	Ref.		183	70.1
CT§	23	22.8	0.84 (0.49–1.44)	0.518	16	17.8	0.58 (0.32–1.07)	0.080	69	26.4
TT§	5	5.0	1.39 (0.45–4.30)	0.564	1	1.1	0.28 (0.04–2.52)	0.229	9	3.4
CC + CT¶			0.90 (0.37–1.50)	0.685			**0.55 (0.30–0.99)†**	**0.045**		
TT‡			1.46 (0.48–4.46)	0.508			0.32 (0.04–2.52)	0.276		

The OR (95% CI) and *p*-value were calculated by logistic regression according the following genetic models: codominant model (MM vs. MM, MM vs. Mm, and MM vs. mm, *i.e.* each genotype was compared with major allele homozygous genotype as reference); dominant model (MM vs. Mm + mm); and recessive model (MM + Mm vs. mm). Bold indicates statistically significant association (* susceptibility or † protection). §, codominant model; ¶, dominant model; ‡, recessive model; M, major allele; m, minor allele; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; OR, odds ratio; CI, confidence interval; Ref., reference.

Table 23

Significant genotype distribution of selected SNPs in myeloid neoplasm patients and controls, and their association with risk of myelodysplastic syndrome and acute myeloid leukemia (*Continued*)

Gene:	MDS				AML				Controls	
dbSNP	*n*	%	OR (95% CI)	*p*-value	*n*	%	OR (95% CI)	*p*-value		
***GPX1*: rs1050450**										
CC§	29	28.7	Ref.		34	37.8	Ref.		80	30.7
CT§	45	44.6	0.88 (0.51–1.51)	0.645	46	51.1	0.77 (0.46–1.29)	0.320	14	54.0
TT§	27	26.7	1.86 (0.98–3.56)	0.060	10	11.1	0.59 (0.26–1.31)	0.194	40	15.3
CC + CT¶			1.10 (0.66–1.82)	0.718			0.73 (0.44–1.20)	0.214		
TT‡			**2.02 (1.16–3.51)***	**0.013**			0.69 (0.33–1.45)	0.326		
***NEIL1*: rs5745920**										
CC§	22	21.8	Ref.		18	20.0	Ref.		68	26.1
CT§	41	40.6	0.92 (0.51–1.66)	0.779	54	60.0	1.48 (0.81–2.71)	0.207	13	52.9
TT§	38	37.6	**2.14 (1.13–4.03)***	**0.019**	18	20.0	1.24 (0.59–2.60)	0.576	55	21.1
CC + CT¶			1.27 (0.73–2.19)	0.400			1.41 (0.79–2.53)	0.251		
TT‡			**2.26 (1.37–3.73)***	**0.001**			0.94 (0.52–1.70)	0.829		
***NFE2L2*: rs13001694**										
TT§	22	21.8	Ref.		26	28.9	Ref.		92	35.2
TC§	54	53.5	1.71 (0.97–3.00)	0.061	44	48.9	1.18 (0.68–2.05)	0.559	13	50.6
CC§	25	24.8	**2.83 (1.42–5.62)***	**0.003**	20	22.2	1.91 (0.95–3.84)	0.068	37	14.2
TT + TC¶			**1.96 (1.14–3.34)***	**0.014**			1.34 (0.80–2.26)	0.272		
CC‡			**1.99 (1.13–3.52)***	**0.018**			1.73 (0.94–3.17)	0.077		
***OGG1*: rs1052133**										
CC§	31	30.7	Ref.		45	50.0	Ref.		120	46.0
CG§	52	51.5	**1.81 (1.08–3.03)***	**0.023**	33	36.7	0.79 (0.47–1.33)	0.380	11	42.5
GG§	18	17.8	**2.32 (1.15–4.70)***	**0.019**	12	13.3	1.07 (0.50–2.26)	0.866	30	11.5
CC + CG¶			**1.92 (1.18–3.13)***	**0.009**			0.85 (0.53–1.38)	0.510		
GG‡			1.67 (0.88–3.15)	0.114			1.19 (0.58–2.43)	0.643		
***SLC19A1*: rs1051266**										
AA§	26	25.7	Ref.		12	13.3	Ref.		86	33.0
AG§	51	50.5	1.28 (0.80–2.39)	0.246	44	48.9	**2.59 (1.29–5.18)***	**0.007**	12	46.7
GG§	24	23.8	1.50 (0.78–2.88)	0.224	34	37.8	**4.60 (2.19–9.65)***	**<0.001**	53	20.3
AA + AG¶			1.42 (0.85–2.37)	0.184			**3.19 (1.65–6.18)***	**0.001**		
GG‡			1.22 (0.71–2.12)	0.472			**2.38 (1.41–4.02)***	**0.001**		
***SOD2*: rs4880**										
TT§	17	16.8	Ref.		23	25.6	Ref.		69	26.4
TC§	47	46.5	**1.78 (1.05–3.01)***	**0.033**	43	47.8	1.26 (0.70–2.26)	0.442	13	51.0
CC§	37	36.6	**2.55 (1.30–4.98)***	**0.006**	24	26.7	1.22 (0.63–2.38)	0.560	59	22.6
TT + TC¶			1.78 (0.99–3.20)	0.056			1.05 (0.61–1.81)	0.870		
CC‡			**1.98 (1.20–3.28)***	**0.007**			1.25 (0.72–2.16)	0.435		

The OR (95% CI) and *p*-value were calculated by logistic regression according the following genetic models: codominant model (MM vs. MM, MM vs. Mm, and MM vs. mm, *i.e.* each genotype was compared with major allele homozygous genotype as reference); dominant model (MM vs. Mm + mm); and recessive model (MM + Mm vs. mm). Bold indicates statistically significant association (* susceptibility or † protection). §, codominant model; ¶, dominant model; ‡, recessive model; M, major allele; m, minor allele; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; OR, odds ratio; CI, confidence interval; Ref., reference.

We also investigated the potential role of selected SNPs in MDS subtypes and IPSS risk groups, as well as in AML classification. Nevertheless, we did not detect any

association of allele and genotype with MDS and AML classification or with IPSS risk groups (data not shown).

6.4.3. Genotypic profile analysis

The majority of proteins are involved in complex networks of structural, functional, and regulatory interactions. Therefore, the combination of several variants within the same pathway could identify high-risk individuals, who may benefit from preventive and/or therapeutic interventions. In order to assess the distribution of multilocus genotypes, we performed a genotypic profile (GP) analysis. GPs were inferred using Arlequin software and were grouped in five different pathways: oxidative stress (*CAT+CYBA+GPX1+MPO+SOD1+SOD2*), KEAP1-NRF2 (*KEAP1+NFE2L2*), base excision repair (*NEIL1+OGG1+XRCC1*), DNA methylation (*DNMT1+DNMT3A+DNMT3B*), and folate metabolism (*MTRR+SLC19A1*). Table 24 shows the nine significant GPs associated with the myeloid neoplasms, distributed as follow: five in MDS and four in AML. These GPs were mutually exclusive. In the oxidative stress pathway we observed a total of 84 GPs, of which 42 (50.0%) were unique to the following groups: nine (10.7%) in MDS, four (4.8%) in AML, and 29 (34.5%) in the control group. Among the significant GPs, the GP3 and GP4 increased the risk of MDS development (GP3: OR=10.72, 95% CI 1.18–97.17, p=0.023; GP4: OR=4.06, 95% CI 1.12–14.70, p=0.032), while the GP1 and GP2 conferred susceptibility to AML (GP1: OR=5.06, 95% CI 1.19–21.62, p=0.029; GP2: OR=3.63, 95% CI 1.23–10.69, p=0.021). In the KEAP1-NRF2 pathway we found eight GPs, but only one – the GP1 – appeared to have a protective effect against MDS development (OR=0.43, 95% CI 0.24–0.76, p=0.004). Regarding the base excision repair pathway, a total of 18 GPs were detected. Two (11.1%) of these GPs were unique in AML group, and the GP1 was the most commonly found in AML and control groups (23.3% and 23.4%, respectively). Only one GP presented a protective effect against MDS development (GP1: OR=0.28, 95% CI 0.13–0.61, p<0.001). In the DNA methylation pathway we observed a total of 20 GPs, but only GP1 was associated with an MDS

predisposition (OR=3.13, 95% CI 1.57–6.25, *p*=0.002). Finally, the folate metabolism pathway had a total of 9 GPs, of which one (11.1%) was exclusively present in the MDS group and one (11.1%) in the AML group. In this pathway, GP1 was found to have a protective effect against AML development (OR=0.36, 95% CI 0.17–0.77, *p*=0.006), while GP2 predisposed to this malignancy (OR=2.03, 95% CI 1.11–3.73, *p*=0.028). Besides the results mentioned previously, there were no other significant associations between GPs and diseases risk.

Table 24.
Significant genotypic profiles distribution of selected SNPs in myeloid neoplasia patients and controls, and its association with risk of myelodysplastic syndrome and acute myeloid leukemia

Pathway: Genotypic profile	MDS			AML			CTL
	%	OR (95% CI)	*P*-value	%	OR (95% CI)	*P*-value	%
Oxidative stress: *CAT + CYBA + GPX1 + MPO + SOD1 + SOD2*							
GP1: CT CC CC AA AA CT	2.0	1.74 (0.29–10.56)	0.621	5.6	**5.06 (1.19–21.62)***	**0.029**	1.1
GP2: CT CT CT GA AA CC	2.0	0.73 (0.15–3.59)	1.000	7.8	**3.63 (1.23–10.69)***	**0.021**	2.7
GP3: CT TT TT AA AA CC	4.0	**10.72 (1.18–97.17)***	**0.023**	0.0	–	–	0.4
GP4: TT CT CT GA AA CC	5.9	**4.06 (1.12–14.70)***	**0.032**	3.3	2.22 (0.49–10.10)	0.379	1.5
KEAP1–NRF2: *KEAP1 + NFE2L2*							
GP1: GG TT	16.8	**0.43 (0.24–0.76)†**	**0.004**	23.3	0.64 (0.37–1.12)	0.142	32.2
Base excision repair: *NEIL1 + OGG1 + XRCC1*							
GP1: CT CC CC	7.9	**0.28 (0.13–0.61)†**	**<0.001**	23.3	1.00 (0.57–1.76)	1.000	23.4
DNA methylation: *DNMT1 + DNMT3A + DNMT3B*							
GP1: CC CC CC	18.8	**3.13 (1.57–6.25)***	**0.002**	11.1	1.69 (0.75–3.81)	0.258	6.9
Folate metabolism: *MTRR + SLC19A1*							
GP1: AA AA	18.8	0.76 (0.43–1.35)	0.398	10.0	**0.36 (0.17–0.77)†**	**0.006**	23.4
GP2: AA GG	18.8	1.55 (0.84–2.86)	0.185	23.3	**2.03 (1.11–3.73)***	**0.028**	13.0

Bold indicates statistically significant association (* susceptibility or † protection). MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; OR, odds ratio; CI, confidence interval; GP, genetic profile.

6.4.4. Prognostic impact

The last question addressed whether selected SNPs had a significant impact on patient's prognosis. We evaluated their impact in disease prognosis by estimating the rate of MDS transformation into AML and the overall survival (OS) of patients

with MDS and AML. The prognostic impact was assessed in MDS and AML patients, for whom follow-up information was available (*n*=96 and *n*=80, respectively). The rate of MDS transformation into AML and the OS were analyzed using the Kaplan-Meier method, and the patients were stratified according to their SNP genotypes. As observed in Figure 38, the *DNMT3A* CC genotype (HR=0.23, 95% CI 0.12–0.94, *p*=0.047) and the *MTRR* AA genotype (HR=0.30, 95% CI 0.10–0.93, *p*=0.045) were significantly associated with a lower rate of AML transformation, in comparison to the *DNMT3A* T carrier genotypes and *MTRR* G carrier genotypes, respectively.

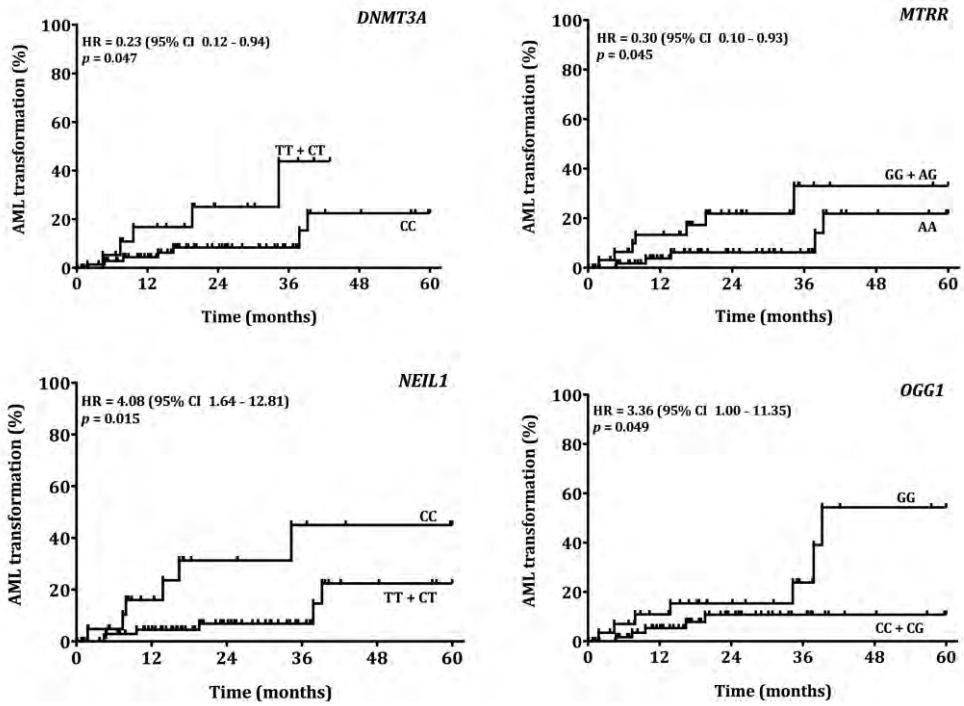


Figure 38. Time to AML transformation curves in myelodysplastic syndrome (MDS) patients, according to *DNMT3A* (rs2424908), *NEIL1* (rs5745920), MTRR (rs162036), and *OGG1* (rs1052133) genotypes. Time to AML transformation analysis was performed by Kaplan Meier method, differences in survival were tested with log rank test, and the hazard ratio (HR) with 95% confidence interval (CI) were calculated using Cox proportional hazard model.

Although, MDS patients with *NEIL1* CC genotype (HR=4.08, 95% CI 1.64–12.81, *p*=0.015) and *OGG1* GG genotype (HR=3.36, 95% CI 1.00–11.35, *p*=0.049) had a significantly higher rate of AML transformation, compared to *NEIL1* T carrier

genotypes and *OGG1* C carrier genotypes, respectively. Moreover, MDS patients carrying *DNMT3A* CC genotype had a significantly longer overall survival time (HR=0.39, 95% CI 0.16–0.94, p=0.043) than those with T carrier genotypes, while patients with *OGG1* GG had a significantly lower overall survival time (HR=3.13, 95% CI 1.21–8.11, p=0.019) than those with C carrier genotypes (Figure 39). Finally, a significantly lower overall survival time was noted in AML patients with *GPX1* CC genotype (HR=1.97, 95% CI 1.04–3.74, p=0.037), in comparison to those with *GPX1* T carrier genotypes. A similar lower overall survival time was observed in AML patients with *KEAP1* TT genotype (HR=12.67, 95% CI 2.09–31.76, p=0.008), when compared to those with *KEAP1* G carrier genotypes.

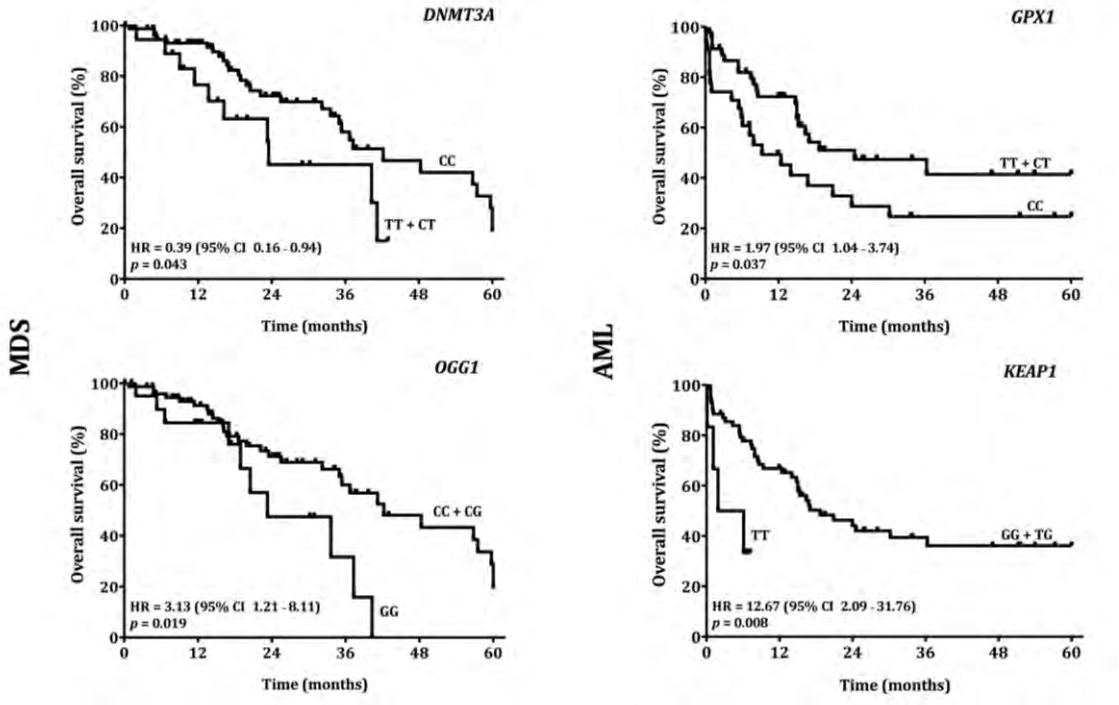


Figure 39. Overall survival curves of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients, according to DNMT3A (rs2424908), OGG1 (rs1052133), GPX1 (rs1050450), and KEAP1 (rs11085735) genotypes. Survival analysis was performed by Kaplan Meier method, differences in survival were tested with log rank test, and the hazard ratio (HR) with 95% confidence interval (CI) were calculated using Cox proportional hazard model.

6.4.5. Genotype-phenotype analysis

In order to evaluate the impact of genetic variants on the levels of oxidative stress (ROS/TAS ratio), DNA damage (8-OHdG), and DNA methylation (5-mC), we analyzed these parameters according to subjects' genotypes (40 MDS patients and 10 controls) for the selected genes. Frist, we analyzed the association of *CAT*, *CYBA*, *GPX1*, *KEAP1*, *MPO*, *NFE2L2*, *SOD1*, and *SOD2* genotypes with oxidative stress. As observed in Figure 40A, subjects with *CYBA* TT genotype (85.7±4.7) exhibited higher oxidative stress level than those with CT (33.0±3.9, $p<0.001$) and CC (34.9±2.7, $p<0.001$). Similarly, *SOD2* CC individuals (52.6±5.1) had higher oxidative stress level than TC (36.9±3.6, $p=0.017$) and TT (28.2±3.6, $p=0.004$). Furthermore, *GPX1* CC individuals (26.8±2.7) had lower oxidative stress level than TT homozygous (41.8±3.6, $p<0.001$).

Next, we analyzed the association of *NEIL1*, *OGG1*, and *XRCC1* genotypes with DNA damage (Figure 40B). *NEIL1* TT subjects (42.0±1.7 ng/ml) showed higher DNA damage than CT (37.1±2.2 ng/ml, $p=0.031$) and CC (33.5±0.8 ng/ml, $p= 0.040$) ones, and *OGG1* GG individuals (40.8±1.7 ng/ml) had higher DNA damage than CC homozygous (34.4±0.8 ng/ml, $p = 0.011$).

Finally, we analyzed the association of *DNMT1*, *DNMT3A*, *DNMT3B*, *MTRR*, and *SLC19A1* genotypes with DNA methylation (Figure 40C). Subjects with *DNMT1* TT (1.09±0.11%) exhibited higher DNA methylation than those with CT (0.66±0.07%, $p<0.001$) and CC (0.42±0.05, $p<0.001$), and *MTRR* GG individuals (1.00±0.13%) had higher DNA methylation than AA homozygous (0.61±0.05%, $p=0.017$). In contrary, *DNMT3A* CC (0.25±0.07%) exhibited lower DNA methylation than CT (0.72±0.12%, $p<0.001$) and TT (0.73±0.06%, $p<0.001$). Similarly, *DNMT3B* TT (0.62±0.03%) had lower DNA methylation than CC (0.91±0.04%, $p=0.009$).

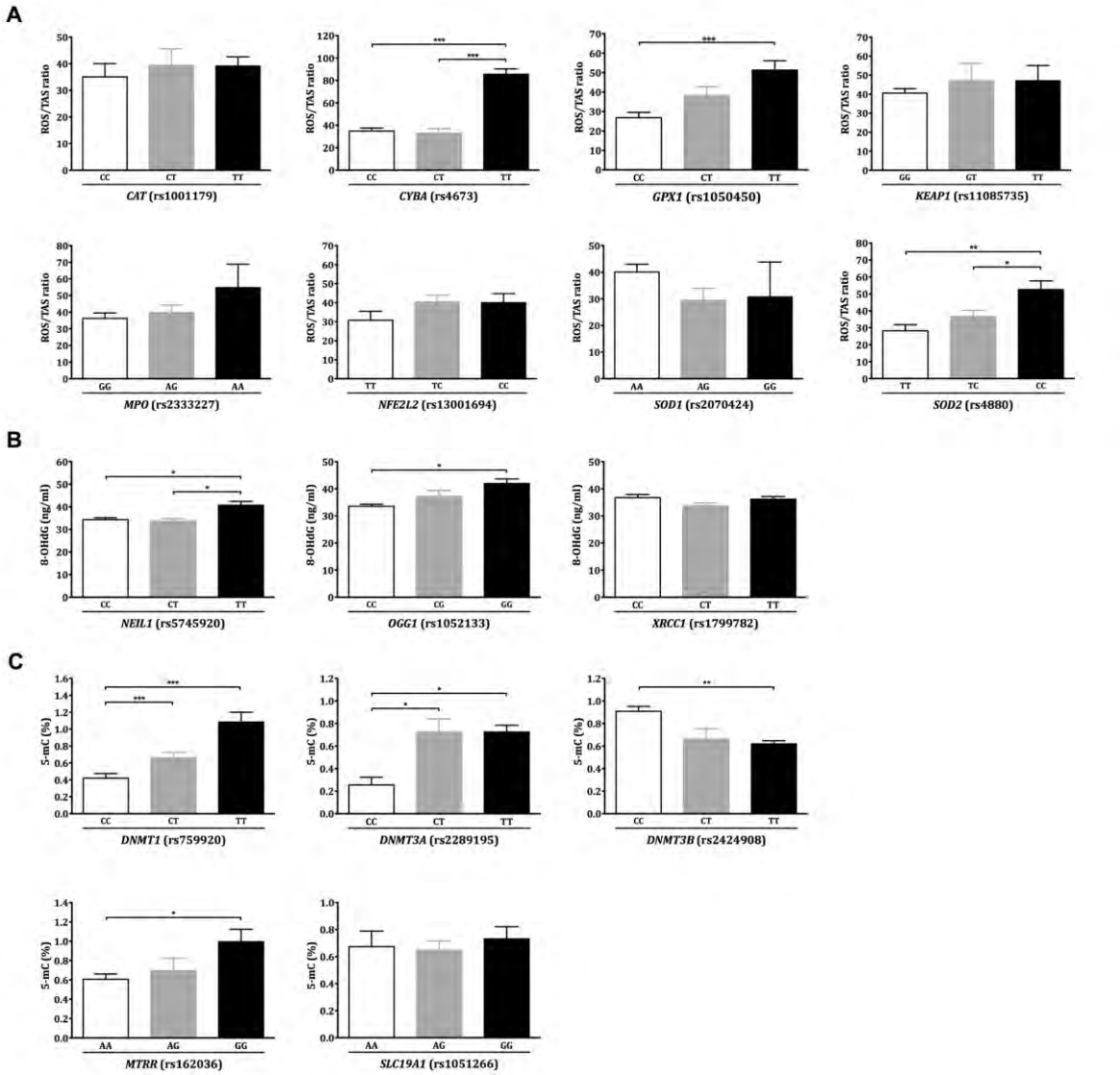


Figure 40. Oxidative stress (A), DNA damage (B), and DNA methylation (C) levels according to individuals' genotypes of selected SNPs. Oxidative stress was evaluated by the ROS/GSH ratio, DNA damage by 8-OHdG levels, and DNA methylation by 5-mC levels in 40 MDS patients and 10 controls. ROS, reactive oxygen species; TAS, total antioxidant status; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 5-mC, 5-methylcytosine; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.5. Discussion

The molecular study of hematological malignancies has been, and will continue to be, fundamental to understanding the genetic mechanisms underlying leukemogenesis, since it allows the identification of susceptibility genes and potential therapeutic targets, as well as the prediction of treatment response. In

this investigation, we were able to identify five genes associated with MDS (*GPX1*, *NEIL1*, *NFE2L2*, *OGG1*, and *SOD2*), two genes associated with AML (*DNMT3B* and *SLC19A1*), and two genes associated with the susceptibility for both diseases (*CYBA* and *DNMT1*).

The oxidative stress has been implicated in the pathogenesis and prognosis of MDS [Gonçalves *et al.,* 2015b; Jankowska *et al.,* 2008]. This cellular state is modulated by antioxidant and base excision repair enzymes, such as superoxide dismutase (SOD) and 8-oxoguanine DNA glycosylase (OGG1), respectively, as well as by transcriptional factors, such as nuclear factor erythroid 2-like 2 (NRF2) [Ambrosone *et al.,* 2005; Sutton *et al.,* 2006; Wang *et al.,* 2009]. In the present study, we observed that *SOD2* C carrier genotypes (CT and CC) and *GPX1* TT genotype increased the susceptibility for MDS development. Few authors have studied the relationship of these antioxidant enzymes with hematological neoplasms, and only Vineis *et al*. [2007] found that the *SOD2* CC genotype increased the risk for myeloid leukemia [Bănescu *et al.,* 2014; Lightfoot *et al.,* 2006; Vineis *et al.,* 2007]. The *SOD2* C and *GPX1* T variants were associated with high activity of MnSOD and with low activity of glutathione peroxidase, respectively. As suggested for prostate cancer [Li *et al.,* 2005; Wang *et al.,* 2009], these variants may induce an oxidative cellular state that leads to higher oxidative DNA damage, which increased the risk of MDS development. This suggestion is supported by the genotype-phenotype studies presented here, indicating that subjects with *SOD2* CC and *GPX1* TT genotypes had higher oxidative stress levels.

In addition, we found that *OGG1* G carrier genotypes (GC and GG) were risk factors for the same disease. Other authors observed similar findings in this neoplastic malignancy [Aktuglu *et al.,* 2014; Jankowska *et al.,* 2008]. The *OGG1* G variant has been associated with chromosomal instability, due to it decrease activity and, therefore, to constitutive insufficiency of DNA repair machinery [Jankowska *et al.,* 2008; Kohno *et al.,* 1998]. According to these observations, we also found that *OGG1* GG genotype had higher DNA damage than *OGG1* CG and CC genotypes. This association may explain the presence of chromosomal breaks, DNA double-strand breaks, and elevated levels of 8-OHdG in MDS patients, and consequently, the

susceptibility effect conferred by this variant [Jankowska *et al.,* 2008; Kohno *et al.,* 1998]. In addition to OGG1, cells have another DNA glycosylases, such as NEIL1, which also repair oxidatively induced DNA lesions; however, unlikely OGG1, NEIL1 catalyzes efficiently cleave DNA-containing oxidatively induced base lesions near single-strand break sites [Roy *et al.,* 2007]. In the present study, we observed that *NEIL1* TT genotype and T allele predisposed to MDS. Some research groups reported that *NEIL1* polymorphisms were associated with increased risk of colorectal [Broderick *et al.,* 2006] and gastric cancer [Goto *et al.,* 2010], but until today no study has addressed the association of *NEIL1* variants with MDS or other hematological neoplasm. According to the functional information on the Functional Single Nucleotide Polymorphism (F-SNP) database [Lee & Shatkay, 2008], the intronic *NEIL1* T variant may cause the loss of *NEIL1* transcription, and, consequently, the inefficient repair of DNA lesions (i.e. the individuals with the *NEIL1* TT genotype had increased DNA damage levels). However, this hypothesis needs to be confirmed with functional studies.

Furthermore, we observed that *NFE2L2* C carrier genotypes (CT and CC) increased susceptibility to develop MDS, compared with TT genotype. An association of polymorphisms in *NFE2L2* with susceptibility and prognosis for breast cancer was found by Hartikainen *et al.* [2012], underlying the complex effect of *NFE2L2* gene in cancer predisposition and progression. In fact, mutations in the *NFE2L2* and *KEAP1* genes have been found in hepatocellular carcinoma [Gu *et al.,* 2013; Yoo *et al.,* 2012], stomach [Yoo *et al.,* 2012], lung [Singh *et al.,* 2006; Sporn & Liby, 2012], and breast cancer [Sjöblom *et al.,* 2006]. However, the impact of *NFE2L2* polymorphisms on hematological malignancies has not been studied yet; and more studies focus on SNP biological consequences are needed to elucidate it carcinogenic potential. Altogether, these results support the hypothesis that oxidative stress is a key player in MDS development.

The hypermethylation of tumor suppressor genes and the global DNA hypomethylation had been implicated as major mechanisms involved in AML development [Ibrahem *et al.,* 2015: Schoofs & Müller-Tidow, 2011; Zheng *et al.,* 2013]. Also, a large number of recurrent genetic abnormalities found in AML

patients occur in genes involved in epigenetic modulators, such as *TET2* and *DNMT3A* [Fathi & Abdel-Wahab, 2012]. In the present study, we observed that *DNMT3B* T carrier genotypes (CT and TT) may offer a protective effect against AML development, while *SLC19A1* G carrier genotypes (AG and GG) increased the risk of this malignancy. Although, the association of *DNMT3B* variants with cancer susceptibility has been reported in the literature [Bao *et al.,* 2011; Lee *et al.,* 2005; Montgomery *et al.,* 2004; Singal *et al.,* 2004; Zheng *et al.,* 2013], it remains unclear [Zhu *et al.,* 2015]. To our best knowledge, only one report studied the association of *DNMT3B* gene with hematological neoplasms [Zheng *et al.,* 2013], finding that C allele could confer a protective effect against AML development in Chinese Han population. In the present study, we found that *DNMT3B* TT genotype was associated with lower DNA methylation, which could explain it protective effect against AML development. Additionally, the SLC19A1 solute carrier is the major mechanism by which folates are delivered to cells [Galbiatti *et al.,* 2011; Hou & Matherly, 2014]. During DNA replication, low folate levels result in the compromised production of thymidine, which induces misincorporation of uracil into DNA sequence, and, consequently, the DNA double-strand breaks. The *SLC19A1* G variant has been associated with increased risk of developing head and neck squamous cell carcinoma [Galbiatti *et al.,* 2011] as well as colorectal cancer [Levine *et al.,* 2011]. However, De Jonge *et al.* [2009] found that the AA genotype and the A allele carriers had an increased susceptibility to acute lymphoblast leukemia [De Jonge *et al.,* 2009]. The exact function of this substitution is not clear [De Jonge *et al.,* 2009; Galbiatti *et al.,* 2011; Koppen *et al.,* 2010; Yee *et al.,* 2010], and we did not found significant differences in DNA methylation levels between individuals with *SLC19A1* AA, AG, and GG genotypes. The presence of a less active form of SLC19A1 leads to a lower folate influx, which will increase the propensity of epigenetic and genetic abnormalities, and thus the risk of AML development.

Several authors have implicated the oxidative stress and the hypermethylation of tumor suppressor genes in myelodysplastic syndrome and acute myeloid leukemia [Gonçalves *et al*., 2015c; Hole *et al.,* 2011; Meldi & Figueroa, 2014]. These are common molecular mechanisms of myeloid neoplasms. Here, we found that *CYBA*

TT genotype carriers had an increased susceptibility to develop MDS and AML. Lan *et al.* [2007] found similar results, associating TT genotype with risk of non-Hodgkin lymphoma, particularly T-cell lymphoma; however, Wang *et al.* [2006] did not observe this association [Lan *et al.,* 2007; Wang *et al.,* 2006]. It has been suggested that the *CYBA* T variant has an increase in NADPH oxidase activity, which induce a higher production of ROS [Bedard *et al.,* 2009; Shimo-Nakanishi *et al.,* 2004]. Similarly, in the present study, we found that *CYBA* TT genotype was associated with higher oxidative stress levels. However, the functional consequence of this variant is controversial, since other studies associated the *CYBA* T variant with reduced activity [Guzik *et al.,* 2000; Schirmer *et al.,* 2008; Wyche *et al.,* 2004]. Furthermore, we identified an association of *DNMT1* genotypes with MDS and AML development. We also observed that *DNMT1* TT genotype increased the predisposition to MDS, while *DNMT1* CT genotype was associated with an increased risk to AML development. It has been reported an association of *DNMT1* variants with breast [Sun *et al.,* 2005; Xiang *et al.,* 2010] and ovarian cancer [Mostowska *et al.,* 2013], but not with gastric [Jiang *et al.,* 2012; Khatami *et al.,* 2009a; Yang *et al.,* 2012], rectal [Curtin *et al.,* 2011], and colon cancer [Khatami *et al.,* 2009b]. The selected *DNMT1* SNP is an intronic variant (T allele) that have been recently associated with a possible enhancer function [Saradalekshmi *et al.,* 2014]. This fact may explain the association of TT and CT genotypes with increased 5-mC levels observed in the present study. However, functional studies of this SNP must be conducted in order to establish a causality effect with cancer. Together, these results support the idea that oxidative stress and aberrant DNA methylation are molecular mechanisms linking MDS and AML.

Myeloid malignancies, such as MDS and AML, emerge from a complex interplay between several DNA changes involving many genes and wide-range environmental factors [Fröhling *et al.,* 2005]. These complex cross-talks affect entire pathways that consequently increase or decrease the risk of developing myeloid malignancies, as well as the disease severity and survival. Although each single genetic variant may confer a small disease risk, the joint action of several variants are likely to have a more significant role in disease development [Jin *et al.,*

2014]. Based on this data, we hypothesize that MDS and AML development may be affected by variants in genes involved in functional pathways: oxidative stress, KEAP1-NRF2, base excision repair, DNA methylation, and folate metabolism. In multilocus genotype analysis, we observed three genotypic profiles of susceptibility for MDS (two in oxidative stress pathway, GP3: CT TT TT AA AA CC and GP4: TT CT CT GA AA CC; and one in DNA methylation pathway, GP1: CC CC CC) and two GPs of protection (one in KEAP1-NFE2L2 pathway, GP1: GG TT; and one in DNA base excision repair pathways, GP1: CT CC CC). Similarly, we identified three GPs of susceptibility for AML (two in oxidative stress pathway, GP1: CT CC CC AA AA CT and GP2: CT CT CT GA AA CC; and one in folate metabolism pathway, GP2: AA GG) and one GP of protection (folate metabolism pathway, GP1: AA AA). When we compared the risk prediction between single and combined genotypes (GPs), we observed that GPs had a higher value of odds ratio, depending on the number of risk alleles. For example, the oxidative stress profile GP3 increased 10.72-fold the risk of MDS development and this GP is composed of three risk genotypes (*CYBA* TT, *GPX1* TT, and *SOD2* CC). Similarly, base excision repair GP1 appear to confer a higher protective effect (0.28-fold) against MDS development than it single genotypes and this GP only had one risk allele (*NEIL1* T). Therefore, the GP analyses may have more implications with clinical usefulness in terms of myeloid neoplasm risk prediction.

In the last few years, AML and MDS were identified as highly heterogeneous diseases with different risk groups, based on molecular features involving gene mutations, non-coding RNAs, and abnormal DNA methylation. Here, we observed that variants in *DNMT3A*, *MTRR, NEIL1*, and *OGG1* genes influence the AML transformation rate of MDS patients. Moreover, we found a significant correlation of *DNMT3A* and *OGG1* variants with the survival of MDS patients. Similarly, *GPX1* and *KEAP1* variants contributed to the AML patient survival. The influence of *DNMT3A* CC genotype in AML transformation rate and survival of MDS patients may be due to the lower DNA methylation observed in individuals with this genotype. This fact is in accordance with previous reports that indicate that DNA methylation of tumor suppressor genes are associated with higher AML

transformation rates and worst survival of MDS patients [Aggerholm *et al.,* 2006; Shen *et al.,* 2010; Tien *et al.,* 2001]. Similarly, *MTRR* AA genotype had lower DNA methylation and AML transformation rate. In addition, DNA repair systems have a critical role in maintaining genome integrity and also had an impact on the risk of developing different types of cancer, as well as in their prognosis [Bănescu *et al.,* 2013]. Here, we found that *OGG1* GG genotype influenced the AML transformation rate and survival of MDS patients. This genotype was associated with higher DNA damage and, therefore, might contribute to the worst prognosis of MDS patients. On the other hand, we observed that *NEIL1* CC genotype increased the AML transformation rate of MDS patients. This genotype was associated with lower DNA damage, leading to a lower probability of genotoxicity. Consequently, *NEIL1* CC genotype may have preventive effects against cancer development, but in cancer cells the higher DNA repair activity appears to confer poor prognosis (for example through resistance to therapy, that ultimately, will be translate in a short survival). The impact of genomic variants of nucleotide excision repair on AML survival has been previously reported [Allan *et al.,* 2004; Bănescu *et al.,* 2014b; Bhatla *et al.,* 2008; Fabiani *et al.,* 2009; Shi *et al.,* 2011], but there are no published data on survival relevance of base excision repair variants in MDS patients. The present study is the first one to correlate *GPX1* and *KEAP1* gene variants with the prognosis of AML patients. The *GPX1* CC genotype may influence AML survival through resistance to chemotherapy. The *GPX1* C variant is associated with increased GPX1 activity that can prevent cell death induced by chemotherapy, contributing to a lower survival. The influence of *KEAP1* TT genotype in AML survival is not clear. Although, our genotype-phenotype study did not found significant differences in oxidative stress levels between *KEAP1* genotypes, functional studies are needed to understand their role in AML survival. These findings suggest that the evaluation of *DNMT3A*, *MTRR*, *OGG1*, *NEIL1*, *GPX1*, and *KEAP1* gene variants could increase the discriminative power of prognostic scoring systems to detect high-risk features. Taken into account that these gene variants seem to be independent of other clinical prognostic factors, they could improve the current score systems for AML and MDS.

In the present study, seven selected SNPs are located in the protein coding region and lead to a change in the translated amino acids (missense variants). The other ones are non-coding SNPs (ncSNPs) located within regulatory regions, like promoters, introns, and UTRs. It is well known that ncSNPs can disrupt gene expression by several mechanisms: 1. altering transcription factor binding sites and microRNAs binding sites; and 2. influencing the strength of enhancers and promoters, and change methylation sites (CpG dinucleotides). Therefore, these mechanisms make the ncSNPs of prime importance to be considered for candidate gene association studies [Patnala *et al.,* 2013; Preskill & Weidhaas, 2013; Prokunina & Alarcón-Riquelme, 2004]. The selection of ncSNPs was based on the fact that variations within and around genes harbor more polygenic effects than intergenic regions; and regulatory genic elements are particularly enriched for polygenic effects [Schork *et al.,* 2013]. With this strategy, we found significant associations of myeloid neoplasms (MDS and AML) with five SNPs located in coding regions and four SNPs in non-coding regions. These findings reinforce the role of ncSNPs in cancer development. However, the present study has some limitations due to sample size and study design (hospital-based case-control). Although the sample size was reduced, the allele frequencies observed in cases and controls were in HWE, suggesting that our subject sampling was sufficiently random. To exclude the probability of finding associations by chance, our results should be confirmed through replication in other populations. Moreover, other variants in linkage disequilibrium with those identified here should be evaluated in order to validate the causative effect.

Altogether, the data presented here suggest that variants in genes involved in oxidative stress (*CYBA, GPX1*, *NFE2L2*, and *SOD2*), DNA base excision repair (*OGG1* and *NEIL1),* DNA methylation (*DNMT1* and *DNMT3B*), and folate metabolism pathways (*SLC19A1*) may play important roles in the susceptibility of MDS and AML. These pathways can also be implicated in the increased propensity to AML transformation observed in MDS patients (*DNMT3A*, *MTRR*, *NEIL1*, and *OGG1*), as well as in the survival of patients with MDS (*DNMT3A* and *OGG1*) and AML (*GPX1* and *KEAP1*). The variants in these genes influenced the predisposition to develop

MDS and AML, as well as the prognosis of patients with these neoplasms through the modulation of oxidative stress, DNA damage, and DNA methylation.

Chapter 7

Acute and chronic exposure to hydrogen peroxide modulates the methylome of normal and malignant hematological cells

Gonçalves, A.C., *Alves, R., Ribeiro, I.P., Pires, A., Jorge, J., Laranjo, M., Botelho, M.F., Nascimento Costa, J.M., Mota-Vieira, L., Carreira, I.M. & Sarmento-Ribeiro, A.B.*

(In preparation, Sept 2015)

7.1. Abstract

Oxidative stress and abnormal DNA methylation have been implicated in hematological neoplasms. These malignancies can simultaneously harbor changes in reactive oxygen species (ROS) levels and DNA methylation pattern. This fact led us to investigate whether acute and chronic exposure to hydrogen peroxide (H_2O_2) could affect the genome and methylome of normal and malignant hematological cells, and to examine the involvement of epigenetic machinery in this process. Acute and chronic exposure to H_2O_2 induced an increase in DNA methylation levels of several tumor suppressor genes (TSG) and a decrease in long interspersed nuclear element-1 (LINE-1) sequences methylation. The increase in TSG methylation levels was accompanied by the up-regulation of a specific gene expression signature, comprising *DNMT1*, *DNMT3A*, *MECP2*, *HDAC1* and *EZH2* genes. Although, H_2O_2 exposure induced hypermethylation of different TSG in a cell line-dependent manner, the increase in *TP73* methylation levels was frequently observed. Furthermore, chronic exposure to H_2O_2 induced LINE-1 hypomethylation associated with increased reduced glutathione content. Moreover, the pre-treatment with N-acetylcysteine, an antioxidant molecule, prevented these events. Overall, this study indicates that chronic exposure and, to a lesser extent, acute exposure to H_2O_2 induces TSG hypermethylation and LINE-1 hypomethylation, and suggests that different mechanistic pathways are involved in these DNA methylation changes.

7.2. Introdution

Excessive cellular reactive oxygen species (ROS) production and/or deficient antioxidant defenses have been found in several diseases, namely in hematopoietic malignancies [Hole *et al*., 2011; Klauning & Kamendulis, 2004; Klauning & Kamendulis, 2011; Valko *et al*., 2007]. This imbalance between oxidants and antioxidants, in favor of the former, leads to a cellular state known as oxidative stress [Klauning & Kamendulis, 2004; Klauning & Kamendulis, 2011; Valko *et al*., 2007]. ROS has been recognized for playing a dual role in living organisms. At low/moderate levels ROS have beneficial effects, activating cellular pathways involved in physiological responses; however, at high concentrations, they induce oxidative stress and consequently adverse modifications in DNA, protein and lipids. These macromolecules damage lead to changes in gene transcription and deregulation of signaling pathways, as well as to chromosome instability, genetic mutation and epigenetic abnormalities [Birben *et al.*, 2012; Klauning & Kamendulis, 2004; Klauning & Kamendulis, 2011; Valko *et al*., 2007]. Hematopoietic cells appear to be particularly vulnerable to oxidative stress. For instance, ROS accumulation in red blood cells results in hemolysis that leads to anemia, while in hematopoietic stem cells increased ROS levels may induce hematological malignancies development [Ghaffari, 2008].

Another characteristic feature of hematopoietic neoplasms is the abnormal DNA methylation pattern. DNA methylation involves a covalent addition of a methyl group at the carbon 5 position of the cytosine ring, and this chemical modification occurs mainly in the CpG dinucleotide, catalyzed by DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B [Das & Singal 2004; Fong *et al*., 2014; Galm *et al*., 2006]. The distribution of CpGs is not uniform in the genome, probably due to their high mutagenic potential, and these dinucleotides are predominantly localized in gene promoters [Galm *et al*., 2006]. DNMT1 is the enzyme responsible for the maintenance of methylation pattern, and has been shown to prefer hemimethylated DNA in comparison with unmethylated strands. DNMT3A and DNMT3B are *de novo* methyltransferases, and methylate previously unmethylated DNA [Akhavan-Niaki & Samadani *et al.*, 2013; Taby & Issa, 2010]. Almost all cancer

types display aberrant hypermethylation in promoter of tumor suppressor genes (TSG), which is associated with transcriptional silencing of these genes [Das & Singal, 2004; Galm *et al*., 2006]. DNA hypomethylation is also observed in several cancer types, however the timing of demethylation varies according to disease stage and/or grade. Hypomethylation can occur in gene-specific and global contexts, and causes chromosomal instability, aberrant gene expression (such as oncogene activation), as well as repetitive sequences [such as long interspersed nuclear element-1 (LINE-1) sequences methylation] activation [Akhavan-Niaki & Samadani *et al.*, 2013].

Since oxidative stress and DNA methylation abnormalities are common in hematopoietic malignancies, it is of interest to study the potential link between them. Some studies demonstrated that ROS is correlated and able to modulate both DNA hypomethylation and hypermethylation [Gonçalves *et al*., 2015c; Kang *et al*., 2012; Wongpaiboonwattana *et al*., 2013; Wu & Ni, 2015]. Moreover, acute and chronic oxidative stress can trigger genetic and epigenetic changes [Johnstone & Baylin, 2010]. However, since chronic stress is able to establish abnormal cell states that can persist even when the exposure is removed, molecular abnormalities are more likely to occur in consequence of chronic stress [Johnstone & Baylin, 2010]. In this context, and for the first time in normal and malignant hematopoietic cells, we investigated whether acute and chronic exposure to oxidative stress can modulate genetic (copy number changes and DNA damage) and epigenetic events (TSG hypermethylation and global hypomethylation).

7.3. Methods

7.3.1. Cell culture

Malignant hematological cell lines HL-60 and K-562 were obtained from American Type Culture Collection (ATCC; LGC Promochem), NB-4 from the German Collection of Microorganisms (DSMZ), and the F-36P cell line from European Collection of Cell Cultures (ECACC; Public Health England). The normal lymphocyte

cell line, the IMC cells, was established in the Cytogenetics and Genomics Laboratory (Faculty of Medicine, University of Coimbra) through Epstein-Barr virus immortalization *in vitro*, as described by others [Neitzel, 1986]. The cell lines were routinely grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640), containing 2 mM L-glutamine, 25 mM HEPES-Na, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen), and supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen). The F-36P cells were cultured in RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum and 10 ng/ml of recombinant IL-3 (Gibco, Invitrogen).

Cells were grown and maintained at 37°C in a humidified atmosphere with 5% CO_2. Initially, growing cells were incubated with different concentrations of H_2O_2 (Sigma-Aldrich) ranging from 25 to 250 µM to establish a dose-response and proliferation curves. For acute exposure, the different cell lines were cultured with 50 µM of H_2O_2 for 48h, and then used in subsequent analyses. As control of acute exposure, cells were pre-treated with 1 mM of NAC for 1h before H_2O_2 addition. For chronic exposure, cells were treated with increasing doses of H_2O_2. The starting H_2O_2 concentration was 1 µM and this concentration was increased every 14 days of culture until a final concentration of 50 µM. The chronic exposure culture was maintained for 6 months. During this period of time, parallel cultures were grown and maintained as a passage matched controls.

7.3.2. Cell viability and proliferation assays

Cell viability and proliferation were assessed by the trypan blue exclusion test. Briefly, cells were treated with different concentrations of H_2O_2 (0, 25 µM, 50 µM, 75 µM, 100 µM, and 250 µM) for 72h. At each 24h, cells were harvested and stained with trypan blue. The number of stained (nonviable) and unstained (viable) cells was counted using a hemocytometer (Neubauer chamber). The viability was

calculated as percentage of viable cells and cell proliferation was determinate by the number of viable cells (density).

7.3.3. Assessment of ROS and GSH

Intracellular ROS levels were measured using 2',7' dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Molecular Probes, Life Technologies Corporation) by fluorimetric assays, as described by others [Li *et al*., 2010]. Briefly, cells were incubated with 5 μM of $DCFH_2$-DA for 45 min at 37°C in a humidified atmosphere of 5% CO_2, in the dark. Next, cells were washed twice with cold PBS by centrifugation at 300g for 5 min, resuspended in the same buffer, and 50×10^3 cells of each condition were distributed in a black 96-well plate, in triplicate. The fluorescence in each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a Synergy™ multi-mode microplate reader (Bio Tek instruments Inc). The GSH content was also measured by fluorimetric assays, using mercury orange (MO) dye (Sigma-Aldrich). For this purpose, cells were incubated with 40 μM of MO during 15 min at RT, in the dark. Cells were then washed twice with cold PBS by centrifugation at 300g for 5 min, resuspended in the same buffer, and 50×10^3 cells of each condition were distributed in a black 96-well plate, in triplicate. The fluorescence in each well was measured at an excitation wavelength of 535 nm and an emission wavelength of 595 nm using the same microplate reader.

7.3.4. 8-hydroxy-2-deoxyguanosine quantification

The 8-OHdG levels were measured using a competitive quantitative ELISA Kit (8-hydroxy-2-deoxyguanosine ELISA Kit, Abcam), according to manufacturer's instructions. The colorimetric intensity was determined spectrophotometrically in

a Synergy™ multi-mode microplate reader, and optical density value was inversely proportional to the amount of 8-OHdG.

7.3.5. Global DNA methylation analysis

5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels were determined in genomic DNA from cells by specific ELISA assays (5-methylcytosine DNA ELISA kit and 5-hydroxymethylcytosine DNA ELISA Kit; Enzo), according to the manufacturer's protocol. Genomic DNA was extracted from whole blood, as previously described by Bartlett & White [2003]. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Global methylation was also assessed by methylation analysis of LINE-1 repetitive elements, using combined bisulphite restriction analysis (COBRA) as previously described [Yang *et al*., 2004]. Briefly, the genomic DNA was treated with sodium bisulphite using the EpiTect Bisulfite Kit (Qiagen). PCR was performed on bisulphite treated DNA using the following primers: 5'-GATCTTTTT-ATTAAAAATATAAAAATTAGT-3' and 5'-GATCCCAAACTAAAATACAATAA-3'. The final PCR product was digested with the *HinfI* restriction enzyme. The digested PCR products were resolved by electrophoresis on 10% polyacrylamide gels, and stained with ethidium bromide. Gel images were acquired using a Gel Doc XR (Bio-Rad), and band intensity was measured by densitometry through QuantityOne software (Bio-Rad). The ratio between the intensity of digested bands and the sum of digested and undigested bands indicated LINE-1 methylation levels.

7.3.6. Methylation-specific multiplex ligation-dependent probe amplification

The MS-MLPA assays (ME001B, MRC-Holland) were performed according to manufacturer's instructions. Briefly, 100 ng of each DNA sample was used. All reactions were carried out in a thermo cycler equipped with a heat lid (ABI 2720, Applied Biosystems). Three control DNA obtained from healthy individuals and a

negative control (no template control) were also included in each MS-MLPA assay. The fluorescently labeled PCR products were separated by capillary electrophoresis (ABI-PRISM 3130 sequencer, Applied Biosystems) and analyzed by Coffalyser.Net software. Duplicate experiments were performed for methylation analysis and copy number analysis. In methylation analysis, samples with a methylation level ≥10% were considered as methylated, and different ranges of methylation were determined: demethylation (0–9%), moderate methylation (10–49%), substantial methylation (50–74%), and extensive methylation (≥75%). In the copy numbers analysis, a mean value 0.20 lower than not exposed cell of each cell line was defined as deletion (loss of copy number) and a mean value 0.20 higher than not exposed cell of each cell line was defined as amplification (gain of copy number).

7.3.7. Gene expression analysis

Total RNA was isolated from cells cultured in the absence and presence of H_2O_2 and/or NAC, as described above, using the Quick-RNA™ MiniPrep (Zymo Research), according to manufacturer's instructions. Real-time quantitative PCR (qPCR) studies were performed in a two-step process. Firstly, total RNA was reverse transcribed to cDNA with SuperScript™ III Reverse Transcriptase kit (Invitrogen, Life Technologies) using a 1:1 mix of random hexamers and oligo-dTs. Secondly, the amplification of target and housekeeping genes was performed using SsoFast™ EvaGreen® Supermixe (BioRad) in a IQ5 Real-Time PCR System (BioRad), in triplicate. Primer sequences for qPCR are listed in Supplementary Table S2. Standard curves were generated for all studied genes using a serially diluted control sample. The standard curves were used to assess the reaction efficiency. For each experiment, a no template control (NTC) was included as negative control. Melting curve analysis and amplicon size verification by electrophoresis was used to confirm the specificity of the qPCR reactions. The relative expression of the target genes was analyzed using the Pfaffl method [Pfaffl, 2001]. The expression levels were normalized to the geometric mean of two

housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and glucuronidase beta (*GUSB*) [Vandesompele *et al*., 2002].

7.3.8. Statistical analysis

Data were expressed as mean ± SEM of the number of independent experiments indicated in the figure legends. Analysis of variance (ANOVA) with Dunnett's post-hoc test was performed to compare experimental data with controls in each cell line. All statistical analyses were two-sided, and a $p<0.05$ was considered statistically significant.

7.4. Results

7.4.1. Genetic and epigenetic characterization of normal and malignant hematological cell lines

In order to explore the possible role of oxidative stress in genetic and epigenetic abnormalities we first analyzed the copy number of 38 TSG as well as the methylation status of 24 of these TSG in normal and malignant hematological cell lines, by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). The TSG main characteristics can be found as supplementary Table S3. As models of hematological neoplasm we used four acute myeloid leukemia cell lines, HL-60, NB-4, K-562 and F-36P cells; and as normal counterpart we used an Epstein-Barr virus-immortalized B lymphocyte cell line (IMC cells). Overall, malignant hematological cell lines carried several deletions (average of 10 deletions per cell line) and amplifications in studied TSG (average of 11 amplifications per cell line). As observed in Figure 41A, all neoplastic cell lines carried deletions within *CDKN2A*, *CDKN2B*, and *HIC1* genes, but NB-4 and K-562 cells had homozygous deletions of *CDKN2A* and *CDKN2B* genes. On the other hand, the normal lymphocyte cell line was wild type for all TGS. The highest number of methylated TSG was observed in NB-4 cells (9 methylated TSG), followed by HL-60

and F-36P cells (8 methylated TSG), while the lowest number of methylated TSG was found in K-562 (4 methylated TSG; Figure 41B). In contrast, all analyzed TSG were unmethylated in the normal cell line.

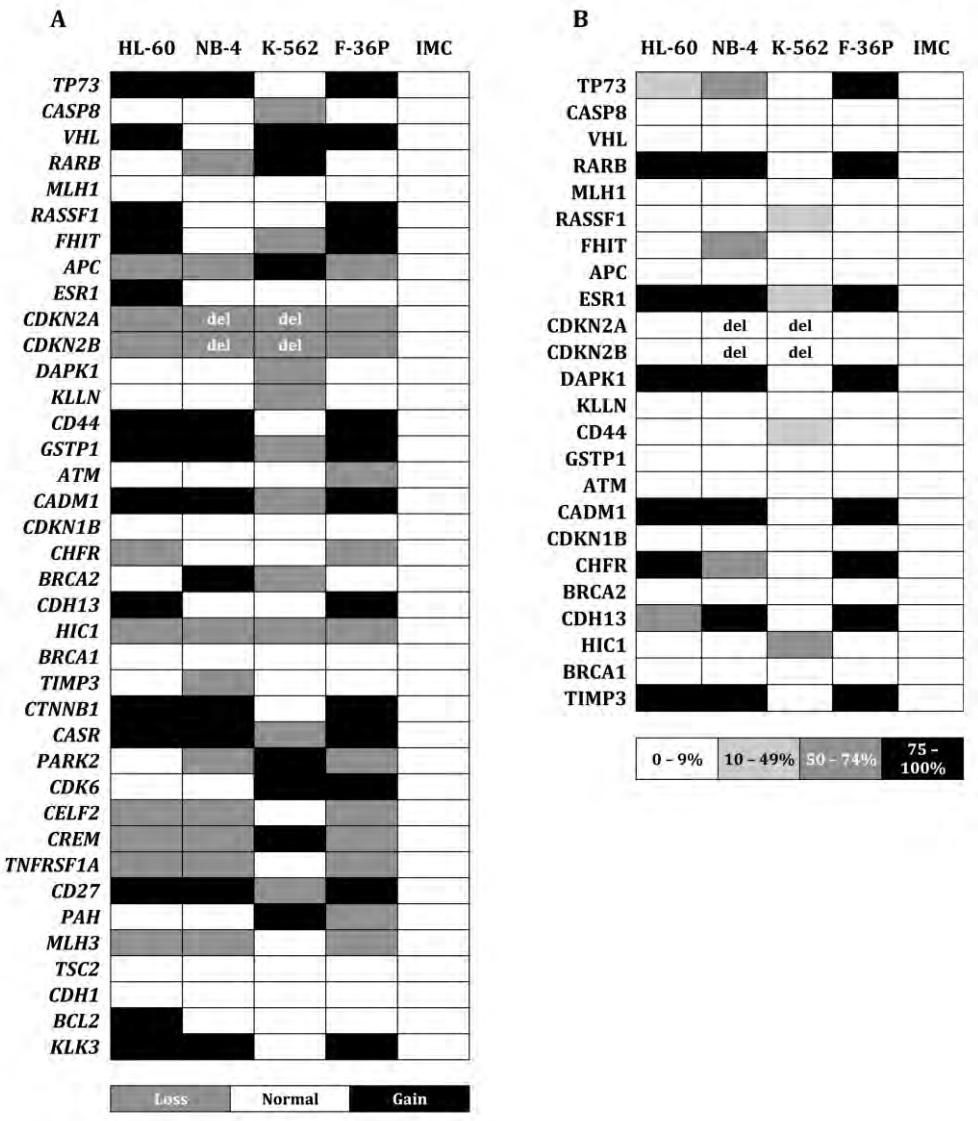


Figure 41. Copy number and methylation status of tumor suppressor genes in HL-60, NB-4, K-562, F-36P and IMC cell lines. Schematic representation of MS-MLPA results showing the changes in copy number of 38 tumor suppressor genes (A) and the methylation status of 24 of these genes (B). The deletions (grey) were defined as a loss ≥0.20 in relation to healthy subjects and the amplifications (black) were defined as a gain of 0.20 in relation to healthy subjects. Samples with a methylation level ≥10% were considered as methylated, and different ranges of methylation were determined: demethylation (0–9%), moderate methylation (10–49%), substantial methylation (50–74%), and extensive methylation (≥75%). del, homozygous deletion.

7.4.2. Cytotoxicity and oxidative stress induction by hydrogen peroxide

In order to choose a sub-lethal H_2O_2 concentration, we performed cytotoxicity assays. Since exposure to 50 μM of H_2O_2 for 48h did not significantly alter the viability and proliferation of the different cell lines (Supplementary Figure S1 and S2), the other experiments were performed using this concentration. Additionally, to confirm that chronic and acute exposure to exogenous H_2O_2 induced oxidative stress, we analyzed the intracellular levels of ROS (Figure 42A) and reduced glutathione (GSH) (Figure 42B). Moreover, since oxidative stress results from the imbalance between free radicals and antioxidants, we also calculated the ROS/GSH ratio (Figure 42C).

The acute exposure to H_2O_2 induced an increase of 1.2-, 1.6-, 1.6- and 1.5-fold of ROS levels in HL-60 (p=0.034), NB-4 (p<0.001), F-36P (p<0.001) and IMC cells (p=0.004), respectively. Similarly, chronic exposure to H_2O_2 increased the ROS levels in all cells [2.2- (p<0.001), 1.7- (p<0.001), 1.6- (p<0.001), 1.4- (p=0.038) and 1.4-fold (p=0.017) in HL-60, NB-4, K-562, F-36P and IMC cells, respectively]. Moreover, acute exposure to H_2O_2 led to 2.5-fold decrease in GSH levels of K-562 cells (p<0.001), while chronic exposure induced a 1.9- (p<0.001), 1.1- (p=0.043), 1.9- (p<0.001) and 4.7-fold (p=0.017) increase in this antioxidant molecule of HL-60, NB-4, F-36P and IMC cell lines, respectively. Finally, as observed in Figure 42C, the ROS/GSH ratio was significantly increased in all cell lines exposed to acute [1.1- (p=0.004), 1.7- (p<0.001), 2.7- (p<0.001) and 1.9-fold (p<0.001) in HL-60, NB-4, K-562 and IMC, respectively], and chronic H_2O_2 [1.2- (p<0.001), 1.5- (p=0.016), 1.4- (p =0.005) and 1.3-fold (p=0.003) in HL-60, NB-4, K-562 and IMC, respectively], except in F-36P cells. In this cell line we observed that chronic exposure led to 1.7-fold decrease in ROS/GSH ratio (p=0.008). The pre-treatment with 1 mM of N-acetylcysteine (NAC) for 1h was able to neutralize the formation of ROS and prevented the establishment of oxidative stress. Moreover, passage-matched controls of the chronically exposed cells were grown and maintained in parallel cultures for all cell lines. The control cultures displayed similar results to those observed in low passage cells (Supplementary Table S4).

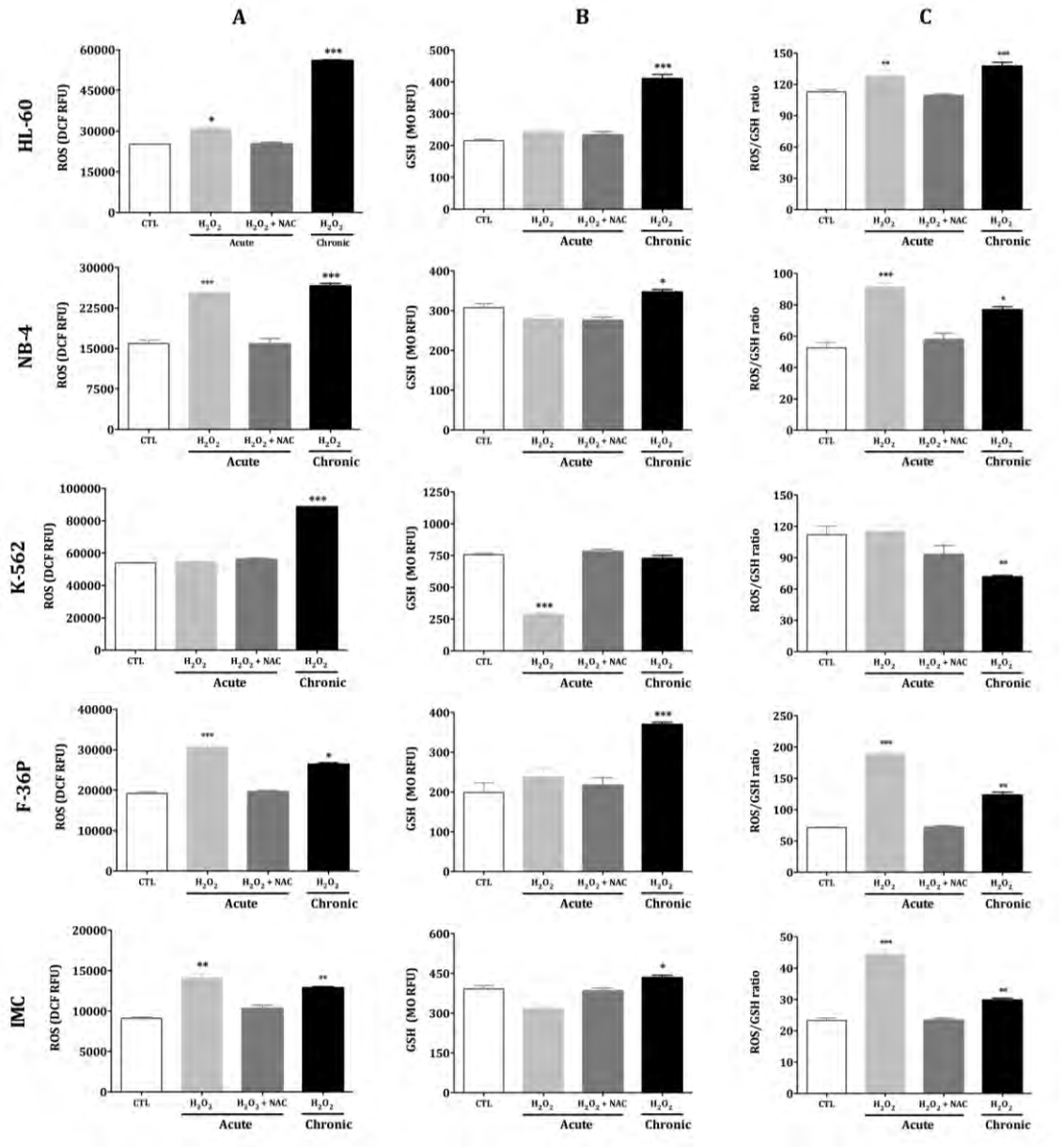


Figure 42. Acute and chronic exposure to hydrogen peroxide influences reactive oxygen species (ROS), reduced glutathione (GSH) and oxidative stress levels in normal and malignant hematopoietic cells. The intracellular levels of ROS (A), GSH (B) and ROS/GSH (C) were analyzed in HL-60, NB-4, K-562, F-36P and IMC cells acutely (48h) and chronically (6 months) exposure to exogenous 50 μM of H_2O_2, as described in methods. As control of acute exposure, cells were pre-treated with 1 mM of N-acetylcysteine (NAC) for 1h before H_2O_2 addition. Statistical analysis was carried out by ANOVA with Dunnett's post-hoc test. Data are shown as mean ± SEM of 9 independent experiments. H_2O_2, hydrogen peroxide; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

7.4.3. Oxidative stress induces DNA damage and copy number changes

The induction of DNA damage and copy numbers changes by chronic and acute exposure to H_2O_2 was also investigated. As observed in Figure 43A, acute exposure to H_2O_2 induced a 2.0- (p=0.007), 1.6- (p=0.009), 1.9- (p<0.001), and 3.5-fold (p<0.001) increased in 8-hydroxy-2-deoxyguanosine (8-OHdG) levels of HL-60, NB-4, K-562, and IMC cells. The chronic exposure to H_2O_2 induced similar results in K-562 (1.7-fold increase, p=0.005) and IMC cells (3.6-fold increase, p<0.001).

Furthermore, the acute and chronic exposure to H_2O_2 induced changes in copy number of cancer-associated genes in all cell lines, except in IMC cells (Figure 43B). Acute exposure to H_2O_2 and, particularly, chronic exposure induced deletions in 2.6% (1/38) and 10.5% (4/38) of studied TSG in HL-60 cells, respectively. In NB-4 cell line, acute exposure to H_2O_2 induced deletions in 7 out of 38 (18.4%) TSG and amplifications in 5 out of 38 (13.2%). The chronic exposure induced only deletions in 6 out of 38 (15.8%) TSG, without any amplification of these genes. The K-562 cells exposed to H_2O_2 mainly suffered additional deletions (acute exposure: 10.5%, 4/38; chronic exposure: 13.2%, 5/38), and only the *TNFRSF1A* gene was amplified. Finally, F-36P cells acutely exposed to H_2O_2 showed additional deletions in 5 out 38 (13.2%) TSG and amplifications in 4 out of 38 (10.5%), while the chronically exposed cells showed deletions in 7 out of 38 (18.4%) TSG. In cells pre-treated with 1 mM N-acetylcysteine (NAC) during 1h, the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG) levels as well as copy number changes in cancer-associated genes were prevented.

7.4.4. Oxidative stress affects global and localized DNA methylation

To elucidate the potential effects of oxidative stress on DNA methylation, global and TSG-specific DNA methylation were evaluated in normal and malignant hematological cell lines acutely and chronically exposed to H_2O_2. In order to analyze global DNA methylation, we assessed the 5-methylcytosine

(5-mC)/5-hydroxymethylcytosine (5-hmC) ratio (Figure 44A) and the LINE-1 methylation levels (Figure 44B).

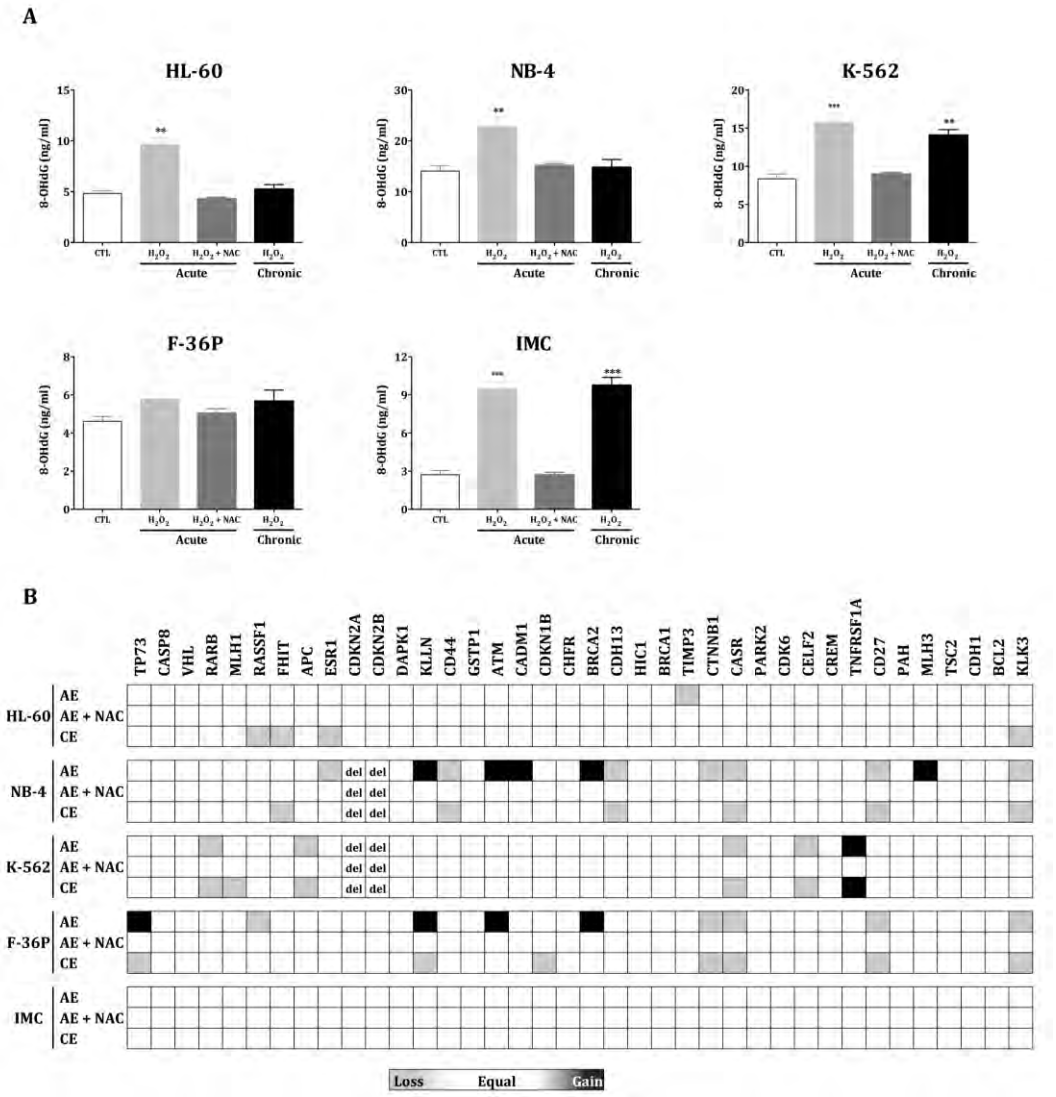


Figure 43. Acute and chronic exposure to hydrogen peroxide induced DNA damage and changes in tumor suppressor gene copy number. The DNA damage (A) was analyzed through 8-hydroxy-2-deoxyguanosine (8-OHdG) levels and the changes in copy number of 38 tumor suppressor genes (B) were analyzed by MS-MLPA in HL-60, NB-4, K-562, F-36P and IMC cells acutely (48h) and chronically (6 months) exposure to exogenous 50 μM of H_2O_2, as described in methods. As control of acute exposure, cells were pre-treated with 1 mM of N-acetylcysteine (NAC) for 1h before H_2O_2 addition. Statistical analysis was carried out by ANOVA with Dunnett's post-hoc test. DNA damage data are shown as mean ± SEM of 3 independent experiments. In tumor suppressor genes copy number analysis, deletions (grey) were defined as a loss ≥0.20 in relation to control cells (not exposed to H_2O_2) and amplifications (black) were defined as a gain of 0.20 in relation to control cells. H_2O_2, hydrogen peroxide; AE, acute exposure; CE, chronic exposure; del, homozygous deletion; **, $p<0.01$; ***, $p<0.001$.

Acute exposure to H_2O_2 led to a 2.0- (p=0.009), 1.9- (p=0.032), 2.9- (p=0.001) and 1.4-fold (p=0.009) increase in 5-mC/5-hmC levels of HL-60, NB-4, K-562 and IMC cells, in comparison to controls.

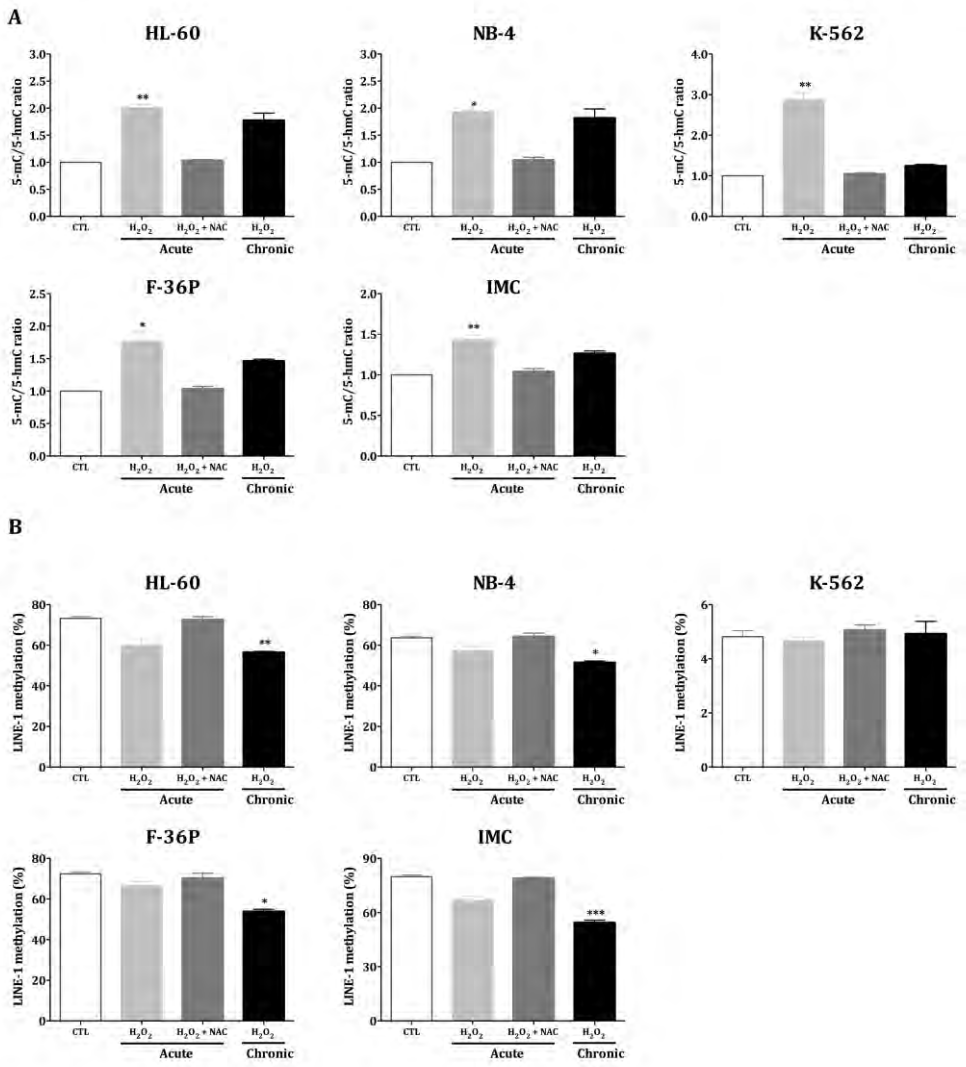


Figure 44. Acute and chronic exposure to hydrogen peroxide changed global DNA methylation. Global DNA methylation was analyzed by 5-methylcytosine (5-mC)/5-hydroxymetylcytosine (5hmC) ratio and LINE-1 methylation (B) in HL-60, NB-4, K-562, F-36P and IMC cells acutely (48h) and chronically (6 months) exposure to exogenous 50 μM of H_2O_2. Statistical analysis was carried out by ANOVA with Dunnett's post-hoc test. Global DNA methylation data are shown as mean ± SEM of 4 independent experiments. Grey box, decrease in methylation levels ≥10% in comparison to controls; Black box, increase in methylation levels ≥10% in comparison to controls; H_2O_2, hydrogen peroxide; *, p<0.05; **, p<0.01; ***, p<0.001.

Under chronic exposure to H_2O_2, LINE-1 methylation levels decreased 1.3- (p=0.006), 1.3- (p=0.019), 1.4- (p=0.016) and 1.5-fold (p<0.001) in HL-60, NB-4, F-36P and IMC cells, respectively, in comparison to their respective controls. The pre-treatment with the antioxidant NAC blocked the increase of 5-mC/5-hmC ratio and the decrease in LINE-1 methylation levels.

In regard to TSG-specific DNA methylation, we analyzed the effect of oxidative stress in the methylation levels of 24 TSG by MS-MLPA. The methylation status was considered changed if a difference ≥10% in the methylation level were observed between exposed and not exposed cells of each cell line, and different ranges of methylation were determined: demethylation (0–9%), moderate methylation (10–49%), substantial methylation (50–74%), and extensive methylation (≥75%). We found that acute and chronic exposure to H_2O_2 induced localized hypermethylation on TSG in all cell lines except F-36P cells.

As observed in Figure 45, chronic exposure to H_2O_2 increased *TP73* methylation levels in HL-60, NB-4, K-562, and IMC cells. In fact, this TSG changed from unmethylated to moderately methylated in K-562 and IMC cells, from moderately to substantially methylated in HL-60 cells and from substantially to extensively methylated in NB-4 cells. In addition to *TP73* modulation, exposure to H_2O_2 increased the methylation levels of other TSG in a cell line and exposure-dependent manner. In HL-60 cells, acute exposure to H_2O_2 increased the methylation levels of *ESR1* and *CADM1* genes, while chronic exposure increased the methylation levels of *ESR1*, *CADM1* and *CDH13* genes.

Furthermore, in K-562 cells chronically exposed to H_2O_2 the methylation status of *RARβ*, *DAPK1*, *CADM1*, *CHFR*, *CDH13* and *TIMP3* genes changed from unmethylated to moderately methylated and the methylation levels of *ESR1* increased 15%; while acute exposure to H_2O_2 changed *CDH13* and *TIMP3* genes from unmethylated to moderately methylated. In the normal lymphocyte cell line (IMC cells), acute exposure to H_2O_2 changed *RARβ* gene from unmethylated to moderately methylated, while chronic exposure changed *RARβ* and *CDH13* genes from

unmethylated to moderately methylated. Furthermore, chronic exposure to H_2O_2 induced a decrease in the methylation levels of *RARβ* in F-36P cells.

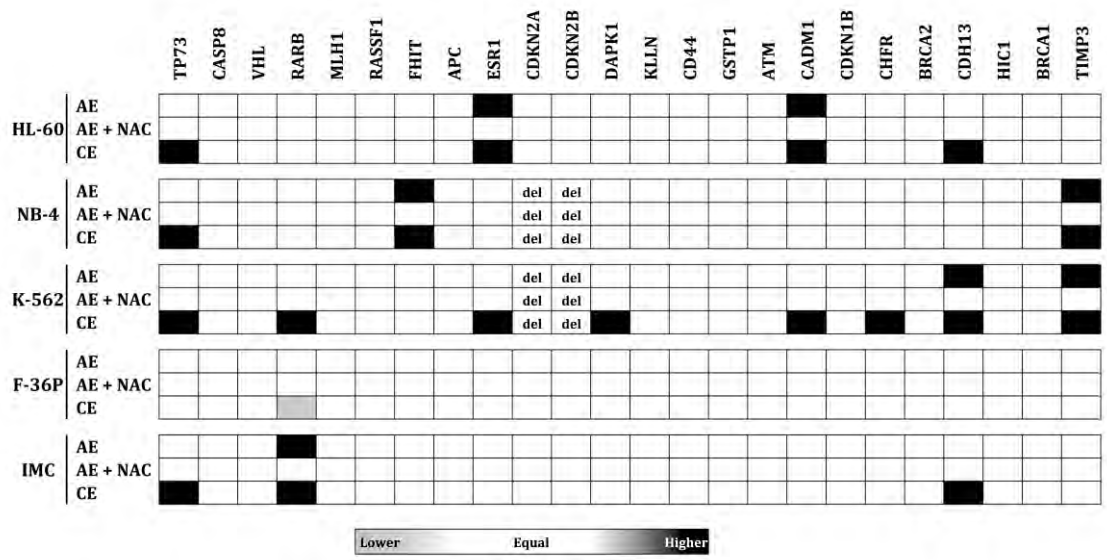


Figure 45. Acute and chronic exposure to hydrogen peroxide changed tumor suppressor gene DNA methylation. The changes induced by hydrogen peroxide (H_2O_2) in the tumor suppressor gene DNA methylation levels were assessed by MS-MLPA in HL-60, NB-4, K-562, F-36P and IMC cells acutely (48h) and chronically (6 months) exposure to exogenous 50 μM of H_2O_2. The methylation status was considered changed if a difference ≥10% in the methylation level were observed between exposed and not exposed cells. As control of acute exposure, cells were pre-treated with 1 mM of N-acetylcysteine (NAC) for 1h before H_2O_2 addition. Statistical analysis was carried out by ANOVA with Dunnett's post-hoc test. Global DNA methylation data are shown as mean ± SEM of 4 independent experiments. Grey box, decrease in methylation levels ≥10% in comparison to controls; Black box, increase in methylation levels ≥10% in comparison to controls; H_2O_2, hydrogen peroxide; AE, acute exposure; CE, chronic exposure; del, homozygous deletion; *, p<0.05; **, p<0.01; ***, p<0.001.

7.4.5. Oxidative stress modulates the expression levels of epigenetic machinery

The expression levels of epigenetic genes may indicate its involvement in the methylation changes observed under oxidative stress. In this context, we analyzed the expression levels of a panel of genes directly involved in the establishment and maintenance of methylation status (Figure 46). We identified common transcriptional responses in cells with H_2O_2-induced methylation, involving

DNMT1, *DNMT3A*, *MECP2*, *EZH2*, and *HDAC1* genes. Under acute exposure to H_2O_2, we observed a significant upregulation of 1.59- to 2.10-fold change in *DNMT1* and between 1.60- and 2.03-fold in *DNMT3A*. The expression levels of *MECP2* were increased under acute exposure to H_2O_2, ranging from 1.51- to 2.22-fold, as well as the expression levels of *EZH2* (ranging between 2.01- to 2.55-fold) and *HDAC1* (ranging between 1.64- and 2.31-fold). Similar results were observed in cells chronically exposed to H_2O_2. Under this condition, *DNMT1* and *DNMT3A* genes were upregulated (fold change ranging from 1.81- to 2.04-fold in *DNMT1* and from 1.71- to 2.18-fold in *DNMT3A*). We also found that chronic exposure to H_2O_2 increased the expression levels of *MECP2* between 1.86- and 2.37-fold, *EZH2* between 2.02- and 2.75-fold as well as *HDAC1* between 1.86- and 2.40-fold. Additionally, the chronic exposure to H_2O_2 induced an upregulation of *DNMT3B* gene (2.97 ± 0.07, $p = 0.001$) in the IMC cells.

7.5. Discussion

The hypothesis that ROS may modulate DNA methylation emerged from observations that cancer cells display aberrant DNA methylation patterns and that these cells are often in an oxidative stress state. In agreement with this theory, here we demonstrated that both acute and chronic exposure to hydrogen peroxide, at a sub-lethal dose, induced oxidative stress and DNA damage, altered DNA methylome and changed TSG copy number in normal as well as in malignant hematological cell lines. The effect of oxidative stress in DNA methylation was demonstrated not only by the increase in DNA methylation level of several TSG, associated with the upregulation of *DNMT1*, *DNMT3A*, *MECP2*, *EZH2* and *HDAC1* genes, but also by the decrease in LINE-1 methylation levels, associated with a significant increase in GSH levels. Moreover, the pre-treatment of cells with the antioxidant NAC prevented these events, confirming the relationship between oxidative stress and DNA methylation.

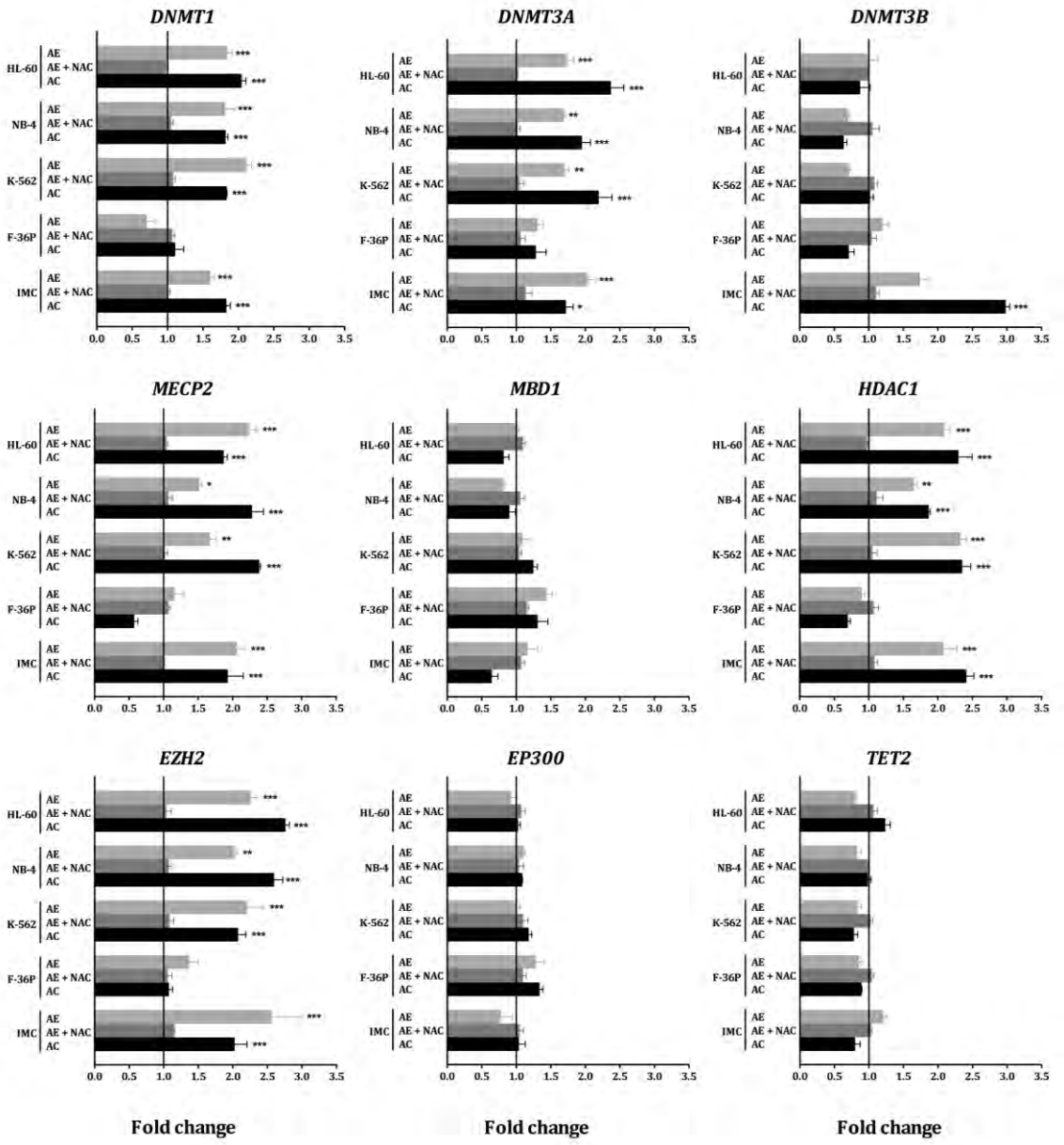


Figure 46. Acute and chronic exposure to hydrogen peroxide modulates gene expression levels of epigenetic machinery and upregulate a specific gene expression signature. The gene expression levels of *DNMT1, DNMT3A, DNMT3B, MECP2, MBD1, HDAC1, EZH2, EP300* and *TET2* genes was evaluated by real time PCR in HL-60, NB-4, K-562, F-36P and IMC cells acutely (48h) and chronically (6 months) exposed to exogenous 50 μM of H_2O_2. As control of acute exposure, cells were pre-treated with 1 mM of N-acetylcysteine (NAC) for 1h before H_2O_2 addition. Statistical analysis was carried out by ANOVA with Dunnett's post-hoc test. Data are shown as mean ± SEM of 3 independent experiments. H_2O_2, hydrogen peroxide; AE, acute exposure; CE, chronic exposure; *,p <0.05; **, p<0.01; ***, p<0.001.

In HL-60, NB-4, K-562 and IMC cells, acute and chronic exposure to hydrogen peroxide affected the DNA methylation of several TSG. In these cell lines exposure to H_2O_2 increased ROS levels and ROS/GSH ratio, and consequently induced a pro-oxidative state. However, in F-36P cells the chronic exposure to this oxidant molecule increased ROS and also GSH levels, inducing a decrease in the ROS/GSH ratio and, consequently, a reduced cellular environment. This cell line was approximately 2.5-fold more resistant to H_2O_2 than HL-60 and K-562 cell and 3.3-fold than NB-4 and IMC cells (Supplementary Figure S1), and this baseline resistance may be responsible for the absence of ROS-induced methylation in F-36P cells. Since TSG methylation was not increased by H_2O_2 exposure in F-36P cells, the changes in the methylation level must be induced only when ROS overwhelmed antioxidant defenses and an oxidative stress environment is established. In agreement with this finding, F-36P cells chronically exposed to H_2O_2 showed a decrease in methylation levels of *RARβ*.

The induction of TSG methylation was mediated by the up-regulation of a specific gene expression signature, comprising a set of genes that encode DNA methyltransferases (*DNMT1* and *DNMT3A*), methyl-CpG-binding proteins (*MECP2*), histone deacetylalases (*HDAC1*), and histone methyltransferases (*EZH2*). Several authors reported the recruitment of DNMT1 and HDAC1 proteins in ROS-induced methylation [Chuang *et al*., 2011; Kang *et al*., 2012; Lim *et al*., 2008; O'Hagan *et al*., 2011; Soberanes *et al*., 2012]. Moreover, O'Hagan *et al*. (2011) demonstrated that hydrogen peroxide treatment induced the formation of a repression complex composed of DNMT1, DNMT3B, EZH2, SIRT1, and γ-H2AX in the context of DNA damage. This author suggested that the repression complex targeted the on-going DNA damage, primarily the 8-OHdG lesions at GC-rich promoters [O'Hagan *et al*., 2011]. In agreement with this hypothesis, we observed a positive association between the increase in TSG methylation and 8-OHdG levels in cells chronically exposed to H_2O_2, and a similar association was found between the increase in TSG methylation and the changes in copy number of the same genes. However, the up-regulation of *DNMT1A* observed here might be specific to hematological cells, since the expression levels of *DNMT1* and *DNMT3A* is higher than *DNMT3B* in

differentiated blood [Mizuno *et al*., 2001] and *DNMT3A* mutations are common events in acute myeloid leukemia [Shih *et al*., 2012]. Furthermore, a previous study demonstrated that inactivation of *RARβ* tumor suppressor gene by methylation was associated with the recruitment of DNMT1 and DNMT3A proteins in NB-4 cells. In this report, Di Croce *et al*. [2002] demonstrated that PML-RAR, an oncogenic transcription factor present in this acute promyelocytic leukemia cell line, is responsible for the recruitment and relocalization of DNMT1, DNMT3A, and HDAC1, as well as for the methylation of target genes, such as *RARβ*. Altogether, these facts suggest that DNMT3A may be the more important *de novo* DNMT in hematological cells.

As mentioned above, exposure to hydrogen peroxide increased the methylation level of several TSG. However, H_2O_2 exposure induced an increase in the *TP73* methylation levels in all cell lines, except in F-36P cells. This cell line presented an extensive methylation of *TP73* (>75%) at baseline, and H_2O_2 did not induce oxidative stress or changes in TSG-specific methylation. The *TP73* gene is a member of the TP53 family with high structural homology to *TP53* gene. The transcription activation of it target genes leads to the induction of cell-cycle arrest and/or apoptosis [Pluta *et al*., 2006]. *TP73* gene locus has two promoters that, by differential splicing and alternative promoter usage, generate two classes of isoforms: TAp73 (tumor suppressor isoforms) and ΔNp73 (oncogenic isoforms). The TAp73 isoforms are activated following DNA damage and promote cell death, whereas the ΔNp73 isoforms act as oncogene leading to cell transformation and promoting cancer development. The balance between TAp73 and ΔNp73 isoforms regulates apoptosis, proliferation, and differentiation [Alexandrova & Moll, 2012; Lai *et al*., 2014; Pluta *et al*., 2006]. Here, we observed that oxidative stress induced hypermethylation of *TP73* gene promoter P1. This methylation change may lead to transcription repression of TAp73 and to preferential expression of ΔNp73. In this context, the hypermethylation of P1 shift the equilibrium between TAp73 and ΔNp73 into the oncogenic activity, contributing to cell transformation and tumorigenesis [Lai *et al*., 2014].

DNA methylation is not only associated with gene expression regulation but also with the methylation of repetitive sequences, such as LINE-1. This type of DNA methylation inhibits the reactivation of these repetitive sequences and consequently prevents chromosomal instability and gene disruption [Portela & Esteller, 2010; Ross *et al*., 2010]. In the present study, we demonstrated that chronic exposure to H_2O_2 also induced LINE-1 hypomethylation in HL-60, NB-4, F-36P, and IMC cells. Similar findings were observed in bladder and urothelial cancer cell lines [Kloypan *et al*., 2015; Wongpaiboonwattana *et al*., 2013]. Furthermore, we found that GSH levels were elevated in cells where ROS-induced LINE-1 hypomethylation was observed. Recently, Kloypan *et al*. [2015] demonstrated that LINE-1 hypomethylation induced by ROS was mediated by depletion of S-adenosylmethionine (SAM) and homocysteine in normal kidney cells and urothelial cancer cell lines. According to this report, SAM – the endogenous universal donor of methyl groups – and homocysteine depletion results from their use in GSH synthesis [Kloypan *et al*., 2015]. The high requirement of GSH in cells exposed to oxidative stress induces a deviation of homocysteine from methionine cycle to GSH synthesis through the transsulfuration pathway [Hitchler & Domann, 2007; Kloypan *et al*., 2015; Lertratanangkoon *et al*., 1997]. Under this condition, homocysteine will be preferentially used in GSH synthesis, inducing a decrease in SAM levels [Hitchler & Domann, 2007]. However, other mechanisms may contribute, at least in part, to LINE-1 hypomethylation induced by ROS. For instance, the formation of 8-OHdG may negatively affect the methylation of adjacent bases by inhibiting MECP2 binding and diminishing the ability of DNMTs to methylate DNA [Valinluck *et al*., 2004], or through the induction of G→T transversions [Lee *et al*., 2002]. Furthermore, ROS contributes to DNA demethylation through passive hydroxylation of 5-methylcytosine [Valinluck *et al*., 2007]. Finally, the lack of association between LINE-1 hypomethylation and 5-mC levels suggests that other mechanisms, such as interactions with transcription factors and repair machinery, may be responsible for the observed LINE-1 hypomethylation [Wilson *et al*., 2007].

The epigenetic events, namely the DNA methylation, can be described as a stable but reversible mechanism, making it a therapeutic target [Das & Singal, 2004]. Furthermore, the changing nature of DNA methylation and the increase in oxidative stress levels seem to be associated with age, and these age-related shifts are highly similar to those observed in cancer [Rang & Boonstra, 2014]. In this context a higher incidence of epigenetic-induced diseases, such as hematological neoplasms, due to increase life expectancy is expected. In the present study we found that the antioxidant NAC were able to prevent the ROS-induced TSG hypermethylation and LINE-1 hypomethylation. Therefore, compounds that reduce oxidative stress may be beneficial in the prevention of epigenetic-associated cancers and/or in its treatment. However, this hypothesis remains to be elucidated.

Different cancer models suggest that high ROS levels can induce either tumor suppressor hypermethylation and/or global DNA hypomethylation [Chuang *et al*., 2011; Kang *et al*., 2012; Kloypan *et al*., 2015; Lertratanangkoon *et al*., 1997; Lim *et al*., 2008; O'Hagan *et al*., 2011; Soberanes *et al*., 2012 Wongpaiboonwattana *et al*., 2013]. However, the influence of ROS on DNA methylation in hematopoietic models has not yet been investigated. We have previously reported that hypermethylation of *P15* and, to a lesser extent, *P16* gene promoters were correlated with ROS levels, as well as with peroxide/GSH ratio, in myelodysplastic syndrome patients [Gonçalves *et al*., 2015c]. Moreover, we also found an association of peroxide levels and peroxide/GSH ratio not only with tumor suppressor gene hypermethylation, but also with the LINE-1 hypomethylation in myeloid neoplasia patients [Chapter 5]. In this context, we used four acute myeloid leukemia cell lines as malignant hematological cells; and since normal myeloid cell lines are not commercially available we established a B lymphocyte cell line, the IMC cells, to use as normal counterpart. Additional to the difference in hematopoietic cell lineage between normal and malignant models, this study presents other limitations that should be mentioned. We found a gene expression signature associated with the ROS-induced hypermethylation of TSG, but we did not explore the expression, co-localization, and activity of the proteins encoded by

these signature genes. Similarly, we observed that oxidative stress-induced LINE-1 hypomethylation was associated with an increased in GSH levels, although we did not prove that SAM was depleted under this pro-oxidant state. However, this study is the first to analyze ROS-induced methylation levels of TSG by a quantitative methodology, like MS-MLPA, and to explore the influence of chronic exposure to H_2O_2 in DNA methylation.

In summary, taken together our data demonstrated, for the first time in normal and malignant hematological cells, that acute and mainly chronic exposure to H_2O_2 induces not only an increase in DNA methylation level of several TSG but also a decrease in LINE-1 methylation levels. The induction of TSG methylation was only observed in cells with a significant increase in ROS/GSH ratio, and was mediated by the up-regulation of a specific gene expression signature, comprising *DNMT1*, *DNMT3A*, *MECP2*, *HDAC1*, and *EZH2* genes. Moreover, we observed that oxidative stress might influence the development and, probably, the progression of hematological neoplasms through the increase of *TP73* methylation levels. Our findings also suggest that chronic exposure to ROS induces an increase in GSH synthesis, probably through the transsulfuration pathway, that leads to LINE-1 hypomethylation. Overall, this study indicates that chronic and, to a lesser extent, acute exposure to H_2O_2 induced TSG hypermethylation and LINE-1 hypomethylation, and suggest that different mechanistic pathways are involved in these DNA methylation changes.

Chapter 8

General discussion &

Concluding remarks

Myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), and acute myeloid leukemia (AML) are clonal disorders of hematopoietic stem or progenitor cells [Fröhling *et al.*, 2005; Kim *et al.*, 2015; Murati *et al.*, 2012]. MDSs are a heterogeneous group of clonal stem cell disorders characterized by dysplasia, impaired differentiation, and ineffective hematopoiesis, which leads to peripheral cytopenias [Adès *et al*., 2014; Issa, 2013; Nimer, 2008]. Likewise, MPNs are a group of neoplasms that arise from genetically altered myeloid stem or progenitor cells [Klco *et al*., 2010; Tefferi *et al*., 2009; Tefferi & Vainchenker, 2011]. These malignancies are characterized by the expansion of one or more hematopoietic cell lineages, with a hypercellular bone marrow due to the overproduction of myeloid cells [Klco *et al*., 2010]. Boths – MDS and MPN – display a high propensity to evolve to AML [Fröhling *et al.*, 2005]. AML is an aggressive malignancy characterized by impaired myeloid differentiation and uncontrolled proliferation of clonal myeloid progenitors that accumulate in peripheral blood, bone marrow, or other tissue [Vardiman *et al.*, 2008]. Similarly to MDS and MPN, AML is a clinically, morphological, and genetically heterogeneous disease involving cells from myeloid lineages [Vardiman *et al.*, 2008]. It is recognized that multiple genetic and epigenetic modifications, which change gene expression, are required for the development of myeloid neoplasms [Kitamura *et al.*, 2014].

During the last few years, many studies have investigated molecular mechanisms underlying myeloid neoplasms. The oxidative stress, one of these mechanisms, is considered an important player in the initiation and progression of these malignancies [Sardina *et al.*, 2012]. The pro-oxidant cellular state is established when ROS levels overwhelm cellular antioxidant defenses [Ghaffari, 2008; Sardina *et al.*, 2012]. ROS can have both beneficial and deleterious effects, and several biological processes are dependent upon appropriate intracellular ROS levels, namely those involved in the activation of signaling pathways such as proliferation, differentiation, and cell death [Ghaffari, 2008; Hasselbalch *et al*., 2014; Imbesi *et al*., 2013]. High levels of ROS may contribute to cancer development through both genetic and epigenetic mechanisms [Wu & Ni, 2015]. At an epigenetic level, both DNA hypermethylation and hypomethylation can be induced by ROS, and these

methylome alterations may provide one way through which ROS exert their deleterious effects [Cuozzo *et al*., 2007; Chung *et al*., 2014; Esteller, 2008; Taby & Issa, 2010; Rang & Boonstra, 2014]. DNA methylation is a reversible epigenetic mechanism that regulates gene expression without altering the gene sequence [Meldi & Figueroa, 2014; Woods & Levine, 2015]. Under normal physiological conditions, CpG islands are unmethylated, but repetitive genomic sequences and introns are hypermethylated [Akhavan-Niaki & Samadani, 2013; Esteller, 2008; Meldi & Figueroa, 2014]. Contrarily, the genome of cancer cells is characterized by global hypomethylation and localized hypermethylation. *CDKN2B* (*P15*) and *CDKN2A* (*P16*) genes are examples of localized hypermethylation that had been described in hematological neoplasms [Esteller, 2008; Galm *et al*., 2006; Karlic *et al*., 2014; Taby & Issa, 2010].

Although the role of ROS in the DNA methylation remains controversial, it is generally accepted that ROS levels increase with age, leading to protein, lipid, and DNA damage [Bejma *et al*., 2000; Driver *et al*., 2000; Hamilton *et al*., 2001; Rang & Boonstra, 2014; Salmon, 2012; Sohal & Orr, 2012]. Similarly, the methylation pattern also changes during lifetime [Bollati *et al*., 2009]. The hypothesis that ROS might modulate the DNA methylation result from the fact that cancer cells display abnormal methylation patterns and are often in a state of oxidative stress [Franco *et al*., 2008]. Moreover, ROS has been shown to be responsible for epigenetic changes in several cancer models [Campos *et al*., 2007; Lim *et al*., 2008; Quan *et al*., 2011; Ziech *et al*., 2011]. Since oxidative stress also increases with age, it is probable that ROS affect DNA methylation in healthy and malignant cells.

The present study evaluated the contribution of oxidative stress and DNA methylation, as well as the role of variants in genes involved in these mechanisms, on myeloid neoplasms development. The ultimate goal of this investigation was to clarify the cross talk between oxidative stress and DNA methylation in hematopoietic cells, and to evaluate their potential clinical utility as diagnostic and prognostic biomarkers of myeloid neoplasms. In order to achieve these goals, we analyzed the relationship between oxidative stress and DNA methylation from three different perspectives: 1. patient-oriented studies on mechanisms of human

disease (Chapter 3 to 5); 2. disease-gene association study (Chapter 6); and 3. *in vitro* mechanistic study (Chapter 7).

In order to address the correlation between oxidative stress and DNA methylation, we first analyzed individually oxidative stress parameters and DNA methylation patterns in patients with MDS and MPN. Our results confirmed that oxidative stress was present not only in bone marrow cells of MDS patients (Chapter 3), but also at a systemic level in patients with MDS and MPN (Chapter 5). Moreover, the accuracy of oxidative stress parameters as diagnostic biomarkers, such as GSH in bone marrow myeloid and nucleated erythroid cells as well as systemic peroxide and 8-OHdG levels, reinforced the hypothesis that oxidative stress is a major event in MDS pathogenesis (Chapter 3 and 5). Similar results in MPN patients also suggested the importance of oxidative stress in this disease (Chapter 5). In regard to DNA methylation, patients with MDS and, to a lesser extent, with MPN had frequently one or more methylated genes, increased levels of 5-mC, decreased 5-hmC/5-mC ratio, and LINE-1 hypomethylation. These results suggest that abnormal hypermethylation of tumor suppressor genes and global hypomethylation of genome are common epigenetic abnormalities in myeloid malignancies (Chapter 5). However, the short follow-up time of our second cohort of myeloid neoplasm patients unable us to validate the role of oxidative stress and DNA methylation in the prediction of disease outcome, namely in the propensity to AML transformation and survival of patients with MDS and MPN, but these analyses will be done in the future.

Furthermore, we found that *KEAP1* gene promoter is frequently hypermethylated in MDS patients. The present study is the first one to investigate the *KEAP1* hypermethylation in hematological neoplasms (Chapter 5). KEAP1 is essential for the regulation of NRF2 activity, and the KEAP1–NRF2 system is one of the critical cytoprotective mechanisms of vertebrates [Geismann *et al*., 2014; Kensler *et al*., 2007; Mitsuishi *et al*., 2012; Niture *et al*., 2014; Rushworth & MacEwan, 2011; Rushworth *et al*., 2012; Singh *et al*., 2006; Stepkowski & Kruszewski, 2011]. Under physiological conditions, NRF2 is constantly ubiquitinated through KEAP1 in the cytoplasm and degraded in the proteasome [Mitsuishi *et al*., 2012; Niture *et al*.,

2014; Rushworth & MacEwan, 2011]. NRF2 stimulates the transcriptional activation of over a hundred cytoprotective and detoxification genes including antioxidants (ferritin, GSR, GCLM and GCLC), phase-I drug oxidation enzymes (NQO1), and cytoprotective enzymes (HO-1) [Rushworth & MacEwan, 2011]. The *KEAP1* hypermethylation has been identified in colorectal [Hanada *et al*., 2012], lung [Muscarella *et al*., 2011a], malignant glioma [Muscarella *et al*., 2011b], and prostate cancers [Zhang *et al*., 2010]. The inhibition of *KEAP1* gene expression results in NRF2 accumulation, leading to increased cell growth and survival, as well as to decreased apoptosis [Mitsuishi *et al*., 2012; Niture *et al*., 2014]. The *KEAP1* hypermethylation leads to constitutive NRF2 activation and, consequently, to high expression of cytoprotective and detoxification genes. This fact contributes to the development of chemoresistance during therapy, due to overexpression of genes that encode drug efflux pumps, detoxification proteins, and antioxidants enzymes [Rushworth & MacEwan, 2011]. Since *KEAP1* hypermethylation can be reversed by decitabine treatment [Hanada *et al*., 2012], epigenetic modulators could be a therapeutic strategy to circumvent chemoresistance associated with *KEAP1* hypermethylation (NRF2 overexpression) in myeloid neoplasm. In this context, *KEAP1* hypermethylation may contribute to the development and progression of myeloid neoplasms, and can have therapeutic implications. However, this hypothesis needs to be confirmed experimentally.

Following the confirmation that oxidative stress and DNA methylation abnormalities were present in these myeloid neoplasms, we investigated the correlation between them. Overall, our results confirm that oxidative stress is correlated with DNA methylation in myeloid patients. We firstly demonstrated that MDS patients with high levels of intracellular peroxides and superoxide anion, as well as those with high ratios of peroxides/GSH and superoxide/GSH had increased methylation frequency of *P15* and *P16* gene promoters. Moreover, oxidative stress levels were able to discriminate MDS patients with methylation from those without, being the peroxides/GSH ratio the more accurate methylation biomarker (Chapter 4). We also found that LINE-1 hypomethylation, 5-mC levels, and tumor suppressor gene hypermethylation was positively correlated with

oxidative stress parameters (peroxide levels and peroxide/GSH ratio). Interestingly, the hypermethylation of tumor suppressor genes did not depend on oxidative stress levels, i.e. patients with one, two, or three methylated genes (independently of the gene) had similar levels of oxidative stress (Chapter 5). Together, these finding supports the hypothesis that oxidative stress is correlated with DNA methylation.

The identification of causal genetic variants is one goal of molecular epidemiology, medicine, and pharmacogenomics. However, since myeloid neoplasms are genetically complex and the identification of their causative gene variants is unlikely, the goal for these complex diseases is, at least, to identify variants that increase the risk of disease development. In myeloid neoplasms, Mendel's rules of inheritance do not apply, but other phenomena are observed, such as reduced penetrance, variable expressivity, polygenic traits, gene-gene interactions, and gene-environment interactions [Craig, 2008]. The identification of genetic risk variants underlying complex human diseases typically applies disease-gene association studies. In this context, we performed an exploratory/hypothesis generating hospital-based case-control study that allowed us to identify five genes associated with MDS (*GPX1, NEIL1, NFE2L2, OGG1*, and *SOD2*), two genes associated with AML (*DNMT3B* and *SLC19A1*), and two genes associated with the susceptibility of both disease (*CYBA* and *DNMT1*) s. These disease-associated variants were located in genes involved in oxidative stress (*CYBA, GPX1, NFE2L2*, and *SOD2*), DNA base excision repair (*OGG1* and *NEIL1*), DNA methylation (*DNMT1* and *DNMT3B*), and folate metabolism pathways (*SLC19A1*; Chapter 6).

As mentioned before, a candidate gene approach was used to select SNPs based on their involvement in oxidative stress, DNA methylation, base excision repair, and folate metabolism pathways. This strategy allowed us to find nine gene variants associated with MDS and AML. Future candidate gene studies should be focus in susceptibility genes identified in the present study. However, the SNP selection needs to be prioritized based on their potential deleterious functional effects. To improve SNP selection, several informatic and WEB-based tools have been developed. These tools allow the prioritization of protein coding regions SNPs, as

well as non-coding and regulatory regions SNPs [Buske *et al.*, 2013; Castellana & Mazza, 2013; Jung & Kim, 2014; Lee & Shatkay, 2008; Ohanian *et al.*, 2012; Wang *et al.*, 2005; Xu & Taylor, 2009; Yuan *et al.*, 2006].

Moreover, to understand the complexity of diseases and traits, new methodologies should be applied. Systems genetics approaches, which focus on networks of interaction between genes and phenotypes, are one example of these new methodologies [Kogelman & Kadarmideen, 2014]. The analysis of networks, functional pathways, and underlying causal genes are able to unravel the biological and genetic background of complex diseases [Jin *et al*., 2014]. According to this, the joint action of several variants is more likely to have a significant role in disease development. In the combined genotype analysis, we found different susceptibility genotypic profiles for MDS and AML development, and these profiles may have more implications with clinical usefulness in terms of myeloid neoplasm risk prediction than single SNP analysis (Chapter 6). However, the exploratory nature of our study (small cohort) did not allow us to establish consistent associations. Since the analysis of single variants within isolated genes is not informative enough to explain the underlying disease mechanisms, future studies must be focus on pathway networks, through the integration of protein–protein interaction networks [Bakir-Gungor *et al*., 2014]. These methods follow a systems biology approach where pairwise or collective effects of genetic variants are evaluated, and the functional consequence of each susceptibility allele is inferred from interactions between proteins pairs [Andrew *et al*., 2012; Hicks *et al*., 2013; Jin *et al*., 2014].

Another goal of medical genetics is to identify prognostic variants. Although prognostic biomarkers in human cancers are, traditionally, obtained from various patient- and disease-related measurable variables, such as the demographic, pathological, and molecular characteristics, genetic variants could be important biomarkers of survival and disease outcome [Savas *et al*., 2013]. Here, we found that variants from *DNMT3A, MTRR, NEIL1*, and *OGG1* genes can be implicated in the increased propensity to AML transformation observed in MDS patients. Moreover, our results suggest that *DNMT3A* and *OGG1* variants influence the survival of MDS

patients, and *GPX1* and *KEAP1* variants modulate the survival of AML patients (Chapter 6). The identification of genetic prognostic variants may improve treatment decisions and clinical outcomes in cancer patients [Savas *et al*., 2013]. Taken together, our findings contribute to a better understanding of the molecular basis of these myeloid neoplasms, since it allows the identification of susceptibility genes and potential therapeutic targets, as well as the prediction of disease outcome.

To provide insights in the underlying mechanisms of DNA methylation changes induced by oxidative stress, we finished this investigation with *in vitro* mechanistic studies. Our results indicated that acute and chronic exposure to hydrogen peroxide, at a sub-lethal dose, induced oxidative stress, altered DNA methylome, induced DNA damage, and changed tumor suppressor genes (TSG) copy number in normal and malignant hematological cell lines (Chapter 7). The effect of oxidative stress in DNA methylation was demonstrated not only by the increase in DNA methylation level of several TSG, but also by the decrease in LINE-1 methylation levels. The TSG hypermethylation was mediated by the up-regulation of epigenetic machinery comprising *DNMT1*, *DNMT3A*, *MECP2*, *HDAC1*, and *EZH2* genes, while LINE-1 hypomethylation was associated with a significant increase in GSH levels. Furthermore, the increase in ROS levels was not sufficient to induce TSG hypermethylation, and this event was only observed in cells with a significant imbalance between ROS and GSH (Chapter 7). This finding corroborates the results obtained in our clinical studies (Chapter 4 and 5), in which peroxides/GSH ratio was a more accurate discriminator of TSG methylation. In agreement with the hypothesis that ROS induced genetic and epigenetic changes, the antioxidant N-acetylcysteine was able to prevent these events (Chapter 7). However, some mechanistic aspects, such as the role of S-adenosylmethionine in LINE-1 hypomethylation and the composition of the DNA repression complex in hematological cells, need to be further addressed.

The involvement of ROS in DNA methylation may have several clinical implications in cancer. Oxidative stress increases exponentially with age, and a remarkable decline of cell repair machinery is also observed in elderly individuals [Cencioni *et*

al., 2013]. Moreover, the gradual increase of oxidative damage and DNA methylation changes in aging is associated with ROS accumulation [Ben-Avraham *et al*., 2012; Cencioni *et al*., 2013]. In this context, the age induced oxidative stress and DNA methylation abnormalities may influence the incidence of myeloid neoplasms, which are predominantly diseases of the elderly. In the last decades, global life expectancy has increased remarkably and the expected increase in the aging population will have a significant impact on the incidence of myeloid neoplasms. However, oxidative stress and DNA methylation may constitute new targets for therapeutic and chemopreventive approaches aiming to slow the aging process and, consequently, age-associated diseases.

In regard to myeloid neoplasms treatment, ROS could represent a potential therapeutic target. Two therapeutic strategies can be applied. One approach is based in the administration of compounds, such as Motexafin, Gadolinium, and β-Lapachone, that amplifies the existing ROS in malignant cells [Cencioni *et al*., 2013; Hole *et al*., 2011]. This therapeutic regime is expected to increase ROS level and, therefore, activate cell death [Hole *et al*., 2011]. Cancer cells, due to their intrinsic oxidative stress state, are more vulnerable to further ROS insults. Experimental systems had demonstrated that cell death induced by exogenous ROS-generating agents occur preferentially in neoplastic cells, and that some of these agents show promising therapeutic activity in clinical studies [Cencioni *et al*., 2013]. The other approach is based in the suppression of ROS [Cencioni *et al*., 2013; Hole *et al*., 2011]. This therapeutic regime is based on the fact that ROS-generating cancers develop a new redox homeostatic state requiring higher ROS levels than their normal counterparts [Cencioni *et al*., 2013; Hole *et al*., 2011]. These strategies use ROS-modulating agents, for example Imexon (depletes and exports GSH outside cancer cells) or Mangafodipir (mimetic of SOD, CAT, and GSR redutase), which induce cancer cell dead and, simultaneously, protect normal cells from oxidative damage [Cencioni *et al*., 2013; Hole *et al*., 2011; Robbins & Zhao, 2014]. In regard to DNA hypermethylation two classes of epigenetic modifiers already have been approved for MDS and AML treatment: DNMT inhibitors, such

as Decitabine and Azacitidine, and HDAC inhibitors, such as Vorinostat and Depsopeptide [Taby & Issa, 2010].

The increase global incidence of cancer, with its associated morbidity, mortality, and high healthcare costs, promoted the interest in development of strategies for disease prevention. In 1976, Sporn defined cancer chemoprevention as the use of natural, synthetic, or biological chemical agents to reverse, suppress, or prevent either the initial phase of carcinogenesis or the progression of neoplastic cells to cancer [Steward & Brown, 2013]. The successes in the chemoprevention of breast, prostate, and colon cancer, stimulated this investigation field and, at this moment, are at least 10 FDA-approved agents to treat precancerous lesions or to reduce cancer risk [Steward & Brown, 2013; William *et al*., 2009]. The chemoprevention underlies in the fact that carcinogenesis is multistep – resulting from genetic and epigenetic alterations –, multipathway, and multifocal [William *et al*., 2009]. In this context, chemoprevention agents should have pleiotropic proprieties.

Several studies have provided evidence that some xenobiotic and natural bioactive compounds found in food and herbs can target the epigenetic machinery [Stefanska *et al*., 2012]. Besides it actions, as epigenetic modulators, these compounds also share antioxidant properties. The increase production of ROS during aging, at least in part, is attributed to decrease activity and protein levels of SIRT1 (silent information regulator 1), a class III histone deacetylase [Cencioni *et al*., 2013; Lavu *et al*., 2008]. This protein is involved in several cellular processes, including metabolism, aging, and cancer [Lavu *et al*., 2008]. Resveratrol, a SIRT1 activator studied as chemopreventive agent, exhibits pleiotropic effects including antioxidant, anti-inflammatory, cardioprotective, and antitumor activities [Huang *et al*., 2011]. At epigenetic level, this compound induces P300 HAT activity and tumor suppressor miRNAs, but also inhibits oncogenic miRNAs [Cencioni *et al*., 2013; Lavu *et al*., 2008; Huang *et al*., 2011]. On the other hand, their high antioxidant properties are responsible for the capacity of decrease aging rate [Marchal *et al*., 2013] and, therefore, may modulate ROS-induced DNA methylation changes. In this context, Resveratrol is a good candidate drug to chemoprevention of myeloid neoplasms.

Additionally to Resveratrol, nutrients and vitamins (e.g. folate and other nutrients of one-carbon metabolism, retinoic acid, and vitamin E), polyphenols and other natural products (e.g. epigallocatechin gallate-3-gallate, parthenolide, and curcumin), sulfur-containing compounds (e.g. sulforaphane), pharmacological agents (such as Celecoxib, Decitabine, and Zebularine), among others, also display chemoprotective effects [Huang *et al*., 2011; Stefanska *et al*., 2012; William *et al*., 2009]. For example, folate deficiency has been associated with the development of several cancers, and malignant transformation in this condition was accompanied by SAM depletion and global DNA hypomethylation, as well as by hypomethylation of oncogenes (oncogenes activation) and hypermethylation of tumor suppressor genes (tumor suppressor genes silencing) [Huang *et al*., 2011; Stefanska *et al*., 2012]. The increase in DNMTs induced by folate deficiency may explain the hypermethylation observed, whereas the stimulation of MBD2 and MBD4 may be responsible for oncogenes hypomethylation, since both proteins have been implicated in active DNA demethylation [Stefanska *et al*., 2012]. Altogether, these facts suggested that folate supplementation might have chemopreventive effects. However, folate chemoprotective effects on DNA methylation and cancer risk seem to be dependent of *MTHFR* gene polymorphisms [Davis & Uthus, 2004; Slattery *et al*., 1999].

Besides the influence of ROS on DNA methylation, other mechanisms may influence DNA methylation. For example, genetic variants are possible sources of DNA methylation diversity. The interindividual variations in DNA methylation pattern are, at least in part, due to the presence of polymorphic variants in the CpG dinucleotides and in the immediately surrounding sequences [Hellman & Chess, 2010; Heyn *et al*., 2013]. SNPs and other genetic variants have an effect on DNA methylation, which in turn may affect the phenotype. Moreover, DNA methylation can be modulated by polymorphisms in the epigenetic machinery and in enzymes from one-carbon metabolism (Chapter 6). However, studies addressing the functional implications of the disease-associated SNPs found in the present investigation should be explored in the future.

The present study intended to better understand the biology of myeloid neoplasms, through the cross talk between oxidative stress and DNA methylation, as well as their genetic variants on the development and progression of myeloid neoplasms.

In **summary**, the major conclusions were:

- Bone marrow cells from MDS patients have increased intracellular peroxides levels and decreased GSH content, when compared with their normal counterpart, suggesting that oxidative stress is implicated in MDS development;

- Blasts appear to be the most affected cells, contributing more to MDS development and progression. However, all blood cell types are affected in a subtype dependent manner;

- RA and RCMD patients have the highest oxidative stress levels, which could contribute to the higher apoptotic rates, translated into cytopenias, observed in these patients; while RAEB-1 and -2 patients are the less oxidative stress affected MDS subtypes. Moreover, low risk patients (low- and int-1-risk groups) show higher intracellular ROS levels than high-risk patients (int-2-risk group);

- Precursor cells from RAEB-1, RAEB-2, and int-2 risk patients have increased superoxide/peroxides ratio and $\Delta\psi_{mit}$, which may explain their cellular proliferative potential. Furthermore, blasts from RCMD patients have the lower $\Delta\psi_{mit}$ simultaneously with a low superoxide/peroxides ratio, contributing to the peripheral cytopenias observed in this MDS subtype;

- MDS patients have lower systemic levels of GSH and TAS, higher ratios of NO/GSH and NO/TAS, as well as higher DNA damage (8-OHdG) levels, while MPN patients have increased levels of lipid peroxidation (MDA). Additionally, patients with both diseases have increased peroxide levels;

- The hypermethylation of *P15*, *P16, DAPK1,* and *KEAP1* genes is a common event in MDS patients, while hypermethylation of *TP53* and *MGMT* gene is absent in these patients. Furthermore, patients with MDS or MPN have increased levels of 5-mC, decreased 5-hmC/5-mC ratio, and LINE-1 hypomethylation;

- Intracellular levels of peroxides, superoxide, and GSH, as well as the superoxide/peroxides ratio may constitute novel biomarkers with value in diagnosis and/or prognosis of MDS. Moreover, peripheral levels of 8-OHdG and 5-mC are accurate MDS diagnostic biomarkers, whereas MDA levels are potentially diagnostic biomarkers for MPN;

- Systemic levels of peroxide, 8-OHdG, and 5-mC, as well as the presence of two or more methylated TSG are independent risk factors for MDS development, while peroxide and MDA are independent risk factors for MPN;

- The hypermethylation of *P15* and *P16* gene promoters is correlated with bone marrow intracellular levels of ROS, as well as with the ratios of peroxides/GSH and superoxide/GSH in MDS patients. Furthermore, this correlation is also observed in peripheral blood analysis, which showed that 5-mC levels, LINE-1 hypomethylation, as well as TSG hypermethylation are associated with peroxide levels and peroxide/GSH ratio in MPN and MDS;

- Variants in *GPX1*, *NEIL1*, *NFE2L2*, *OGG1*, and *SOD2* genes influence MDS predisposition, whereas *DNMT3B* and *SLC19A1* variants predispose to AML; the susceptibility for both diseases are associated with *CYBA* and *DNMT1* variants;

- Variants in *DNMT3A*, *MTRR*, *NEIL1*, and *OGG1* genes influence the AML transformation rate of MDS patients, whereas *MTRR*, *KEAP1*, and *GPX1* variants modulate the survival of patients with MDS and AML;

- Acute and chronic exposure to H_2O_2 increase DNA methylation levels of several tumor suppressor genes, in a cell line-dependent manner. However,

an increase in *TP73* methylation levels is observed in all cell lines. The TSG hypermethylation is mediated by the up-regulation of a specific gene expression signature, comprising *DNMT1*, *DNMT3A*, *MECP2*, *HDAC1*, and *EZH2* genes. Furthermore, chronic exposure to H_2O_2 induces LINE-1 hypomethylation associated with increased GSH content;

– The pre-treatment with N-acetylcysteine, an antioxidant molecule, prevents aberrant DNA methylation events, reinforcing the hypothesis that oxidative stress influences DNA methylation and suggesting a potential chemoprevention effect of this compound.

To concluded, the present study reflects the complexity of myeloid malignancies and points to a possible link between oxidative stress and DNA methylation, which besides the relevance in the development and progression of these neoplasms, could also constitute new diagnostic and prognostic biomarkers, as well as new potential therapeutic and/or chemoprevention targets.

Chapter 9

References

Abdel-Wahab, O., Adli, M., LaFave, L.M., Gao, J., Hricik, T., Shih, A.H., Pandey, S., Patel, J.P., Chung, Y.R., Koche, R., Perna, F., Zhao, X., Taylor, J.E., Park, C.Y., Carroll, M., Melnick, A., Nimer, S.D., Jaffe, J.D., Aifantis, I., Bernstein, B.E., et al (2012) ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. *Cancer cell*, **22,** 180–93.

Adès, L., Itzykson, R. & Fenaux, P. (2014) Myelodysplastic syndromes. *Lancet*, **383,** 2239–52.

Afanas'ev, I. (2014) New nucleophilic mechanisms of ros-dependent epigenetic modifications: comparison of aging and cancer. *Aging and disease*, **5,** 52–62.

Aggerholm, A., Holm, M.S., Guldberg, P., Olesen, L.H. & Hokland, P. (2006) Promoter hypermethylation of p15INK4B, HIC1, CDH1, and ER is frequent in myelodysplastic syndrome and predicts poor prognosis in early-stage patients. *European Journal of Haematology*, **76,** 23–32.

Agirre, X., Novo, F.J., Calasanz, M.J., Larráyoz, M.J., Lahortiga, I., Valgañón, M. García-Delgado, M., Vizmanos, J.L. (2003) TP53 is frequently altered by methylation, mutation, and/or deletion in acute lymphoblastic leukaemia. *Molecular Carcinogenesis*, **38,** 201–208.

Akalin, A., Garrett-Bakelman, F.E., Kormaksson, M., Busuttil, J., Zhang, L., Khrebtukova, I., Milne, T.A., Huang, Y., Biswas, D., Hess, J.L., Allis, C.D., Roeder, R.G., Valk, P.J.M., Löwenberg, B., Delwel, R., Fernandez, H.F., Paietta, E., Tallman, M.S., Schroth, G.P., Mason, C.E., et al (2012) Base-pair resolution DNA methylation sequencing reveals profoundly divergent epigenetic landscapes in acute myeloid leukemia. *PLoS genetics*, **8,** e1002781.

Akhavan-Niaki, H. & Samadani, A.A. (2013) DNA methylation and cancer development: molecular mechanism. *Cell biochemistry and biophysics*, **67,** 501–13.

Aktuglu, M.B., Ayer, M., Bireller, E.S., Rencuzogullari, C., Acik, H., Karaali, Z., Alioglu, T., Yigit, N., Velet, M., Atalay, E., Ure, O.S. & Cakmakoglu, B. (2014) Investigation of DNA repair gene variants on myelodysplastic syndromes in a Turkish population. *Medical Oncology*, **31,** 174.

Alexandrova, E.M. & Moll, U.M. (2012) Role of p53 family members p73 and p63 in human hematological malignancies. *Leukemia & lymphoma*, **53,** 2116–29.

Allan, J.M., Smith, A.G., Wheatley, K., Hills, R.K., Travis, L.B., Hill, D.A., Swirsky, D.M., Morgan, G.J. & Wild, C.P. (2004) Genetic variation in XPD predicts treatment outcome and risk of acute myeloid leukemia following chemotherapy. *Blood*, **104,** 3872–7.

Almeida, S., Sarmento-Ribeiro, A.B., Januário, C., Rego, a C. & Oliveira, C.R. (2008) Evidence of apoptosis and mitochondrial abnormalities in peripheral blood cells of Huntington's disease patients. *Biochemical and biophysical research communications*, **374,** 599–603.

Ambrosone, C.B., Ahn, J., Singh, K.K., Rezaishiraz, H., Furberg, H., Sweeney, C., Coles, B. & Trovato, A. (2005) Polymorphisms in genes related to oxidative stress (MPO, MnSOD, CAT) and survival after treatment for breast cancer. *Cancer research*, **65,** 1105–11.

Andrew, A.S., Hu, T., Gu, J., Gui, J., Ye, Y., Marsit, C.J., Kelsey, K.T., Schned, A.R., Tanyos, S.A., Pendleton, E.M., Mason, R.A., Morlock, E. V, Zens, M.S., Li, Z., Moore, J.H., Wu, X. & Karagas, M.R. (2012) HSD3B and gene-gene interactions in a pathway-based analysis of genetic susceptibility to bladder cancer. *PloS one*, **7,** e51301.

Antico Arciuch, V.G., Elguero, M.E., Poderoso, J.J. & Carreras, M.C. (2012) Mitochondrial regulation of cell cycle and proliferation. *Antioxidants & redox signaling*, **16,** 1150–80.

Arslan, S., Pinarbasi, H. & Silig, Y. (2011) Myeloperoxidase G-463A polymorphism and risk of lung and prostate cancer in a Turkish population. *Molecular Medicine Reports*, **4,** 87–92.

Babushok, D. V. & Bessler, M. (2015) Genetic predisposition syndromes: When should they be considered in the work-up of MDS? *Best Practice & Research Clinical Haematology*, **28,** 55–68.

Bakir-Gungor, B., Egemen, E. & Sezerman, O.U. (2014) PANOGA: a web server for identification of SNP-targeted pathways from genome-wide association study data. *Bioinformatics (Oxford, England)*, **30,** 1287–9.

Baldeiras, I., Santana, I., Proença, M.T., Garrucho, M.H., Pascoal, R., Rodrigues, A., Duro, D. & Oliveira, C.R. (2010) Oxidative damage and progression to Alzheimer's disease in patients with mild cognitive impairment. *Journal of Alzheimer's Disease*, **21,** 1165–77.

Baldus, C.D., Thiede, C., Soucek, S., Bloomfield, C.D., Thiel, E. & Ehninger, G. (2006) BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with normal cytogenetics: prognostic implications. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **24,** 790–7.

Bănescu, C., Tilinca, M., Benedek, E.L., Demian, S., Macarie, I., Duicu, C. & Dobreanu, M. (2013) XRCC3 Thr241Met polymorphism and risk of acute myeloid leukemia in a Romanian population. *Gene*, **526,** 478–83.

Bănescu, C., Trifa, A.P., Voidăzan, S., Moldovan, V.G., Macarie, I., Benedek Lazar, E., Dima, D., Duicu, C. & Dobreanu, M. (2014a) CAT, GPX1, MnSOD, GSTM1, GSTT1, and GSTP1

genetic polymorphisms in chronic myeloid leukemia: a case-control study. *Oxidative medicine and cellular longevity*, **2014,** 875861.

Bănescu, C., Duicu, C., Trifa, A.P. & Dobreanu, M. (2014b) XRCC1 Arg194Trp and Arg399Gln polymorphisms are significantly associated with shorter survival in acute myeloid leukemia. *Leukemia & lymphoma*, **55,** 365–70.

Bao, Q., He, B., Pan, Y., Tang, Z., Zhang, Y., Qu, L., Xu, Y., Zhu, C., Tian, F. & Wang, S. (2011) Genetic variation in the promoter of DNMT3B is associated with the risk of colorectal cancer. *International Journal of Colorectal Disease*, **26,** 1107–12.

Barham, D. & Trinder, P. (1972) An improved colour reagent for the determination of blood glucose by the oxidase system. *The Analyst*, **97,** 142–5.

Bartlett, J.M.S. & White, A. (2003) Extraction of DNA from whole blood. *Methods in molecular biology (Clifton, N.J.)*, **226,** 29–32.

Bedard, K., Attar, H., Bonnefont, J., Jaquet, V., Borel, C., Plastre, O., Stasia, M.J., Antonarakis, S.E. & Krause, K.H. (2009) Three common polymorphisms in the CYBA gene form a haplotype associated with decreased ROS generation. *Human Mutation*, **30,** 1123–33.

Bejar, R., Lord, a., Stevenson, K., Bar-Natan, M., Perez-Ladaga, a., Zaneveld, J., Wang, H., Caughey, B., Stojanov, P., Getz, G., Garcia-Manero, G., Kantarjian, H., Chen, R., Stone, R.M., Neuberg, D., Steensma, D.P. & Ebert, B.L. (2014) TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood*, **124,** 2705–12.

Bejma, J., Ramires, P. & Ji, L.L. (2000) Free radical generation and oxidative stress with ageing and exercise: differential effects in the myocardium and liver. *Acta physiologica Scandinavica*, **169,** 343–51.

Belickova, M., Merkerova, M.D., Stara, E., Vesela, J., Sponerova, D., Mikulenkova, D., Brdicka, R., Neuwirtova, R., Jonasova, A. & Cermak, J. (2013) DNA repair gene variants are associated with an increased risk of myelodysplastic syndromes in a Czech population. *Journal of hematology & oncology*, **6,** 9.

Ben-Avraham, D., Muzumdar, R.H. & Atzmon, G. (2012) Epigenetic genome-wide association methylation in aging and longevity. *Epigenomics*, **4,** 503–9.

Bennemann, K., Galm, O., Wilop, S., Schubert, C., Brümmendorf, T.H. & Jost, E. (2012) Epigenetic dysregulation of secreted frizzled-related proteins in myeloproliferative neoplasms complements the JAK2V617F-mutation. *Clinical epigenetics*, **4,** 12.

Bergman, Y. & Cedar, H. (2013) DNA methylation dynamics in health and disease. *Nature structural & molecular biology*, **20,** 274–81.

Bhatla, D., Gerbing, R.B., Alonzo, T.A., Mehta, P.A., Deal, K., Elliott, J., Meshinchi, S., Geiger, H., Perentesis, J.P., Lange, B.J. & Davies, S.M. (2008) DNA repair polymorphisms and outcome of chemotherapy for acute myelogenous leukemia: a report from the Children's Oncology Group. *Leukemia*, **22,** 265–72.

Bhattacharyya, S., Yu, Y., Suzuki, M., Campbell, N., Mazdo, J., Vasanthakumar, A., Bhagat, T.D., Nischal, S., Christopeit, M., Parekh, S., Steidl, U., Godley, L., Maitra, A., Greally, J.M. & Verma, A. (2013) Genome-wide hydroxymethylation tested using the HELP-GT assay shows redistribution in cancer. *Nucleic Acids Research*, **41,** 1–10.

Bigarella, C.L., Liang, R. & Ghaffari, S. (2014) Stem cells and the impact of ROS signaling. *Development,* **22,** 4206–18.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S. & Kalayci, O. (2012) Oxidative stress and antioxidant defense. *The World Allergy Organization journal*, **5,** 9–19.

Bodoor, K., Haddad, Y., Alkhateeb, A., Al-Abbadi, A., Dowairi, M., Magableh, A., Bsoul, N. & Ghabkari, A. (2014) DNA hypermethylation of cell cycle (p15 and p16) and apoptotic (p14, p53, DAPK and TMS1) genes in peripheral blood of leukemia patients. *Asian Pacific Journal of Cancer Prevention*, 15, 75–84.

Boissel, N., Nibourel, O., Renneville, A., Gardin, C., Reman, O., Contentin, N., Bordessoule, D., Pautas, C., de Revel, T., Quesnel, B., Huchette, P., Philippe, N., Geffroy, S., Terre, C., Thomas, X., Castaigne, S., Dombret, H. & Preudhomme, C. (2010) Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **28,** 3717–23.

Boland, M.L., Chourasia, A.H. & Macleod, K.F. (2013) Mitochondrial dysfunction in cancer. *Frontiers in oncology*, **3,** 292.

Bollati, V., Fabris, S., Pegoraro, V., Ronchetti, D., Mosca, L., Deliliers, G.L., Motta, V., Bertazzi, P.A., Baccarelli, A. & Neri, A. (2009) Differential repetitive DNA methylation in multiple myeloma molecular subgroups. *Carcinogenesis*, **30,** 1330–5.

Borger, D.R., Tanabe, K.K., Fan, K.C., Lopez, H.U., Fantin, V.R., Straley, K.S., Schenkein, D.P., Hezel, A.F., Ancukiewicz, M., Liebman, H.M., Kwak, E.L., Clark, J.W., Ryan, D.P., Deshpande, V., Dias-Santagata, D., Ellisen, L.W., Zhu, A.X. & Iafrate, A.J. (2012) Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. *The oncologist*, **17,** 72–9.

Bourgeais, J., Gouilleux-Gruart, V. & Gouilleux, F. (2013) Oxidative metabolism in cancer: A STAT affair? *Jak-Stat*, **2,** e25764.

Bowen, D. & Peddie, C. (2002) Mitochondrial oxygen consumption and ineffective haematopoiesis in patients with myelodysplastic syndromes. *British journal of haematology*, **118,** 345–6.

Bracht, J.R. (2014) Beyond transcriptional silencing: Is methylcytosine a widely conserved eukaryotic DNA elimination mechanism? *BioEssays*, **36,** 346–52.

Brenet, F., Moh, M., Funk, P., Feierstein, E., Viale, A.J., Socci, N.D. & Scandura, J.M. (2011) DNA methylation of the first exon is tightly linked to transcriptional silencing. *PloS one*, **6,** e14524.

Broderick, P., Bagratuni, T., Vijayakrishnan, J., Lubbe, S., Chandler, I. & Houlston, R.S. (2006) Evaluation of NTHL1, NEIL1, NEIL2, MPG, TDG, UNG and SMUG1 genes in familial colorectal cancer predisposition. *BMC cancer*, **6,** 243.

Brookes, E. & Shi, Y. (2014) Diverse Epigenetic Mechanisms of Human Disease. Annu Rev Genet, **48,** 237-68.

Brunning, R., Orazi, A., Germing, U., Le Beau, M.M., Porwit, A., Baumann, I., Vardiman, J.W. & Hellstrom-Lindberg, E. (2008) Myelodysplastic syndromes/neoplasms. In *WHO classification of tumours of haematopoietic and lymphoid tissues*, Swerdlow, S.H., Campo, E., Harris, NL., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. & Vardiman J.W. (4th ed) pp 88–103. Lyon: International Agency for Research on Cancer.

Buckland, P.R. (2006) The importance and identification of regulatory polymorphisms and their mechanisms of action. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, **1762,** 17–28.

Bujko, M., Musialik, E., Olbromski, R., Przestrzelska, M., Libura, M., Pastwińska, A., Juszczyński, P., Zwierzchowski, L., Baranowski, P. & Siedlecki, J.A. (2014) Repetitive genomic elements and overall DNA methylation changes in acute myeloid and childhood B-cell lymphoblastic leukemia patients. *International Journal of Hematology*, **100,** 79–87.

Burnett, A.K. & Venditti, A. (2011) Acute myeloid leukaemia. In *Postgraduate Haematology*, Hoffbrand, V., Catovsky, D., Tuddenham, E.G.D. & Green, A.R., (6th ed) pp 415–32. Oxford: Blackwell Publishing.

Buske, O.J., Manickaraj, A., Mital, S., Ray, P.N. & Brudno, M. (2013) Identification of deleterious synonymous variants in human genomes. *Bioinformatics*, **29,** 1843–50.

Busque, L., Patel, J.P., Figueroa, M.E., Vasanthakumar, A., Provost, S., Hamilou, Z., Mollica, L., Li, J., Viale, A., Heguy, A., Hassimi, M., Socci, N., Bhatt, P.K., Gonen, M., Mason, C.E., Melnick, A., Godley, L.A., Brennan, C.W., Abdel-Wahab, O. & Levine, R.L. (2012)

Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nature genetics*, **44,** 1179–81.

Bystrom, L.M. & Rivella, S. (2014) Cancer cells with irons in the fire. *Free Radical Biology and Medicine*, **79,** 337–42.

Calvo, X., Nomdedeu, M., Navarro, A., Tejero, R., Costa, D., Muñoz, C., Pereira, A., Peña, O., Risueño, R.M., Monzó, M., Esteve, J. & Nomdedeu, B. (2014) High levels of global DNA methylation are an independent adverse prognostic factor in a series of 90 patients with de novo myelodysplastic syndrome. *Leukemia Research*, **38,** 874–81.

Campbell, P.J. & Green, A.R. (2010) Myeloproliferative neoplasms. In *Postgraduate Haematology*, Hoffbrand, V., Catovsky, D., Tuddenham, E.G.D. & Green, A.R., (6th ed) pp 686–709. Oxford: Blackwell Publishing.

Campos, A.C.E., Molognoni, F., Melo, F.H.M., Galdieri, L.C., Carneiro, C.R.W., D'Almeida, V., Correa, M. & Jasiulionis, M.G. (2007) Oxidative stress modulates DNA methylation during melanocyte anchorage blockade associated with malignant transformation. *Neoplasm (New York, N.Y.)*, **9,** 1111–21.

Cancer Genome Atlas Research Network. (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *The New England journal of medicine*, **368,** 2059–74.

Carbonell, A.L.I., Salhab, R.M., Giampaoli, V., Cendoroglo, M.S. & Chauffaille, M.D.L. (2015) A model for the functional assessment of elderly with myeloid neoplasms. *Revista Brasileira de Hematologia e Hemoterapia*, **37,** 109–14.

Castellana, S. & Mazza, T. (2013) Congruency in the prediction of pathogenic missense mutations: State-of-the-art web-based tools. *Briefings in Bioinformatics*, **14,** 448–59.

Castro, R., Rivera, I., Struys, E.A., Jansen, E.E.W., Ravasco, P., Camilo, M.E., Blom, H.J., Jakobs, C. & Tavares de Almeida, I. (2003) Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. *Clinical chemistry*, **49,** 1292–6.

Cazzola, M. (2010) IDH1 and IDH2 mutations in myeloid neoplasms - novel paradigms and clinical implications. *Haematologica*, **95,** 1623–7.

Cazzola, M., Porta, M.G. Della & Malcovati, L. (2013) The genetic basis of myelodysplasia and its clinical relevance. *Blood*, **122,** 4021–35.

Cencioni, C., Spallotta, F., Martelli, F., Valente, S., Mai, A., Zeiher, A. & Gaetano, C. (2013) Oxidative Stress and Epigenetic Regulation in Ageing and Age-Related Diseases. *International Journal of Molecular Sciences*, **14,** 17643–63.

Cerda, S. & Weitzman, S.A. (1997) Influence of oxygen radical injury on DNA methylation. *Mutation research*, **386,** 141–52.

Chao, M.P., Seita, J. & Weissman, I.L. (2008) Establishment of a normal hematopoietic and leukemia stem cell hierarchy. *Cold Spring Harbor Symposia on Quantitative Biology*, **73,** 439–49.

Chen, H., Sandler, D.P., Taylor, J.A., Shore, D.L., Liu, E., Bloomfield, C.D. & Bell, D.A. (1996) Increased risk for myelodysplastic syndromes in individuals with glutathione transferase theta 1 (GSTT1) gene defect. *Lancet*, **347,** 295–7.

Chen, T.-C., Hou, H.-A., Chou, W.-C., Tang, J.-L., Kuo, Y.-Y., Chen, C.-Y., Tseng, M.-H., Huang, C.-F., Lai, Y.-J., Chiang, Y.-C., Lee, F.-Y., Liu, M.-C., Liu, C.-W., Liu, C.-Y., Yao, M., Huang, S.-Y., Ko, B.-S., Hsu, S.-C., Wu, S.-J., Tsay, W., et al (2014) Dynamics of ASXL1 mutation and other associated genetic alterations during disease progression in patients with primary myelodysplastic syndrome. *Blood cancer journal*, **4,** e177.

Chen, Y., Zheng, T., Lan, Q., Kim, C., Qin, Q., Foss, F., Chen, X., Holford, T., Leaderer, B., Boyle, P., Wang, C., Dai, M., Liu, Z., Ma, S., Chanock, S.J., Rothman, N. & Zhang, Y. (2013) Polymorphisms in DNA repair pathway genes, body mass index, and risk of non-Hodgkin lymphoma. *American Journal of Hematology*, **88,** 606–11.

Chevassut, T.J.T. & Mufti, G.J. (2011) The myelodysplastic syndromes. In *Postgraduate Haematology*, Hoffbrand, V., Catovsky, D., Tuddenham, E.G.D. & Green, A.R., (6th ed) pp 503–29. Oxford: Blackwell Publishing.

Chia, N., Wang, L., Lu, X., Senut, M.-C., Brenner, C. & Ruden, D.M. (2011) Hypothesis: environmental regulation of 5-hydroxymethylcytosine by oxidative stress. *Epigenetics*, **6,** 853–6.

Chim, C.S., Chan, W.W.L. & Kwong, Y.L. (2008) Epigenetic dysregulation of the DAP kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway in acute leukaemias. *Journal of clinical pathology*, **61,** 844–7.

Chim, C.S., Wan, T.S., Fung, T.K. & Wong, K.F. (2010) Methylation of TET2, CBL and CEBPA in Ph-negative myeloproliferative neoplasms. *Journal of clinical pathology*, **63,** 942–6.

Chodavarapu, R.K., Feng, S., Bernatavichute, Y. V, Chen, P.-Y., Stroud, H., Yu, Y., Hetzel, J.A., Kuo, F., Kim, J., Cokus, S.J., Casero, D., Bernal, M., Huijser, P., Clark, A.T., Krämer, U., Merchant, S.S., Zhang, X., Jacobsen, S.E. & Pellegrini, M. (2010) Relationship between nucleosome positioning and DNA methylation. *Nature*, **466,** 388–92.

Choi, S.-W. & Friso, S. (2010) Epigenetics: A New Bridge between Nutrition and Health. *Advances in Nutrition: An International Review Journal*, **1,** 8–16.

Christman, J.K., Sheikhnejad, G., Marasco, C.J. & Sufrin, J.R. (1995) 5-Methyl-2'-deoxycytidine in single-stranded DNA can act in cis to signal de novo DNA methylation. *Proceedings of the National Academy of Sciences of the United States of America*, **92,** 7347–51.

Chuang, J.C. & Jones, P.A. (2007) Epigenetics and microRNAs. *Pediatric Research*, **61,** 24–9.

Chuang, J.Y., Chang, W.C. & Hung, J.J. (2011) Hydrogen peroxide induces Sp1 methylation and thereby suppresses cyclin B1 via recruitment of Suv39H1 and HDAC1 in cancer cells. *Free Radical Biology and Medicine*, **51,** 2309–18.

Chung, Y.J., Robert, C., Gough, S.M., Rassool, F. V. & Aplan, P.D. (2014) Oxidative stress leads to increased mutation frequency in a murine model of myelodysplastic syndrome. *Leukemia Research*, **38,** 95–102.

Chung, Y.R., Schatoff, E. & Abdel-Wahab, O. (2012) Epigenetic alterations in hematopoietic malignancies. *International Journal of Hematology*, **96,** 413–27.

Circu, M.L. & Aw, T.Y. (2010) Reactive oxygen species, cellular redox systems, and apoptosis. *Free radical biology & medicine*, **48,** 749–62.

Clapier, C.R. & Cairns, B.R. (2009) The biology of chromatin remodeling complexes. *Annual review of biochemistry*, **78,** 273–304.

Claus, R. & Lübbert, M. (2003) Epigenetic targets in hematopoietic malignancies. *Oncogene*, **22,** 6489–96.

Cleary, C. & Kralovics, R. (2013) Molecular basis and clonal evolution of myeloproliferative neoplasms. *Clinical chemistry and laboratory medicine*, **51,** 1889–96.

Cortelezzi, A., Cattaneo, C., Cristiani, S., Duca, L., Sarina, B., Deliliers, G.L., Fiorelli, G. & Cappellini, M.D. (2000) Non-transferrin-bound iron in myelodysplastic syndromes: a marker of ineffective erythropoiesis? *The hematology journal : the official journal of the European Haematology Association / EHA*, **1,** 153–8.

Cortesão, E.C., Gonçalves, A.C., Sousa, M.I., Moucho, C., Rito, L., Magalhães, E., Espadana, A.I., Pereira, M.A., Teixeira, A., Nascimento Costa, J.N., Sarmento, A.B. (2008) Evaluation of Apoptotric Markers in Myelodysplasic Syndrome Patients. *Blood (ASH Annual Meeting Abstracts)*, **112,** 5094.

Cortessis, V.K., Thomas, D.C., Joan Levine, a., Breton, C. V., Mack, T.M., Siegmund, K.D., Haile, R.W. & Laird, P.W. (2012) Environmental epigenetics: Prospects for studying epigenetic mediation of exposure-response relationships. *Human Genetics*, **131,** 1565–89.

Craig, J. (2008) Complex diseases: Research and applications. *Nature Education*, **1,** 184.

Crider, K.S., Yang, T.P., Berry, R.J. & Bailey, L.B. (2012) Folate and DNA Methylation : A Review of Molecular Mechanisms and the Evidence for Folate ' s Role. *American Society for Nutrition*, **3,** 21–38.

Cuozzo, C., Porcellini, A., Angrisano, T., Morano, A., Lee, B., Di Pardo, A., Messina, S., Iuliano, R., Fusco, A., Santillo, M.R., Muller, M.T., Chiariotti, L., Gottesman, M.E. & Avvedimento, E. V (2007) DNA damage, homology-directed repair, and DNA methylation. *PLoS genetics*, **3,** e110.

Curtin, K., Ulrich, C.M., Samowitz, W.S., Wolff, R.K., Duggan, D.J., Makar, K.W., Caan, B.J. & Slattery, M.L. (2011) Candidate pathway polymorphisms in one-carbon metabolism and risk of rectal tumor mutations. *International journal of molecular epidemiology and genetics*, **2,** 1–8.

Curtin, N.J. (2012) DNA repair dysregulation from cancer driver to therapeutic target. *Nature reviews. Cancer*, **12,** 801–17.

Dao, T., Cheng, R.Y.S., Revelo, M.P., Mitzner, W. & Tang, W. (2014) Hydroxymethylation as a Novel Environmental Biosensor. *Current environmental health reports*, **1,** 1–10.

Das, P.M. & Singal, R. (2004) DNA methylation and cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **22,** 4632–42.

Davis, C.D. & Uthus, E.O. (2004) DNA methylation, cancer susceptibility, and nutrient interactions. *Experimental biology and medicine (Maywood, N.J.)*, **229,** 988–95.

Dawson, M. a. & Kouzarides, T. (2012) Cancer epigenetics: From mechanism to therapy. *Cell*, **150,** 12–27.

De Jonge, R. De, Tissing, W.J.E., Hooijberg, J.H., Jansen, G., Kaspers, G.J.L., Lindemans, J., Peters, G.J., Pieters, R., de Jonge, R., Tissing, W.J.E., Hooijberg, J.H., Jansen, G., Kaspers, G.J.L., Lindemans, J., Peters, G.J. & Pieters, R. (2009) Polymorphisms in folate-related genes and risk of pediatric acute lymphoblastic leukemia. *Distribution*, **113,** 2284–9.

De Leenheer, a P., De Bevere, V.O. & Claeys, A.E. (1979) Measurement of alpha-, beta-, and gamma tocopherol in serum by liquid chromatography. *Clinical chemistry*, **25,** 425–8.

De Sousa, M.C., Vieira, R.B., Dos Santos, D.S., Carvalho, C.A.T., Camargo, S.E.A., Mancini, M.N.G. & de Oliveira, L.D. (2015) Antioxidants and biomarkers of oxidative damage in the saliva of patients with Down's syndrome. *Archives of oral biology*, **60,** 600–5.

De Souza, G.F., Ribeiro, H.L., De Sousa, J.C., Heredia, F.F., De Freitas, R.M., Martins, M.R.A., Goncalves, R.P., Pinheiro, R.F. & Magalhaes, S.M.M. (2015) HFE gene mutation and oxidative damage biomarkers in patients with myelodysplastic syndromes and its relation to transfusional iron overload: an observational cross-sectional study. *BMJ Open*, **5,** e006048–e006048.

Delatte, B., Deplus, R. & Fuks, F. (2014) Playing TETris with DNA modifications. *The EMBO journal*, **33,** 1198–211.

Deneberg, S., Grövdal, M., Karimi, M., Jansson, M., Nahi, H., Corbacioglu, a, Gaidzik, V., Döhner, K., Paul, C., Ekström, T.J., Hellström-Lindberg, E. & Lehmann, S. (2010) Gene-specific and global methylation patterns predict outcome in patients with acute myeloid leukemia. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, **24,** 932–41.

Dervieux, T., Kremer, J., Lein, D.O., Capps, R., Barham, R., Meyer, G., Smith, K., Caldwell, J. & Furst, D.E. (2004) Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. *Pharmacogenetics*, **14,** 733–9.

Di Croce, L., Raker, V.A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., Minucci, S. & Pelicci, P.G. (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science (New York, N.Y.)*, **295,** 1079–82.

Döhner, H., Estey, E.H., Amadori, S., Appelbaum, F.R., Büchner, T., Burnett, A.K., Dombret, H., Fenaux, P., Grimwade, D., Larson, R.A., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G.J., Sanz, M.A., Sierra, J., Tallman, M.S., Löwenberg, B. & Bloomfield, C.D. (2010) Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*, **115,** 453–74.

Dong, L.M., Potter, J.D., White, E., Ulrich, C.M., Cardon, L.R. & Peters, U. (2008) The Role of Polymorphisms in Candidate Genes. **299,** 2423–36.

Dong, S., Liu, Y. & Chen, J. (2014) MTHFR gene polymorphism and risk of myeloid leukemia: a meta-analysis. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, **35,** 8913–9.

Donkena, K.V., Young, C.Y.F. & Tindall, D.J. (2010) Oxidative stress and DNA methylation in prostate cancer. *Obstetrics and gynecology international*, **2010,** 302051.

Driver, A.S., Kodavanti, P.R. & Mundy, W.R. (2010) Age-related changes in reactive oxygen species production in rat brain homogenates. *Neurotoxicology and teratology*, **22,** 175–81.

Dunna, N.R., Naushad, S.M., Vuree, S., Anuradha, C., Sailaja, K., Surekha, D., Rao, D.R. & Vishnupriya, S. (2014) Association of thymidylate synthase 5'-UTR 28bp tandem repeat and serine hydroxymethyltransfarase C1420T polymorphisms with susceptibility to acute leukemia. *Asian Pacific journal of cancer prevention : APJCP*, **15,** 1719–23.

Durmus, A., Mentese, A., Yilmaz, M., Sumer, A., Akalin, I., Topal, C. & Alver, A. (2014) The Thrombotic Events in Polycythemia Vera Patients May Be Related to Increased Oxidative Stress. *Medical Principles and Practice*, **23,** 253–8.

Durmus, A., Mentese, A., Yilmaz, M., Sumer, A., Akalin, I., Topal, C. & Alver, A. (2013) Increased oxidative stress in patients with essential thrombocythemia. **17,** 2860–6.

Dzierzak, E. (2011) Stem cell and haemopoiesis. In *Postgraduate Haematology*, Hoffbrand, V., Catovsky, D., Tuddenham, E.G.D. & Green, A.R., (6th ed) pp 1–11. Oxford: Blackwell Publishing.

Economopoulou, P., Pappa, V., Papageorgiou, S., Dervenoulas, J. & Economopoulos, T. (2011) Abnormalities of DNA repair mechanisms in common hematological malignancies. *Leukemia & lymphoma*, **52,** 567–82.

Ehrlich, M. (2002) DNA methylation in cancer: too much, but also too little. *Oncogene*, **21,** 5400–13.

Ehrlich, M. (2009) DNA hypomethylation in cancer cells. *Epigenomics*, **1,** 239–59.

Eny, K.M., El-Sohemy, A., Cornelis, M.C., Sung, Y.K. & Bae, S.C. (2005) Catalase and PPARgamma2 genotype and risk of systemic lupus erythematosus in Koreans. *Lupus*, **14,** 351–5.

Erichsen, H.C. & Chanock, S.J. (2004) SNPs in cancer research and treatment. *British journal of cancer*, **90,** 747–51.

Eriksson, A., Lennartsson, A. & Lehmann, S. (2015) Epigenetic aberrations in acute myeloid leukemia: early key events during leukemogenesis. *Experimental Hematology*, **43,** 609–24.

Esposito, M.T. & So, C.W.E. (2014) DNA damage accumulation and repair defects in acute myeloid leukemia: implications for pathogenesis, disease progression, and chemotherapy resistance. *Chromosoma*, **123,** 545–61.

Esteller, M. (2008) Epigenetics in cancer. *The New England journal of medicine*, **358,** 1148–59.

Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S.N., Hidalgo, O.F., Vanaclocha, V., Baylin, S.B. & Herman, J.G. (2000) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *The New England journal of medicine*, **343,** 1350–4.

Excoffier, L. & Lischer, H.E.L. (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular ecology resources*, **10,** 564–7.

Fabiani, E., D'Alò, F., Scardocci, A., Greco, M., Di Ruscio, A., Criscuolo, M., Fianchi, L., Pagano, L., Hohaus, S., Leone, G. & Voso, M.T. (2009) Polymorphisms of detoxification and DNA repair enzymes in myelodyplastic syndromes. *Leukemia Research*, **33,** 1068–71.

Fabiani, E., Leone, G., Giachelia, M., D'alo', F., Greco, M., Criscuolo, M., Guidi, F., Rutella, S., Hohaus, S. & Voso, M.T. (2010) Analysis of genome-wide methylation and gene expression induced by 5-aza-2'-deoxycytidine identifies BCL2L10 as a frequent methylation target in acute myeloid leukemia. *Leukemia & lymphoma*, **51,** 2275–84.

Fabris, S., Bollati, V., Agnelli, L., Morabito, F., Motta, V., Cutrona, G., Matis, S., Recchia, A.G., Gigliotti, V., Gentile, M., Deliliers, G.L., Bertazzi, P.A., Ferrarini, M., Neri, A. & Baccarelli, A. (2011) Biological and clinical relevance of quantitative global methylation of repetitive DNA sequences in chronic lymphocytic leukemia. *Epigenetics*, 6, 188–194.

Farquhar, M.J. & Bowen, D.T. (2003) Stress and the Myelodysplastic Syndromes. *International Journal of Hematology*, **77,** 342–50.

Fathi, A.T., & Abdel-Wahab, O. (2012) Mutations in epigenetic modifiers in myeloid malignancies and the prospect of novel epigenetic-targeted therapy. *Advances in Hematology*, **2012,** 469592.

Fenaux, P. & Rose, C. (2009) Impact of iron overload in myelodysplastic syndromes. *Blood reviews*, **23 Suppl 1,** S15–9.

Ficz, G. & Gribben, J.G. (2014) Loss of 5-hydroxymethylcytosine in cancer: Cause or consequence? *Genomics*, **104,** 352–7.

Figueroa, M.E., Abdel-Wahab, O., Lu, C., Ward, P.S., Patel, J., Shih, A., Li, Y., Bhagwat, N., Vasanthakumar, A., Fernandez, H.F., Tallman, M.S., Sun, Z., Wolniak, K., Peeters, J.K., Liu, W., Choe, S.E., Fantin, V.R., Paietta, E., Löwenberg, B., Licht, J.D., et al (2010) Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation. *Cancer Cell*, **18,** 553–67.

Fong, C.Y., Morison, J. & Dawson, M. A. (2014) Epigenetics in the hematologic malignancies. *Haematologica*, **99,** 1772–83.

Fontenay, M., Cathelin, S., Amiot, M., Gyan, E. & Solary, E. (2006) Mitochondria in hematopoiesis and hematological diseases. *Oncogene*, **25,** 4757–67.

Franco, R., Schoneveld, O., Georgakilas, A.G. & Panayiotidis, M.I. (2008) Oxidative stress, DNA methylation and carcinogenesis. *Cancer Letters*, **266,** 6–11.

Fröhling, S., Scholl, C., Gilliland, D.G. & Levine, R.L. (2005) Genetics of myeloid malignancies: pathogenetic and clinical implications. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **23,** 6285–95.

Fuks, F., Burgers, W.A., Brehm, A., Hughes-Davies, L. & Kouzarides, T. (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature genetics*, **24,** 88–91.

Galbiatti, A.L.S., Ruiz, M.T., Rezende Pinto, D., Raposo, L.S., Maníglia, J.V., Pavarino-Bertelli, E.C. & Goloni-Bertollo, E.M. (2011) A80G polymorphism of reduced folate carrier 1 (RFC1) gene and head and neck squamous cell carcinoma etiology in Brazilian population. *Molecular Biology Reports*, **38,** 1071–8.

Galm, O., Herman, J.G. & Baylin, S.B. (2006) The fundamental role of epigenetics in hematopoietic malignancies. *Blood Reviews*, **20,** 1–13.

Garcia-Manero, G. (2010) Prognosis of myelodysplastic syndromes. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, **2010,** 330–7.

Geismann, C., Arlt, A. & Sebens, S. (2014) Cytoprotection ' gone astray ': Nrf2 and its role in cancer, **7,** 1497–518.

Gelsi-Boyer, V., Brecqueville, M., Devillier, R., Murati, A., Mozziconacci, M.-J. & Birnbaum, D. (2012) Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases. *Journal of hematology & oncology*, **5,** 12.

Ghaffari, S. (2008) Oxidative stress in the regulation of normal and neoplastic hematopoiesis. *Antioxidants & redox signaling*, **10,** 1923–40.

Ghoti, H., Amer, J., Winder, A., Rachmilewitz, E. & Fibach, E. (2007) Oxidative stress in red blood cells, platelets and polymorphonuclear leukocytes from patients with myelodysplastic syndrome. *European journal of haematology*, **79,** 463–7.

Ghoti, H., Fibach, E., Merkel, D., Perez-Avraham, G., Grisariu, S. & Rachmilewitz, E.A. (2010) Changes in parameters of oxidative stress and free iron biomarkers during treatment with deferasirox in iron-overloaded patients with myelodysplastic syndromes. *Haematologica*, **95,** 1433–4.

Gianni, D., Bohl, B., Courtneidge, S.A. & Bokoch, G.M. (2008) The involvement of the tyrosine kinase c-Src in the regulation of reactive oxygen species generation mediated by NADPH oxidase-1. *Molecular biology of the cell*, **19,** 2984–94.

Glebova, K., Veiko, N., Kostyuk, S., Izhevskaya, V. & Baranova, A. (2013) Oxidized extracellular DNA as a stress signal that may modify response to anticancer therapy. *Cancer Letters*, 356, 22–33.

Goldberg, D. & Spooner, R. (1983) Glutathione reductase. In: Bergmeyer, H.; Bergmeyer, J.; Grassl, M., ed. *Methods of enzymatic analysis* (3rd ed.) pp 258-65. Weinheim: Verlag Chemie.

Gonçalves, A.C., Alves, R., Pires, A., Jorge, J., Mota-Vieira, L., Nascimento-Costa J.M., Sarmento-Ribeiro, A.B. (2015a) Chronic exposure to oxidative stress inducers influence methylation status in normal and neoplastic hematological cells. *Revista Portuguesa de Pneumologia*, **21** (Esp Cong 1), 18.

Gonçalves, A.C., Cortesão, E., Oliveiros, B., Alves, V., Espadana, A.I., Rito, L., Magalhães, E., Lobão, M.J., Pereira, A., Nascimento Costa, J.M., Mota-Vieira, L. & Sarmento-Ribeiro, A.B. (2015b) Oxidative stress and mitochondrial dysfunction play a role in myelodysplastic syndrome development, diagnosis, and prognosis: A pilot study. *Free radical research*, **49,** 1081–94.

Gonçalves, A.C., Cortesão, E., Oliveiros, B., Alves, V., Espadana, A.I., Rito, L., Magalhães, E., Pereira, S., Pereira, A., Costa, J.M.N., Mota-Vieira, L. & Sarmento-Ribeiro, A.B. (2015c) Oxidative stress levels are correlated with P15 and P16 gene promoter methylation in myelodysplastic syndrome patients. *Clinical and experimental medicine*. [Epub ahead of print]

Gonzalez-Gomez, P., Bello, M.J., Lomas, J., Arjona, D., Alonso, M.E., Amiñoso, C., Lopez-Marin, I., Anselmo, N.P., Sarasa, J.L., Gutierrez, M., Casartelli, C. & Rey, J.A. (2003) Aberrant methylation of multiple genes in neuroblastic tumours. relationship with MYCN amplification and allelic status at 1p. *European journal of cancer (Oxford, England : 1990)*, **39,** 1478–85.

Gorrini, C., Harris, I.S. & Mak, T.W. (2013) Modulation of oxidative stress as an anticancer strategy. *Nature reviews. Drug discovery*, **12,** 931–47.

Goto, M., Shinmura, K., Tao, H., Tsugane, S. & Sugimura, H. (2010) Three novel NEIL1 promoter polymorphisms in gastric cancer patients. *World journal of gastrointestinal oncology*, **2,** 117–20.

Gough, D.J., Corlett, A., Schlessinger, K., Wegrzyn, J., Larner, A.C. & Levy, D.E. (2009) Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science (New York, N.Y.)*, **324,** 1713–6.

Greenberg, P., Cox, C., LeBeau, M.M., Fenaux, P., Morel, P., Sanz, G., Sanz, M., Vallespi, T., Hamblin, T., Oscier, D., Ohyashiki, K., Toyama, K., Aul, C., Mufti, G. & Bennett, J. (1997) International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*, **89,** 2079–88.

Greenberg, P.L. (2012) Molecular and genetic features of myelodysplastic syndromes. *International journal of laboratory hematology*, **34,** 215–22.

Greenberg, P.L. (2013) The multifaceted nature of myelodysplastic syndromes: clinical, molecular, and biological prognostic features. *Journal of the National Comprehensive Cancer Network : JNCCN*, **11,** 877–84.

Greenberg, P.L., Young, N.S. & Gattermann, N. (2002) Myelodysplastic syndromes. *American Society of Hematology. Education Program*, **136–61.**

Griesshammer, M., Gisslinger, H., Mesa, R. (2015) Current and future treatment options for polycythemia vera. *Annals of Hematology*, **94,** 901-10.

Grove, C.S. & Vassiliou, G.S. (2014) Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer? *Disease models & mechanisms*, **7,** 941–51.

Gu, D.-L., Chen, Y.-H., Shih, J.-H., Lin, C.-H., Jou, Y.-S. & Chen, C.-F. (2013) Target genes discovery through copy number alteration analysis in human hepatocellular carcinoma. *World journal of gastroenterology : WJG*, **19,** 8873–9.

Guan, H., Ji, M., Hou, P., Liu, Z., Wang, C., Shan, Z., Teng, W. & Xing, M. (2008) Hypermethylation of the DNA mismatch repair gene hMLH1 and its association with lymph node metastasis and T1799A BRAF mutation in patients with papillary thyroid cancer. *Cancer*, **113,** 247–55.

Gutiyama, L.M., Coutinho, D.F., Lipkin, M. V & Zalcberg, I.R. (2012) Myeloid neoplasms: what molecular analyses are telling us. *ISRN oncology*, **2012,** 321246.

Guzik, T.J., West, N.E.J., Black, E., McDonald, D., Ratnatunga, C., Pillai, R. & Channon, K.M. (2000) Functional Effect of the C242T Polymorphism in the NAD(P)H Oxidase p22phox Gene on Vascular Superoxide Production in Atherosclerosis. *Circulation*, **102,** 1744–1747.

Haferlach, T., Nagata, Y., Grossmann, V., Okuno, Y., Bacher, U., Nagae, G., Schnittger, S., Sanada, M., Kon, a, Alpermann, T., Yoshida, K., Roller, a, Nadarajah, N., Shiraishi, Y., Shiozawa, Y., Chiba, K., Tanaka, H., Koeffler, H.P., Klein, H.-U., Dugas, M., et al (2014) Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia*, **28,** 241–7.

Hamdy, M.S., El-Haddad, A.M., Bahaa El-Din, N.M., Makhlouf, M.M. & Abdel-Hamid, S.M. (2011) RAD51 and XRCC3 gene polymorphisms and the risk of developing acute myeloid leukemia. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*, **59,** 1124–30.

Hamilton, M.L., Van Remmen, H., Drake, J.A., Yang, H., Guo, Z.M., Kewitt, K., Walter, C.A. & Richardson, A. (2001) Does oxidative damage to DNA increase with age? *Proceedings of the National Academy of Sciences of the United States of America*, **98,** 10469–74.

Hanada, N., Takahata, T., Zhou, Q., Ye, X., Sun, R., Itoh, J., Ishiguro, A., Kijima, H., Mimura, J., Itoh, K., Fukuda, S. & Saijo, Y. (2012) Methylation of the KEAP1 gene promoter region in human colorectal cancer. *BMC cancer*, **12,** 66.

Handy, D.E. & Loscalzo, J. (2012) Redox regulation of mitochondrial function. *Antioxidants & redox signaling*, **16,** 1323–67.

Hartikainen, J.M., Tengström, M., Kosma, V.-M., Kinnula, V.L., Mannermaa, A. & Soini, Y. (2012) Genetic polymorphisms and protein expression of NRF2 and Sulfiredoxin predict survival outcomes in breast cancer. *Cancer research*, **72,** 5537–46.

Hasselbalch, H.C., Thomassen, M., Hasselbalch Riley, C., Kjær, L., Stauffer Larsen, T., Jensen, M.K., Bjerrum, O.W., Kruse, T. a. & Skov, V. (2014) Whole Blood Transcriptional Profiling Reveals Deregulation of Oxidative and Antioxidative Defence Genes in Myelofibrosis and Related Neoplasms. Potential Implications of Downregulation of Nrf2 for Genomic Instability and Disease Progression. *PLoS ONE*, **9,** e112786.

Hayes, P. & Knaus, U.G. (2013) Balancing reactive oxygen species in the epigenome: NADPH oxidases as target and perpetrator. *Antioxidants & redox signaling*, **18,** 1937–45.

Hellman, A. & Chess, A. (2010) Extensive sequence-influenced DNA methylation polymorphism in the human genome. *Epigenetics & chromatin*, **3,** 11.

Hepburn, P.A., Margison, G.P. & Tisdale, M.J. (1991) Enzymatic methylation of cytosine in DNA is prevented by adjacent O6-methylguanine residues. *The Journal of biological chemistry*, **266,** 7985–7.

Heyn, H., Moran, S., Hernando-herraez, I., Res, G., Sayols, S., Gomez, A., Sandoval, J., Monk, D., Hata, K., Marques-bonet, T., Wang, L. & Esteller, M. (2013) DNA methylation contributes to natural human variation DNA methylation contributes to natural human variation. **23,** 1363–72.

Hicks, C., Kumar, R., Pannuti, A., Backus, K., Brown, A., Monico, J. & Miele, L. (2013) An Integrative Genomics Approach for Associating GWAS Information with Triple-Negative Breast Cancer. *Cancer informatics*, **12,** 1–20.

Hitchler, M.J. & Domann, F.E. (2007) An epigenetic perspective on the free radical theory of development. *Free radical biology & medicine*, **43,** 1023–36.

Hitchler, M.J., Wikainapakul, K., Yu, L., Powers, K., Attatippaholkun, W. & Domann, F.E. (2006) Epigenetic regulation of manganese superoxide dismutase expression in human breast cancer cells. *Epigenetics*, **1,** 163–71.

Ho, M.S.H., Medcalf, R.L., Livesey, S.A. & Traianedes, K. (2015) The dynamics of adult haematopoiesis in the bone and bone marrow environment. *British journal of haematology*, **170,** 472–86.

Hofmann, W.-K., Takeuchi, S., Takeuchi, N., Thiel, E., Hoelzer, D. & Koeffler, H.P. (2006) Comparative analysis of hypermethylation of cell cycle control and DNA-mismatch

repair genes in low-density and CD34+ bone marrow cells from patients with myelodysplastic syndrome. *Leukemia research*, **30,** 1347–53.

Hole, P.S., Darley, R.L. & Tonks, A. (2011) Do reactive oxygen species play a role in myeloid leukemias? *Blood*, **117,** 5816–26.

Hole, P.S., Zabkiewicz, J., Munje, C., Newton, Z., Pearn, L., White, P., Marquez, N., Hills, R.K., Burnett, A.K., Tonks, A. & Darley, R.L. (2013) Overproduction of NOX-derived ROS in AML promotes proliferation and is associated with defective oxidative stress signaling. *Blood*, **122,** 3322–30.

Holz-Schietinger, C., Matje, D.M., Harrison, M.F. & Reich, N.O. (2011) Oligomerization of DNMT3A controls the mechanism of de novo DNA methylation. *The Journal of biological chemistry*, **286,** 41479–88.

Honda, M., Yamada, Y., Tomonaga, M., Ichinose, H. & Kamihira, S. (2000) Correlation of urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, and clinical features of hematological disorders: a pilot study. *Leukemia research*, **24,** 461–8.

Hopfer, O., Nolte, F., Mossner, M., Komor, M., Kmetsch, A., Benslasfer, O., Reissmann, M., Nowak, D., Hoelzer, D., Thiel, E. & Hofmann, W.-K. (2012) Epigenetic dysregulation of GATA1 is involved in myelodysplastic syndromes dyserythropoiesis. *European journal of haematology*, **88,** 144–53.

Horton, S.J. & Huntly, B.J.P. (2012) Recent advances in acute myeloid leukemia stem cell biology. *Haematologica*, **97,** 966–74.

Hou, H., Chou, W. & Tien, H. (2010) Genetic Alterations and Their Clinical Implications in Acute Myeloid Leukemia, Myeloid Leukemia - Basic Mechanisms of Leukemogenesis, Dr Steffen Koschmieder (Ed.), ISBN: 978-953-307-789-5.

Hou, Z. & Matherly, L.H. (2014) Biology of the major facilitative folate transporters SLC19A1 and SLC46A1. *Current topics in membranes*, **73,** 175–204.

Huang, J., Plass, C. & Gerhauser, C. (2011) Cancer chemoprevention by targeting the epigenome. *Current drug targets*, **12,** 1925–56.

Huang, L., Deng, D., Peng, Z., Ye, F., Xiao, Q., Zhang, B., Ye, B., Mo, Z., Yang, X. & Liu, Z. (2015) Polymorphisms in the methylenetetrahydrofolate reductase gene (MTHFR) are associated with susceptibility to adult acute myeloid leukemia in a Chinese population. *Cancer epidemiology*, **39,** 328–33.

Hurt, E.M., Thomas, S.B., Peng, B. & Farrar, W.L. (2007) Molecular consequences of SOD2 expression in epigenetically silenced pancreatic carcinoma cell lines. *British journal of cancer*, **97,** 1116–23.

Hurtado-Nedelec, M., Csillag, M.J.G., Boussetta, T., Belambri, S.A., Fay, M., Cassinat, B., Gougerot, M.A.P., Dang, P.M.C. & El-Benna, J. (2013) Increased reactive oxygen species production and p47phox phosphorylation in neutrophils from myeloproliferative disorders patients with JAK2 (V617F) mutation. *Haematologica*, **98,** 1517–24.

Ibrahem, L., Mahfouz, R., Elhelw, L., Abdsalam, E.M. & Soliman, R. (2015) Prognostic significance of DNMT3A mutations in patients with acute myeloid leukemia. *Blood Cells, Molecules, and Diseases*, **54,** 84–9.

Imbesi, S., Musolino, C., Allegra, A., Saija, A., Morabito, F., Calapai, G. & Gangemi, S. (2013) Oxidative stress in oncohematologic diseases: an update. *Expert review of hematology*, **6,** 317–25.

Inoue, N., Kawashima, S., Kanazawa, K., Yamada, S., Akita, H. & Yokoyama, M. (1998) Polymorphism of the NADH/NADPH oxidase p22 phox gene in patients with coronary artery disease. *Circulation*, **97,** 135–7.

Invernizzi, R. (2010) Myelodysplastic Syndromes Iron Overload , Oxidative Damage and Ineffective Erythropoiesis in Myelodysplastic Syndromes. **,** 34–8.

Iorio, M. V & Croce, C.M. (2012) microRNA involvement in human cancer. *Carcinogenesis*, **33,** 1126–33.

Issa, J.-P. (2013) The myelodysplastic syndrome as a prototypical epigenetic disease. *Blood*, **121,** 3811–7.

Itzykson, R., Kosmider, O. & Fenaux, P. (2013) Somatic mutations and epigenetic abnormalities in myelodysplastic syndromes. *Best Practice and Research: Clinical Haematology*, **26,** 355–64.

James, S.J., Melnyk, S., Pogribna, M., Pogribny, I.P. & Caudill, M.A. (2002) Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology. *The Journal of nutrition*, **132,** 2361S–6S.

Jan, M. & Majeti, R. (2013) Clonal evolution of acute leukemia genomes. *Oncogene*, **32,** 135–40.

Jankowska, A M., Gondek, L.P., Szpurka, H., Nearman, Z.P., Tiu, R. V & Maciejewski, J.P. (2008) Base excision repair dysfunction in a subgroup of patients with myelodysplastic syndrome. *Leukemia*, **22,** 551–8.

Jhanwar, S.C. (2015) Genetic and epigenetic pathways in myelodysplastic syndromes: A brief overview. *Advances in biological regulation*, **58,** 28–37.

Jhaveri, M.S., Wagner, C. & Trepel, J.B. (2001) Impact of extracellular folate levels on global gene expression. *Molecular pharmacology*, **60,** 1288–95.

Jiang, J., Jia, Z., Cao, D., Jin, M.S., Kong, F., Suo, J. & Cao, X. (2012) Polymorphisms of the DNA Methyltransferase 1 Associated with Reduced Risks of Helicobacter pylori Infection and Increased Risks of Gastric Atrophy. *PLoS ONE*, **7,** 7–12.

Jin, L., Zuo, X.-Y., Su, W.-Y., Zhao, X.-L., Yuan, M.-Q., Han, L.-Z., Zhao, X., Chen, Y.-D. & Rao, S.-Q. (2014) Pathway-based analysis tools for complex diseases: a review. *Genomics, proteomics & bioinformatics*, **12,** 210–20.

Johnstone, S.E. & Baylin, S.B. (2010) Stress and the epigenetic landscape: a link to the pathobiology of human diseases? *Nature reviews. Genetics*, **11,** 806–12.

Jones, D.P. (2008) Radical-free biology of oxidative stress. *American journal of physiology. Cell physiology*, **295,** C849–68.

Jones, D.P. (2006) Redefining oxidative stress. *Antioxidants & redox signaling*, **8,** 1865–79.

Jost, E., do O, N., Dahl, E., Maintz, C.E., Jousten, P., Habets, L., Wilop, S., Herman, J.G., Osieka, R. & Galm, O. (2007) Epigenetic alterations complement mutation of JAK2 tyrosine kinase in patients with BCR/ABL-negative myeloproliferative disorders. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, **21,** 505–10.

Jung, S. & Kim, S. (2014) EDDY: A novel statistical gene set test method to detect differential genetic dependencies. *Nucleic Acids Research*, **42,** 1–13.

Kang, J. & Pervaiz, S. (2012) Mitochondria: redox metabolism and dysfunction. *Biochemistry research international*, **2012,** 896751.

Kang, K.A., Zhang, R., Kim, G.Y., Bae, S.C. & Hyun, J.W. (2012) Epigenetic changes induced by oxidative stress in colorectal cancer cells: methylation of tumor suppressor RUNX3. *Tumor Biology*, **33,** 403–12.

Kanwal, R. & Gupta, S. (2012) Epigenetic modifications in cancer. *Clinical Genetics*, **81,** 303–11.

Karlic, H., Herrmann, H., Varga, F., Thaler, R., Reitermaier, R., Spitzer, S., Ghanim, V., Blatt, K., Sperr, W.R., Valent, P. & Pfeilstöcker, M. (2014) The role of epigenetics in the regulation of apoptosis in myelodysplastic syndromes and acute myeloid leukemia. *Critical reviews in oncology/hematology*, **90,** 1–16.

Karlic, R., Chung, H.-R., Lasserre, J., Vlahovicek, K. & Vingron, M. (2010) Histone modification levels are predictive for gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, **107,** 2926–31.

Kaufman, D. S. & Scadden, D. T. (2013) Cellular basis of hematopoiesis and stem cell transplantation. *American Society of Hematology Self-Assessment Program*, ***2013*****,** 321–33.

Kaushansky, K. (2006) Lineage-specific hematopoietic growth factors. *The New England journal of medicine*, **354,** 2034–45.

Kensler, T.W., Wakabayashi, N. & Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annual review of pharmacology and toxicology*, **47,** 89–116.

Khan, H., Vale, C., Bhagat, T. & Verma, A. (2013) Role of DNA Methylation in the Pathogenesis and Treatment of Myelodysplastic Syndromes. *Seminars in Hematology*, **50,** 16–37.

Khatami, F., Noorinayer, B., Ghiasi, S., Mohebi, R., Hashemi, M. & Zali, M.R. (2009a) Lack of effects of single nucleotide polymorphisms of the DNA methyltransferase 1 gene on gastric cancer in Iranian patients: a case control study. *Asian Pacific journal of cancer prevention : APJCP*, **10,** 1177–82.

Khatami, F., Noorinayer, B., Mohebi, S.R., Ghiasi, S., Mohebi, R., Hashemi, M. & Zali, M.R. (2009b) Effects of amino acid substitution polymorphisms of two DNA methyltransferases on susceptibility to sporadic colorectal cancer. *Asian Pacific journal of cancer prevention : APJCP*, **10,** 1183–8.

Kikuchi, S., Kobune, M., Iyama, S., Sato, T., Murase, K., Kawano, Y., Takada, K., Ono, K., Hayashi, T., Miyanishi, K., Sato, Y., Takimoto, R. & Kato, J. (2012) Prognostic significance of serum ferritin level at diagnosis in myelodysplastic syndrome. *International journal of hematology*, **95,** 527–34.

Kim, Y.-J.Y.Y., Jekarl, D.W., Kim, J., Kwon, A., Choi, H., Lee, S., Kim, Y.-J.Y.Y., Kim, H.-J., Kim, Y.-J.Y.Y., Oh, I.-H. & Kim, M. (2015) Genetic and epigenetic alterations of bone marrow stromal cells in myelodysplastic syndrome and acute myeloid leukemia patients. *Stem Cell Research*, **14,** 177–84.

Kitamura, T., Inoue, D., Okochi-Watanabe, N., Kato, N., Komeno, Y., Lu, Y., Enomoto, Y., Doki, N., Uchida, T., Kagiyama, Y., Togami, K., Kawabata, K.C., Nagase, R., Horikawa, S., Hayashi, Y., Saika, M., Fukuyama, T., Izawa, K., Oki, T., Nakahara, F., et al. (2014) The molecular basis of myeloid malignancies. *Proceedings of the Japan Academy. Series B, Physical and biological sciences*, **90,** 389–404.

Kitkumthorn, N. & Mutirangura, A. (2011) Long interspersed nuclear element-1 hypomethylation in cancer: Biology and clinical applications. *Clinical Epigenetics*, **2,** 315–30.

Klaunig, J.E. & Kamendulis, L.M. (2004) The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol*, **44,** 239–67.

Klaunig, J.E., Kamendulis, L.M. & Hocevar, B.A. (2010) Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic pathology*, **38,** 96–109.

Klaunig, J.E., Wang, Z., Pu, X. & Zhou, S. (2011) Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicology and Applied Pharmacology*, **254,** 86–99.

Klco, J.M., Vij, R., Kreisel, F.H., Hassan, A. & Frater, J.L. (2010) Molecular pathology of myeloproliferative neoplasms. *American journal of clinical pathology*, **133,** 602–15.

Klepin, H.D., Rao, A. V & Pardee, T.S. (2014) Acute myeloid leukemia and myelodysplastic syndromes in older adults. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **32,** 2541–52.

Kloypan, C., Srisa-Art, M., Mutirangura, A. & Boonla, C. (2015) LINE-1 hypomethylation induced by reactive oxygen species is mediated via depletion of S-adenosylmethionine. *Cell biochemistry and function*. [Epub ahead of print]

Ko, M., Huang, Y., Jankowska, A.M., Pape, U.J., Tahiliani, M., Bandukwala, H.S., An, J., Lamperti, E.D., Koh, K.P., Ganetzky, R., Liu, X.S., Aravind, L., Agarwal, S., Maciejewski, J.P. & Rao, A. (2010) Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature*, **468,** 839–43.

Kogelman, L.J. & Kadarmideen, H.N. (2014) Weighted Interaction SNP Hub (WISH) network method for building genetic networks for complex diseases and traits using whole genome genotype data. *BMC systems biology*, **8 Suppl 2,** S5.

Kohli, R.M. & Zhang, Y. (2013) TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, **502,** 472–9.

Kohno, T., Shinmura, K., Tosaka, M., Tani, M., Kim, S.R., Sugimura, H., Nohmi, T., Kasai, H. & Yokota, J. (1998) Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. *Oncogene*, **16,** 3219–25.

Koppen, I.J.N., Hermans, F.J.R. & Kaspers, G.J.L. (2010) Folate related gene polymorphisms and susceptibility to develop childhood acute lymphoblastic leukaemia. *British Journal of Haematology*, **148,** 3–14.

Kroeze, L.I., Aslanyan, M.G., Van Rooij, A., Koorenhof-Scheele, T.N., Massop, M., Carell, T., Boezeman, J.B., Marie, J.P., Halkes, C.J.M., De Witte, T., Huls, G., Suciu, S., Wevers, R. a., Van Der Reijden, B. a. & Jansen, J.H. (2014) Characterization of acute myeloid leukemia based on levels of global hydroxymethylation. *Blood*, **124,** 1110–8.

Kroeze, L.I., van der Reijden, B.A. & Jansen, J.H. (2015) 5-hydroxymethylcytosine: An epigenetic mark frequently deregulated in cancer. *Biochimica et Biophysica Acta*, **1855**, 144–154.

Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E. & Nishimura, S. (1987) Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. *Nature*, **327,** 77–9.

Kumagai, T., Tefferi, A., Jones, L. & Koeffler, H.P. (2005) Methylation analysis of the cell cycle control genes in myelofibrosis with myeloid metaplasia. *Leukemia Research*, **29,** 511–5.

Lai, J., Nie, W., Zhang, W., Wang, Y., Xie, R., Wang, Y., Gu, J., Xu, J., Song, W., Yang, F., Huang, G., Cao, P. & Guan, X. (2014) Transcriptional regulation of the p73 gene by Nrf-2 and promoter CpG methylation in human breast cancer. *Oncotarget*, **5,** 6909–22.

Lan, Q., Zheng, T., Shen, M., Zhang, Y., Wang, S.S., Zahm, S.H., Holford, T.R., Leaderer, B., Boyle, P. & Chanock, S. (2007) Genetic polymorphisms in the oxidative stress pathway and susceptibility to non-Hodgkin lymphoma. *Human genetics*, **121,** 161–8.

Langemeijer, S.M.C., Jansen, J.H., Hooijer, J., van Hoogen, P., Stevens-Linders, E., Massop, M., Waanders, E., van Reijmersdal, S. V, Stevens-Kroef, M.J.P.L., Zwaan, C.M., van den Heuvel-Eibrink, M.M., Sonneveld, E., Hoogerbrugge, P.M., van Kessel, A.G. & Kuiper, R.P. (2011) TET2 mutations in childhood leukemia. *Leukemia*, **25,** 189–92.

Larsson, C.A., Cote, G. & Quintás-Cardama, A. (2013) The changing mutational landscape of acute myeloid leukemia and myelodysplastic syndrome. *Molecular cancer research : MCR*, **11,** 815–27.

Lavu, S., Boss, O., Elliott, P.J. & Lambert, P.D. (2008) Sirtuins--novel therapeutic targets to treat age-associated diseases. *Nature reviews. Drug discovery*, **7,** 841–53.

Lee, D.-H., O'Connor, T.R. & Pfeifer, G.P. (2002) Oxidative DNA damage induced by copper and hydrogen peroxide promotes CG → TT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells. *Nucleic acids research*, **30,** 3566–73.

Lee, M.-N., Tseng, R.-C., Hsu, H.-S., Chen, J.-Y., Tzao, C., Ho, W.L. & Wang, Y.-C. (2007) Epigenetic inactivation of the chromosomal stability control genes BRCA1, BRCA2, and XRCC5 in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, **13,** 832–8.

Lee, P.H. & Shatkay, H. (2008) F-SNP: Computationally predicted functional SNPs for disease association studies. *Nucleic Acids Research*, **36,** 820–4.

Lee, S.J., Jeon, H.-S., Jang, J.-S., Park, S.H., Lee, G.Y., Lee, B.-H., Kim, C.H., Kang, Y.M., Lee, W.K., Kam, S., Park, R.W., Kim, I.-S., Cho, Y.L., Jung, T.H. & Park, J.Y. (2005) DNMT3B polymorphisms and risk of primary lung cancer. *Carcinogenesis*, **26,** 403–9.

Lensch, M.W. & Daley, G.Q. (2004) Origins of mammalian hematopoiesis: in vivo paradigms and in vitro models. *Current topics in developmental biology*, **60,** 127–96.

Lertratanangkoon, K., Wu, C.J., Savaraj, N. & Thomas, M.L. (1997) Alterations of DNA methylation by glutathione depletion. *Cancer letters*, **120,** 149–56.

Levine, A.J., Lee, W., Figueiredo, J.C., Conti, D. V, Vandenberg, D.J., Davis, B.D., Edlund, C.K., Henning, S.M., Heber, D., Stern, M.C. & Haile, R.W. (2011) Variation in folate pathway genes and distal colorectal adenoma risk: a sigmoidoscopy-based case-control study. *Cancer causes & control : CCC*, **22,** 541–52.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., et al (2010) DNMT3A mutations in acute myeloid leukemia. *The New England journal of medicine*, **363,** 2424–33.

Li, H., Kantoff, P.W., Giovannucci, E., Leitzmann, M.F., Gaziano, J.M., Stampfer, M.J. & Ma, J. (2005) Manganese Superoxide Dismutase Polymorphism, Prediagnostic Antioxidant Status, and Risk of Clinical Significant Prostate Cancer. *Cancer Research*, **65,** 2498–504.

Li, L., Qiu, P., Chen, B., Lu, Y., Wu, K., Thakur, C., Chang, Q., Sun, J. & Chen, F. (2014) Reactive oxygen species contribute to arsenic-induced EZH2 phosphorylation in human bronchial epithelial cells and lung cancer cells. *Toxicology and Applied Pharmacology*, **276,** 165–70.

Li, L., Yang, L., Zhang, Y., Xu, Z., Qin, T., Hao, Y. & Xiao, Z. (2011) Detoxification and DNA repair genes polymorphisms and susceptibility of primary myelodysplastic syndromes in Chinese population. *Leukemia research*, **35,** 762–5.

Li, W.-M., Liu, H.-T., Li, X.-Y., Wu, J.-Y., Xu, G., Teng, Y.-Z., Ding, S.-T. & Yu, C. (2010) The effect of tetramethylpyrazine on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells. *Basic & clinical pharmacology & toxicology*, **106,** 45–52.

Li, Y., Wen, H., Xi, Y., Tanaka, K., Wang, H., Peng, D., Ren, Y., Jin, Q., Dent, S.Y.R., Li, W., Li, H. & Shi, X. (2014) AF9 YEATS Domain Links Histone Acetylation to DOT1L-Mediated H3K79 Methylation. *Cell*, **159,** 558–71.

Liao, R., Xu, Y., Chen, M., Chen, X., Zhan, X. & Sun, J. (2013) Molecular mechanism of microRNA involvement in genesis of myelodysplastic syndrome and its transformation to acute myeloid leukemia. *Hematology (Amsterdam, Netherlands)*, **18,** 191–7.

Liddiard, K., Hills, R., Burnett, a K., Darley, R.L. & Tonks, a (2010) OGG1 is a novel prognostic indicator in acute myeloid leukaemia. *Oncogene*, **29,** 2005–12.

Lightfoot, T.J., Skibola, C.F., Smith, A.G., Forrest, M.S., Adamson, P.J., Morgan, G.J., Bracci, P.M., Roman, E., Smith, M.T. & Holly, E. a. (2006) Polymorphisms in the oxidative stress genes, superoxide dismutase, glutathione peroxidase and catalase and risk of non-Hodgkin's lymphoma. *Haematologica*, **91,** 1222–7.

Lim, S.-O.O., Gu, J.-M.M., Kim, M.S., Kim, H.-S.S., Park, Y.N.M., Park, C.K., Cho, J.W., Park, Y.N.M. & Jung, G. (2008) Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. *Gastroenterology*, **135,** 2128–40.

Lin, C.-C., Hou, H.-A., Chou, W.-C., Kuo, Y.-Y., Wu, S.-J., Liu, C.-Y., Chen, C.-Y., Tseng, M.-H., Huang, C.-F., Lee, F.-Y., Liu, M.-C., Liu, C.-W., Tang, J.-L., Yao, M., Huang, S.-Y., Hsu, S.-C., Ko, B.-S., Tsay, W., Chen, Y.-C. & Tien, H.-F. (2014a) SF3B1 mutations in patients with myelodysplastic syndromes: the mutation is stable during disease evolution. *American journal of hematology*, **89,** E109–15.

Lin, C.-C., Hou, H.-A., Chou, W.-C., Kuo, Y.-Y., Liu, C.-Y., Chen, C.-Y., Lai, Y.-J., Tseng, M.-H., Huang, C.-F., Chiang, Y.-C., Lee, F.-Y., Liu, M.-C., Liu, C.-W., Tang, J.-L., Yao, M., Huang, S.-Y., Ko, B.-S., Wu, S.-J., Tsay, W., Chen, Y.-C., et al (2014b) IDH mutations are closely associated with mutations of DNMT3A, ASXL1 and SRSF2 in patients with myelodysplastic syndromes and are stable during disease evolution. *American journal of hematology*, **89,** 137–44.

Lin, M.T. & Beal, M.F. (2006) Alzheimer's APP mangles mitochondria. *Nature medicine*, **12,** 1241–3.

Lindsley, R.C. & Ebert, B.L. (2013) The biology and clinical impact of genetic lesions in myeloid malignancies. *Blood*, **122,** 3741–8.

London, S.J., Lehman, T.A. & Taylor, J.A. (1997) Myeloperoxidase genetic polymorphism and lung cancer risk. *Cancer research*, **57,** 5001–3.

Lu, S.C. (2009)Regulation of glutathione synthesis. *Molecular aspects of medicine*, **30,** 42–59.

Luczak, M.W. & Jagodziński, P.P. (2006) The role of DNA methylation in cancer development. *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society*, **44,** 143–54.

Ly, A., Hoyt, L., Crowell, J. & Kim, Y.-I. (2012) Folate and DNA Methylation. *Antioxidants & Redox Signaling*, **17,** 302–26.

Marchal, J., Pifferi, F. & Aujard, F. (2013) Resveratrol in mammals: effects on aging biomarkers, age-related diseases, and life span. *Annals of the New York Academy of Sciences*, **1290,** 67–73.

Mardis, E.R., Ding, L., Dooling, D.J., Larson, D.E., McLellan, M.D., Chen, K., Koboldt, D.C., Fulton, R.S., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Locke, D.P., Magrini, V.J., Abbott, R.M., Vickery, T.L., Reed, J.S., Robinson, J.S., Wylie, T., Smith, S.M., Carmichael, L., et al (2009) Recurring mutations found by sequencing an acute myeloid leukemia genome. *The New England journal of medicine*, **361,** 1058–66.

Marian, A.J. (2012) Molecular genetic studies of complex phenotypes. *Translational Research*, **159,** 64–79.

Martinez-Climent, J.A., Fontan, L., Gascoyne, R.D., Siebert, R. & Prosper, F. (2010) Lymphoma stem cells: enough evidence to support their existence? *Haematologica*, **95,** 293–302.

Marty, C., Lacout, C., Droin, N., Le Couédic, J.-P., Ribrag, V., Solary, E., Vainchenker, W., Villeval, J.-L. & Plo, I. (2013) A role for reactive oxygen species in JAK2 V617F myeloproliferative neoplasm progression. *Leukemia*, **27,** 2187–95.

Mascarenhas, J., Roper, N., Chaurasia, P. & Hoffman, R. (2011) Epigenetic abnormalities in myeloproliferative neoplasms: A target for novel therapeutic strategies. *Clinical Epigenetics*, **2,** 197–212.

Matthes, T.W., Meyer, G., Samii, K. & Beris, P. (2000) Increased apoptosis in acquired sideroblastic anaemia. *British journal of haematology*, **111,** 843–52.

Matynia, A.P., Szankasi, P., Shen, W. & Kelley, T.W. (2015) Molecular Genetic Biomarkers in Myeloid Malignancies. *Archives of Pathology & Laboratory Medicine*, **139,** 140825052632001.

Maunakea, A.K., Chepelev, I. & Zhao, K. (2010) Epigenome mapping in normal and disease States. *Circulation research*, **107,** 327–39.

Maynard, S., Schurman, S.H., Harboe, C., de Souza-Pinto, N.C. & Bohr, V.A. (2009) Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis*, **30,** 2–10.

Mazzio, E. a. & Soliman, K.F.A. (2012) Basic concepts of epigenetics: Impact of environmental signals on gene expression. *Epigenetics*, **7,** 119–30.

Medeiros, B.C., Kohrt, H.E., Gotlib, J., Coutre, S.E., Zhang, B., Arber, D.A. & Zehnder, J.L. (2012) Tailored temozolomide therapy according to MGMT methylation status for elderly patients with acute myeloid leukemia. *American Journal of Hematology*, **87**, 45–50.

Mehdipour, P., Santoro, F. & Minucci, S. (2015) Epigenetic alterations in acute myeloid leukemias. *The FEBS journal*, **282,** 1786–800.

Meldi, K.M. & Figueroa, M.E. (2014) Epigenetic deregulation in myeloid malignancies. *Translational Research*, **165,** 102–14.

Méplan, C., Dragsted, L.O., Ravn-Haren, G., Tjønneland, A., Vogel, U. & Hesketh, J. (2013) Association between Polymorphisms in Glutathione Peroxidase and Selenoprotein P Genes, Glutathione Peroxidase Activity, HRT Use and Breast Cancer Risk. *PLoS ONE*, **8,** e73316.

Meyer, S.C. & Levine, R.L. (2014) Translational implications of somatic genomics in acute myeloid leukaemia. *The Lancet Oncology*, **15,** e382–94.

Michaud, E.J., van Vugt, M.J., Bultman, S.J., Sweet, H.O., Davisson, M.T. & Woychik, R.P. (1994) Differential expression of a new dominant agouti allele (Aiapy) is correlated with methylation state and is influenced by parental lineage. *Genes & development*, **8,** 1463–72.

Milosevic, J.D. & Kralovics, R. (2013) Genetic and epigenetic alterations of myeloproliferative disorders. *International Journal of Hematology*, **97,** 183–97.

Milosevic, J.D., Puda, A., Malcovati, L., Berg, T., Hofbauer, M., Stukalov, A., Klampfl, T., Harutyunyan, A.S., Gisslinger, H., Gisslinger, B., Burjanivova, T., Rumi, E., Pietra, D., Elena, C., Vannucchi, A.M., Doubek, M., Dvorakova, D., Robesova, B., Wieser, R., Koller, E., et al (2012) Clinical significance of genetic aberrations in secondary acute myeloid leukemia. *American journal of hematology*, **87,** 1010–6.

Min, J.Y., Lim, S.O. & Jung, G. (2010) Downregulation of catalase by reactive oxygen species via hypermethylation of CpG island II on the catalase promoter. *FEBS Letters*, **584,** 2427–32.

Mishra, M. V, Bisht, K.S., Sun, L., Muldoon-Jacobs, K., Awwad, R., Kaushal, A., Nguyen, P., Huang, L., Pennington, J.D., Markovina, S., Bradbury, C.M. & Gius, D. (2008) DNMT1 as a molecular target in a multimodality-resistant phenotype in tumor cells. *Molecular cancer research : MCR*, **6,** 243–49.

Mitsuishi, Y., Motohashi, H. & Yamamoto, M. (2012) The Keap1-Nrf2 system in cancers: stress response and anabolic metabolism. *Frontiers in oncology*, **2,** 200.

Mizuno, S., Chijiwa, T., Okamura, T., Akashi, K., Fukumaki, Y., Niho, Y. & Sasaki, H. (2001) Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood*, **97,** 1172–9.

Moen, E.L., Mariani, C.J., Jeff-eke, M., Litwin, E., Nikitas, J.N. & Godley, L.A. (2015) New themes in the biological functions of 5-methylcytosine and. **263,** 36–49.

Montgomery, K.G., Liu, M.C.P., Eccles, D.M. & Campbell, I.G. (2004) The DNMT3B C → T promoter polymorphism and risk of breast cancer in a British population: a case-control study. *Breast cancer research : BCR*, **6,** R390–94.

Moore, L.D., Le, T. & Fan, G. (2013) DNA Methylation and Its Basic Function. *Neuropsychopharmacology*, **38,** 23–38.

Mostowska, A., Sajdak, S., Pawlik, P., Lianeri, M. & Jagodzinski, P.P. (2013) DNMT1, DNMT3A and DNMT3B gene variants in relation to ovarian cancer risk in the Polish population. *Molecular Biology Reports*, **40,** 4893–9.

Mufti, G.J. (2004) Pathobiology, classification, and diagnosis of myelodysplastic syndrome. *Best Practice & Research Clinical Haematology*, **17,** 543–57.

Murata, A., Baba, Y., Ishimoto, T., Miyake, K., Kosumi, K., Harada, K., Kurashige, J., Iwagami, S., Sakamoto, Y., Miyamoto, Y., Yoshida, N., Yamamoto, M., Oda, S., Watanabe, M., Nakao, M. & Baba, H. (2015) TET family proteins and 5-hydroxymethylcytosine in esophageal squamous cell carcinoma. *Oncotarget*, **6,** 23372–82.

Murati, A., Brecqueville, M., Devillier, R., Mozziconacci, M.-J., Gelsi-Boyer, V. & Birnbaum, D. (2012) Myeloid malignancies: mutations, models and management. *BMC Cancer*, **12,** 304.

Muscarella, L.A., Parrella, P., D'Alessandro, V., la Torre, A., Barbano, R., Fontana, A., Tancredi, A., Guarnieri, V., Balsamo, T., Coco, M., Copetti, M., Pellegrini, F., De Bonis, P., Bisceglia, M., Scaramuzzi, G., Maiello, E., Valori, V.M., Merla, G., Vendemiale, G. & Fazio, V.M. (2011a) Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer. *Epigenetics*, **6,** 710–9.

Muscarella, L.A., Barbano, R., D'Angelo, V., Copetti, M., Coco, M., Balsamo, T., la Torre, A., Notarangelo, A., Troiano, M., Parisi, S., Icolaro, N., Catapano, D., Valori, V.M., Pellegrini, F., Merla, G., Carella, M., Fazio, V.M. & Parrella, P. (2011b) Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient's outcome. *Epigenetics*, **6,** 317–25.

Musolino, C., Allegra, A., Saija, A., Alonci, A., Russo, S., Spatari, G., Penna, G., Gerace, D., Cristani, M., David, A., Saitta, S. & Gangemi, S. (2012) Changes in advanced oxidation protein products, advanced glycation end products, and s-nitrosylated proteins, in patients affected by polycythemia vera and essential thrombocythemia. *Clinical Biochemistry*, **45,** 1439–43.

Naoe, T., Takeyama, K., Yokozawa, T., Kiyoi, H., Seto, M., Uike, N., Ino, T., Utsunomiya, A., Maruta, A., Jin-nai, I., Kamada, N., Kubota, Y., Nakamura, H., Shimazaki, C., Horiike, S., Kodera, Y., Saito, H., Ueda, R., Wiemels, J. & Ohno, R. (2000) Analysis of genetic polymorphism in NQO1, GST-M1, GST-T1, and CYP3A4 in 469 Japanese patients with therapy-related leukemia/ myelodysplastic syndrome and de novo acute myeloid leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research*, **6,** 4091–5.

Neitzel, H. (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Human genetics*, **73,** 320–6.

Nikoloski, G., Van Der Reijden, B. a. & Jansen, J.H. (2012) Mutations in epigenetic regulators in myelodysplastic syndromes. *International Journal of Hematology*, **95,** 8–16.

Nimer, S.D. (2008) Myelodysplastic syndromes. *Blood*, **111,** 4841–51.

Nishida, N., Arizumi, T., Takita, M., Kitai, S., Yada, N., Hagiwara, S., Inoue, T., Minami, Y., Ueshima, K., Sakurai, T. & Kudo, M. (2013) Reactive oxygen species induce epigenetic instability through the formation of 8-hydroxydeoxyguanosine in human hepatocarcinogenesis. *Digestive diseases (Basel, Switzerland)*, **31,** 459–66.

Niture, S.K., Khatri, R. & Jaiswal, A.K. (2014) Regulation of Nrf2 - An update. *Free Radical Biology and Medicine*, **66,** 36–44.

Novotna, B., Bagryantseva, Y., Siskova, M. & Neuwirtova, R. (2009) Oxidative DNA damage in bone marrow cells of patients with low-risk myelodysplastic syndrome. *Leukemia Research*, **33,** 340–3.

O'Brien, E., Prideaux, S. & Chevassut, T. (2014) The epigenetic landscape of acute myeloid leukemia. *Advances in hematology*, **2014,** 103175.

O'Connor, J.E., Kimler, B.F., Morgan, M.C. & Tempas, K.J. (1988) A flow cytometric assay for intracellular nonprotein thiols using mercury orange. *Cytometry*, **9,** 529–32.

O'Hagan, H.M., Wang, W., Sen, S., DeStefano Shields, C., Lee, S.S., Zhang, Y.W., Clements, E.G., Cai, Y., Van Neste, L., Easwaran, H., Casero, R.A., Sears, C.L. & Baylin, S.B. (2011) Oxidative Damage Targets Complexes Containing DNA Methyltransferases, SIRT1, and Polycomb Members to Promoter CpG Islands. *Cancer Cell*, **20,** 606–19.

Odenike, O., Thirman, M.J., Artz, A.S., Godley, L.A., Larson, R.A. & Stock, W. (2011) Gene mutations, epigenetic dysregulation, and personalized therapy in myeloid neoplasm: Are we there yet? *Seminars in Oncology*, **38,** 196–214.

Ohanian, M., Otway, R. & Fatkin, D. (2012) Heuristic methods for finding pathogenic variants in gene coding sequences. *Journal of the American Heart Association*, **1,** 1–15.

Ohgami, R.S. & Arber, D. A. (2015) The diagnostic and clinical impact of genetics and epigenetics in acute myeloid leukemia. *International Journal of Laboratory Hematology*, **37,** 122–32.

Oki, Y. & Issa, J.-P.J. (2010) Epigenetic mechanisms in AML - a target for therapy. *Cancer treatment and research*, **145,** 19–40.

Olinski, R. (2003) Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development? *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **531,** 177–90.

Orkin, S.H. & Zon, L.I. (2008) Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell*, **132,** 631–44.

Orrenius, S., Gogvadze, V. & Zhivotovsky, B. (2007) Mitochondrial oxidative stress: implications for cell death. *Annual review of pharmacology and toxicology*, **47,** 143–83.

Paglia, D.E. & Valentine, W.N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of laboratory and clinical medicine*, **70,** 158–69.

Park, L.K., Friso, S. & Choi, S.-W. (2012) Nutritional influences on epigenetics and age-related disease. *The Proceedings of the Nutrition Society*, **71,** 75–83.

Parker, J.E. & Mufti, G.J. (2001) The role of apoptosis in the pathogenesis of the myelodysplastic syndromes. *International journal of hematology*, **73,** 416–28.

Parker, J.E., Mufti, G.J., Rasool, F., Mijovic, a, Devereux, S. & Pagliuca, a (2000) The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. *Blood*, **96,** 3932–8.

Paschka, P., Schlenk, R.F., Gaidzik, V.I., Habdank, M., Krönke, J., Bullinger, L., Späth, D., Kayser, S., Zucknick, M., Götze, K., Horst, H.-A., Germing, U., Döhner, H. & Döhner, K. (2010) IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **28,** 3636–43.

Patchsung, M., Boonla, C., Amnattrakul, P., Dissayabutra, T., Mutirangura, A. & Tosukhowong, P. (2012) Long interspersed nuclear element-1 hypomethylation and oxidative stress: correlation and bladder cancer diagnostic potential. *PloS one*, **7,** e37009.

Patnala, R., Clements, J. & Batra, J. (2013) Candidate gene association studies: a comprehensive guide to useful in silico tools. *BMC genetics*, **14,** 39.

Peddie, C.M., Wolf, C.R., McLellan, L.I., Collins, A.R. & Bowen, D.T. (1997) Oxidative DNA damage in CD34+ myelodysplastic cells is associated with intracellular redox changes and elevated plasma tumour necrosis factor-alpha concentration. *British journal of haematology*, **99,** 625–31.

Pellagatti, A. & Boultwood, J. (2015) The molecular pathogenesis of the myelodysplastic syndromes. *European journal of haematology*, **95,** 3–15.

Pervaiz, S. & Clement, M.-V. (2007) Superoxide anion: oncogenic reactive oxygen species? *The international journal of biochemistry & cell biology*, **39,** 1297–304.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, **29,** e45.

Pieczenik, S.R. & Neustadt, J. (2007) Mitochondrial dysfunction and molecular pathways of disease. *Experimental and molecular pathology*, **83,** 84–92.

Pimková, K., Chrastinová, L., Suttnar, J., Štikarová, J., Kotlín, R., Čermák, J. & Dyr, J.E. (2014) Plasma levels of aminothiols, nitrite, nitrate, and malondialdehyde in myelodysplastic syndromes in the context of clinical outcomes and as a consequence of iron overload. *Oxidative Medicine and Cellular Longevity*, **2014,** 416028.

Pisoschi, A.M. & Pop, A. (2015) The role of antioxidants in the chemistry of oxidative stress: a Review. *European Journal of Medicinal Chemistry*, **97,** 55–74.

Platzbecker, U., Meredyth-Stewart, M. & Ehninger, G. (2007) The pathogenesis of myelodysplastic syndromes (MDS). *Cancer Treatment Reviews*, **33,** S53–8.

Pluta, A., Nyman, U., Joseph, B., Robak, T., Zhivotovsky, B. & Smolewski, P. (2006) The role of p73 in hematological malignancies. *Leukemia*, **20,** 757–66.

Portela, A. & Esteller, M. (2010) Epigenetic modifications and human disease. *Nature biotechnology*, **28,** 1057–68.

Preskill, C. & Weidhaas, J.B. (2013) SNPs in microRNA binding sites as prognostic and predictive cancer biomarkers. *Critical reviews in oncogenesis*, **18,** 327–40.

Prokunina, L. & Alarcón-Riquelme, M.E. (2004) Regulatory SNPs in complex diseases: their identification and functional validation. *Expert reviews in molecular medicine*, **6,** 1–15.

Przybylowska, K., Kabzinski, J., Sygut, A., Dziki, L., Dziki, A. & Majsterek, I. (2013) An association selected polymorphisms of XRCC1, OGG1 and MUTYH gene and the level of efficiency oxidative DNA damage repair with a risk of colorectal cancer. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, **745-746,** 6–15.

Qian, J., Yao, D.-M., Lin, J., Wang, Y.-L., Han, L.-X., Xu, W.-R. & Wu, C.-Y. (2010) Methylation of DAPK1 promoter: frequent but not an adverse prognostic factor in myelodysplastic syndrome. *International journal of laboratory hematology*, **32,** 74–81.

Quan, X., Lim, S.O. & Jung, G. (2011) Reactive oxygen species downregulate catalase expression via methylation of a CpG Island in the Oct-1 promoter. *FEBS Letters*, **585,** 3436–41.

Quesnel, B., Guillerm, G., Vereecque, R., Wattel, E., Preudhomme, C., Bauters, F., Vanrumbeke, M. & Fenaux, P. (1998) Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. *Blood*, **91,** 2985–90.

Rang, F.J. & Boonstra, J. (2014) Causes and Consequences of Age-Related Changes in DNA Methylation: A Role for ROS? *Biology*, **3,** 403–25.

Ranjan, A., Penninga, E., Jelsig, A.M., Hasselbalch, H.C. & Bjerrum, O.W. (2013) Inheritance of the chronic myeloproliferative neoplasms. A systematic review. *Clinical genetics*, **83,** 99–107.

Rasool, M., Farooq, S., Malik, A., Shaukat, A., Manan, A., Asif, M., Sani, S., Qazi, M.H., Kamal, M.A., Iqbal, Z. & Hussain, A. (2015) Assessment of circulating biochemical markers and antioxidative status in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients. *Saudi Journal of Biological Sciences*, **22,** 106–11.

Relling, M. V, Gardner, E.E., Sandborn, W.J., Schmiegelow, K., Pui, C.-H., Yee, S.W., Stein, C.M., Carrillo, M., Evans, W.E. & Klein, T.E. (2011) Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clinical pharmacology and therapeutics*, **89,** 387–91.

Rhyasen, G.W. & Starczynowski, D.T. (2012) Deregulation of microRNAs in myelodysplastic syndrome. *Leukemia*, **26,** 13–22.

Ribas, V., García-Ruiz, C. & Fernández-Checa, J.C. (2014) Glutathione and mitochondria. *Frontiers in pharmacology*, **5,** 151.

Rieger, M.A. & Schroeder, T. (2012) Hematopoiesis. *Cold Spring Harbor perspectives in biology*, **4,** a008250.

Rinaldi, L. & Benitah, S.A. (2014) Epigenetic regulation of adult stem cell function. *The FEBS journal*, **282,** 1589–604.

Robb, L. (2007) Cytokine receptors and hematopoietic differentiation. *Oncogene*, **26,** 6715–23.

Robbins, D. & Zhao, Y. (2014) Manganese superoxide dismutase in cancer prevention. *Antioxidants & redox signaling*, **20,** 1628–45.

Rodic, N. & Burns, K.H. (2013) Long interspersed element-1 (LINE-1): passenger or driver in human neoplasms? *PLoS genetics*, **9,** e1003402.

Rodrigues, M.S., Reddy, M.M. & Sattler, M. (2008) Cell cycle regulation by oncogenic tyrosine kinases in myeloid neoplasms: from molecular redox mechanisms to health implications. *Antioxidants & redox signaling*, **10,** 1813–48.

Roman-Gomez, J., Jimenez-Velasco, A., Agirre, X., Castillejo, J. a., Navarro, G., San Jose-Eneriz, E., Garate, L., Cordeu, L., Cervantes, F., Prosper, F., Heiniger, A. & Torres, A. (2008) Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia. *Leukemia Research*, **32,** 487–90.

Römermann, D., Hasemeier, B., Metzig, K., Göhring, G., Schlegelberger, B., Länger, F., Kreipe, H. & Lehmann, U. (2008) Global increase in DNA methylation in patients with myelodysplastic syndrome. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, **22,** 1954–6.

Ropero, S. & Esteller, M. (2007) The role of histone deacetylases (HDACs) in human cancer. *Molecular Oncology*, **1,** 19–25.

Ross, J.P., Rand, K.N. & Molloy, P.L. (2010) Hypomethylation of repeated DNA sequences in cancer. *Epigenomics*, **2,** 245–69.

Rowe, J.M. (2014) The increasing genomic complexity of acute myeloid leukemia. *Best Practice & Research Clinical Haematology*, **27,** 209–13.

Roy, L.M., Jaruga, P., Wood, T.G., McCullough, A.K., Dizdaroglu, M. & Lloyd, R.S. (2007) Human polymorphic variants of the NEIL1 DNA glycosylase. *Journal of Biological Chemistry*, **282,** 15790–8.

Rushworth, S. a. & MacEwan, D.J. (2011) The role of Nrf2 and cytoprotection in regulating chemotherapy resistance of human leukemia cells. *Cancers*, **3,** 1605–21.

Rushworth, S.A., Zaitseva, L., Murray, M.Y., Shah, N.M., Bowles, K.M. & MacEwan, D.J. (2012) The high Nrf2 expression in human acute myeloid leukemia is driven by NF-κB and underlies its chemo-resistance. *Blood*, **120,** 5188–98.

Saigo, K., Takenokuchi, M., Hiramatsu, Y., Tada, H., Hishita, T., Takata, M., Misawa, M., Imoto, S. & Imashuku, S. (2011) Oxidative stress levels in myelodysplastic syndrome patients: their relationship to serum ferritin and haemoglobin values. *The Journal of international medical research*, **39,** 1941–5.

Sallmyr, A., Fan, J. & Rassool, F.V. (2008) Genomic instability in myeloid malignancies: Increased reactive oxygen species (ROS), DNA double strand breaks (DSBs) and error-prone repair. *Cancer Letters*, **270,** 1–9.

Salmon, A.B. (2012) Oxidative stress in the etiology of age-associated decline in glucose metabolism. *Longevity & healthspan*, **1,** 7.

Santini, V., Melnick, A., Maciejewski, J.P., Duprez, E., Nervi, C., Cocco, L., Ford, K.G. & Mufti, G. (2013) Epigenetics in focus: Pathogenesis of myelodysplastic syndromes and the role of hypomethylating agents. *Critical Reviews in Oncology/Hematology*, **88,** 231–45.

Saradalekshmi, K.R., Neetha, N.V., Sathyan, S., Nair, I. V., Nair, C.M. & Banerjee, M. (2014) DNA methyl transferase (DNMT) gene polymorphisms could be a primary event in epigenetic susceptibility to schizophrenia. *PLoS ONE*, **9,** 1–8.

Sardina, J.L., López-Ruano, G., Sánchez-Sánchez, B., Llanillo, M. & Hernández-Hernández, A. (2012) Reactive oxygen species: are they important for haematopoiesis? *Critical reviews in oncology/hematology*, **81,** 257–74.

Sarmento-Ribeiro, A.B., Proença, M.T., Sousa, I., Pereira, A., Guedes, F., Teixeira, A. & Oliveira, C.R. (2012) A possible role for oxidation stress in lymphoid leukaemias and therapeutic failure. *Leukemia research*, **36,** 1041–8.

Savas, S., Liu, G. & Xu, W. (2013) Special considerations in prognostic research in cancer involving genetic polymorphisms. *BMC medicine*, **11,** 149.

Saxonov, S., Berg, P. & Brutlag, D.L. (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proceedings of the National Academy of Sciences*, **103,** 1412–7.

Schildgen, V., Wulfert, M. & Gattermann, N. (2011) Impaired mitochondrial gene transcription in myelodysplastic syndromes and acute myeloid leukemia with myelodysplasia-related changes. *Experimental hematology*, **39,** 666–75.e1.

Schirmer, M., Hoffmann, M., Kaya, E., Tzvetkov, M. & Brockmöller, J. (2008) Genetic polymorphisms of NAD(P)H oxidase: variation in subunit expression and enzyme activity. *The pharmacogenomics journal*, **8,** 297–304.

Schoofs, T. & Müller-Tidow, C. (2011) DNA methylation as a pathogenic event and as a therapeutic target in AML. *Cancer Treatment Reviews*, **37,** S13–8.

Schoofs, T., Berdel, W.E. & Müller-Tidow, C. (2014) Origins of aberrant DNA methylation in acute myeloid leukemia. *Leukemia*, **28,** 1–14.

Schork, A.J., Thompson, W.K., Pham, P., Torkamani, A., Roddey, J.C., Sullivan, P.F., Kelsoe, J.R., O'Donovan, M.C., Furberg, H., Schork, N.J., Andreassen, O. a. & Dale, A.M. (2013) All SNPs Are Not Created Equal: Genome-Wide Association Studies Reveal a Consistent Pattern of Enrichment among Functionally Annotated SNPs. *PLoS Genetics*, **9,** e1003449.

Scopim-Ribeiro, R., Machado-Neto, J.A., Campos, P.D.M., Silva, C.A.M., Favaro, P., Lorand-Metze, I., Costa, F.F., Saad, S.T.O. & Traina, F. (2014) Ten-Eleven-Translocation 2 (TET2) is downregulated in myelodysplastic syndromes. *European Journal of Haematology*, **94,** 413-8.

Sedelnikova, O.A., Redon, C.E., Dickey, J.S., Nakamura, A.J., Georgakilas, A.G. & Bonner, W.M. Role of oxidatively induced DNA lesions in human pathogenesis. *Mutation research*, **704,** 152–9.

Seedhouse, C., Faulkner, R., Ashraf, N., Das-Gupta, E. & Russell, N. (2004) Polymorphisms in genes involved in homologous recombination repair interact to increase the risk of developing acute myeloid leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research*, **10,** 2675–80.

Seedhouse, C.H., Das-Gupta, E.P. & Russell, N.H. (2003) Methylation of the hMLH1 promoter and its association with microsatellite instability in acute myeloid leukemia. *Leukemia*, **17,** 83–8.

Sheikhha, M.H., Tobal, K. & Liu Yin, J.A. (2002) High level of microsatellite instability but not hypermethylation of mismatch repair genes in therapy-related and secondary acute myeloid leukaemia and myelodysplastic syndrome. *British journal of haematology*, **117,** 359–65.

Shen, L., Kantarjian, H., Guo, Y., Lin, E., Shan, J., Huang, X., Berry, D., Ahmed, S., Zhu, W., Pierce, S., Kondo, Y., Oki, Y., Jelinek, J., Saba, H., Estey, E. & Issa, J.-P.J. (2010) DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **28,** 605–13.

Shi, J.-Y., Ren, Z.-H., Jiao, B., Xiao, R., Yun, H.-Y., Chen, B., Zhao, W.-L., Zhu, Q., Chen, Z. & Chen, S.-J. (2011) Genetic variations of DNA repair genes and their prognostic significance in patients with acute myeloid leukemia. *International journal of cancer. Journal international du cancer*, **128,** 233–8.

Shi, X., Zhang, Y., Zheng, J. & Pan, J. (2012) Reactive oxygen species in cancer stem cells. *Antioxidants & redox signaling*, **16,** 1215–28.

Shih, A.H. & Levine, R.L. (2011) Molecular biology of myelodysplastic syndromes. *Seminars in oncology*, **38,** 613–20.

Shih, A.H., Abdel-Wahab, O., Patel, J.P. & Levine, R.L. (2012) The role of mutations in epigenetic regulators in myeloid malignancies. *Nature Reviews Cancer*, **12,** 599–612.

Shimo-Nakanishi, Y., Hasebe, T., Suzuki, A., Mochizuki, H., Nomiyama, T., Tanaka, Y., Nagaoka, I., Mizuno, Y. & Urabe, T. (2004) Functional effects of NAD(P)H oxidase p22(phox) C242T mutation in human leukocytes and association with thrombotic cerebral infarction. *Atherosclerosis*, **175,** 109–15.

Shin, M.G., Kajigaya, S., Levin, B.C. & Young, N.S. (2003) Mitochondrial DNA mutations in patients with myelodysplastic syndromes. *Blood*, **101,** 3118–25.

Shlush, L.I., Zandi, S., Mitchell, A., Chen, W.C., Brandwein, J.M., Gupta, V., Kennedy, J.A., Schimmer, A.D., Schuh, A.C., Yee, K.W., McLeod, J.L., Doedens, M., Medeiros, J.J.F., Marke, R., Kim, H.J., Lee, K., McPherson, J.D., Hudson, T.J., Brown, A.M.K., Yousif, F., et al (2014) Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*, **506,** 328–33.

Singal, R., Ferdinand, L., Das, P.M., Reis, I.M. & Schlesselman, J.J. (2004) Polymorphisms in the methylenetetrahydrofolate reductase gene and prostate cancer risk. *International journal of oncology*, **25,** 1465–71.

Singh, A., Misra, V., Thimmulappa, R.K., Lee, H., Ames, S., Hoque, M.O., Herman, J.G., Baylin, S.B., Sidransky, D., Gabrielson, E., Brock, M. V & Biswal, S. (2006) Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS medicine*, **3,** e420.

Sirhan, S., Busque, L., Foltz, L., Grewal, K., Hamm, C., Laferriere, N., Laneuville, P., Leber, B., Liew, E., Olney, H.J., Prchal, J., Porwit, A. & Gupta, V. (2015) Evolving Therapeutic Options for Polycythemia Vera: Perspectives of the Canadian Myeloproliferative Neoplasms (MPN) Group. *Clinical Lymphoma Myeloma and Leukemia*. [Epub ahead of print].

Sjöblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N., Szabo, S., Buckhaults, P., Farrell, C., Meeh, P., Markowitz, S.D., Willis, J., Dawson, D., Willson, J.K. V, Gazdar, A.F., Hartigan, J., et al (2006) The

consensus coding sequences of human breast and colorectal cancers. *Science (New York, N.Y.)*, **314,** 268–74.

Skoda, R.C., Duek, A. & Grisouard, J. (2015) Pathogenesis of myeloproliferative neoplasms. *Experimental Hematology*, **43,** 599–608.

Skokowa, J., Steinemann, D., Katsman-Kuipers, J.E., Zeidler, C., Klimenkova, O., Klimiankou, M., Unalan, M., Kandabarau, S., Makaryan, V., Beekman, R., Behrens, K., Stocking, C., Obenauer, J., Schnittger, S., Kohlmann, A., Valkhof, M.G., Hoogenboezem, R., Göhring, G., Reinhardt, D., Schlegelberger, B., et al (2014) Cooperativity of RUNX1 and CSF3R mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis. *Blood*, **123,** 2229–37.

Slattery, M.L., Potter, J.D., Samowitz, W., Schaffer, D. & Leppert, M. (1999) Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, **8,** 513–8.

Slupianek, A., Hoser, G., Majsterek, I., Bronisz, A., Malecki, M., Blasiak, J., Fishel, R. & Skorski, T. (2002) Fusion tyrosine kinases induce drug resistance by stimulation of homology-dependent recombination repair, prolongation of G(2)/M phase, and protection from apoptosis. *Molecular and cellular biology*, **22,** 4189–201.

Smith, B.D. & Sung, L. (2013) Acute myeloid leukemia. *American Society of Hematology Self-Assessment Program*, ***2013*****,** 481–90.

Smith, M.T., Wang, Y., Kane, E., Rollinson, S., Wiemels, J.L., Roman, E., Roddam, P., Cartwright, R. & Morgan, G. (2001) Low NAD(P)H:quinone oxidoreductase 1 activity is associated with increased risk of acute leukemia in adults. *Blood*, **97,** 1422–6.

Soberanes, S., Gonzalez, A., Urich, D., Chiarella, S.E., Radigan, K. a, Osornio-Vargas, A., Joseph, J., Kalyanaraman, B., Ridge, K.M., Chandel, N.S., Mutlu, G.M., De Vizcaya-Ruiz, A. & Budinger, G.R.S. (2012) Particulate matter Air Pollution induces hypermethylation of the p16 promoter Via a mitochondrial ROS-JNK-DNMT1 pathway. *Scientific reports*, **2,** 275.

Sohal, R.S. & Orr, W.C. (2012) The redox stress hypothesis of aging. *Free radical biology & medicine*, **52,** 539–55.

Solomon, P.R., Munirajan, A.K., Tsuchida, N., Muthukumarasamy, K., Rathinavel, A., Selvam, G.S. & Shanmugam, G. (2008) Promoter hypermethylation analysis in myelodysplastic syndromes: diagnostic & prognostic implication. *The Indian journal of medical research*, **127,** 52–7.

Sorour, A., Ayad, M.W. & Kassem, H. (2013) The genotype distribution of the XRCC1, XRCC3, and XPD DNA repair genes and their role for the development of acute myeloblastic leukemia. *Genetic testing and molecular biomarkers*, **17,** 195–201.

Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H. & LLeonart, M.E. (2013) Oxidative stress and cancer: an overview. *Ageing research reviews*, **12,** 376–90.

Sporn, M.B. & Liby, K.T. (2012) NRF2 and cancer: the good, the bad and the importance of context. *Nature reviews. Cancer*, **12,** 564–71.

Steensma, D.P. (2015) Myelodysplastic Syndromes. *Mayo Clinic Proceedings*, **90,** 969–983.

Steensma, D.P. & Gattermann, N. (2013) When is iron overload deleterious, and when and how should iron chelation therapy be administered in myelodysplastic syndromes? *Best Practice and Research: Clinical Haematology*, **26,** 431–44.

Stefanska, B., Karlic, H., Varga, F., Fabianowska-Majewska, K. & Haslberger, A. (2012) Epigenetic mechanisms in anti-cancer actions of bioactive food components--the implications in cancer prevention. *British journal of pharmacology*, **167,** 279–97.

Stępkowski, T.M. & Kruszewski, M.K. (2011) Molecular cross-talk between the NRF2/KEAP1 signaling pathway, autophagy, and apoptosis. *Free radical biology & medicine*, **50,** 1186–95.

Steward, W.P. & Brown, K. (2013) Cancer chemoprevention: a rapidly evolving field. *British journal of cancer*, **109,** 1–7.

Sun, M.-Y., Yang, X.-X., Xu, W.-W., Yao, G.-Y., Pan, H.-Z. & Li, M. (2012) polymorphisms with breast cancer risk in Han Chinese women from South China. *Genet Mol Res*, **11,** 4330–41.

Sutton, A., Nahon, P., Pessayre, D., Rufat, P., Poiré, A., Ziol, M., Vidaud, D., Barget, N., Ganne-Carrié, N., Charnaux, N., Trinchet, J.-C., Gattegno, L. & Beaugrand, M. (2006) Genetic polymorphisms in antioxidant enzymes modulate hepatic iron accumulation and hepatocellular carcinoma development in patients with alcohol-induced cirrhosis. *Cancer research*, **66,** 2844–52.

Swerdlow, S.H.; Campo, E.; Harris, N.L.; Jaffe, E.S.; Pileri, S.A.; Stein, H.; Thiele, J.; Vardiman, J.W. Who Classification of Tumours of Hematopoietic and Lymphoid Tissues, 4th ed.; International Agency for Research on Cancer: Lyon, France, 2008.

Taby, R. & Issa, J.-P. (2010) Cancer epigenetics. *CA: a cancer journal for clinicians*, **60,** 376–92.

Tada, M., Yokosuka, O., Fukai, K., Chiba, T., Imazeki, F., Tokuhisa, T. & Saisho, H. (2005) Hypermethylation of NAD(P)H: quinone oxidoreductase 1 (NQO1) gene in human hepatocellular carcinoma. *Journal of hepatology*, **42,** 511–9.

Tan, I.B., Ngeow, J. & Tan, P. (2010) Role of Polymorphisms in Cancer Susceptibility. In: *Encyclopedia of Life Sciences (ELS)*. John Wiley & Sons, Ltd: Chichester.

Tarng, D.C., Tsai, T.J., Chen, W.T., Liu, T.Y. & Wei, Y.H. (2001) Effect of human OGG1 1245C-->G gene polymorphism on 8-hydroxy-2'-deoxyguanosine levels of leukocyte DNA among patients undergoing chronic hemodialysis. *Journal of the American Society of Nephrology : JASN*, **12,** 2338–47.

Tefferi, A. & Barbui, T. (2015) Polycythemia vera and essential thrombocythemia: 2015 update on diagnosis, risk-stratification and management. *American journal of hematology*, **90,** 162–73.

Tefferi, A. & Vainchenker, W. (2011) Myeloproliferative neoplasms: Molecular pathophysiology, essential clinical understanding, and treatment strategies. *Journal of Clinical Oncology*, **29,** 573–82.

Tefferi, A. & Vardiman, J.W. (2008) Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia*, **22,** 14–22.

Tefferi, A. & Vardiman, J.W. (2009) Myelodysplastic syndromes. *The New England journal of medicine*, **361,** 1872–85.

Tefferi, A., Skoda, R. & Vardiman, J.W. (2009) Myeloproliferative neoplasms: contemporary diagnosis using histology and genetics. *Nature reviews. Clinical oncology*, **6,** 627–37.

Thannickal, V.J. & Fanburg, B.L. (2000) Reactive oxygen species in cell signaling. *American journal of physiology. Lung cellular and molecular physiology*, **279,** L1005–28.

Thol, F., Damm, F., Lüdeking, A., Winschel, C., Wagner, K., Morgan, M., Yun, H., Göhring, G., Schlegelberger, B., Hoelzer, D., Lübbert, M., Kanz, L., Fiedler, W., Kirchner, H., Heil, G., Krauter, J., Ganser, A. & Heuser, M. (2011) Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, **29,** 2889–96.

Tien, H.F., Tang, J.L., Tsay, W., Liu, M.C., Lee, F.Y., Wang, C.H., Chen, Y.C. & Shen, M.C. (2001) Methylation of the $p15^{INK4B}$ gene in myelodysplastic syndrome: It can be detected early at diagnosis or during disease progression and is highly associated with leukaemic transformation. *British Journal of Haematology*, **112,** 148–54.

Titheradge, M.A. (1998) The enzymatic measurement of nitrate and nitrite. *Methods in molecular biology (Clifton, N.J.)*, **100,** 83–91.

Titmarsh, G.J., Duncombe, A.S., Mcmullin, M.F., O'Rorke, M., Mesa, R., De Vocht, F., Horan, S., Fritschi, L., Clarke, M. & Anderson, L. a. (2014) How common are myeloproliferative neoplasms? A systematic review and meta-analysis. *American Journal of Hematology*, **89,** 581–7.

Toyokuni, S. (2008) Molecular mechanisms of oxidative stress-induced carcinogenesis: From epidemiology to oxygenomics. *IUBMB Life*, **60,** 441–7.

Tsai, S.-C., Shih, L.-Y., Liang, S.-T., Huang, Y.-J., Kuo, M.-C., Huang, C.-F., Shih, Y.-S., Lin, T.-H., Chiu, M.-C. & Liang, D.-C. (2015) Biological Activities of RUNX1 Mutants Predict Secondary Acute Leukemia Transformation from Chronic Myelomonocytic Leukemia and Myelodysplastic Syndromes. *Clinical cancer research : an official journal of the American Association for Cancer Research*, **21,** 3541–51.

Turk, P.W., Laayoun, A., Smith, S.S. & Weitzman, S.A. (1995) DNA adduct 8-hydroxyl-2'-deoxyguanosine (8-hydroxyguanine) affects function of human DNA methyltransferase. *Carcinogenesis*, **16,** 1253–5.

Vainchenker, W. & Constantinescu, S.N. (2012) JAK/STAT signaling in hematological malignancies. *Oncogene,* **32,** 1–13.

Valencia, A., Cervera, J., Such, E., Ibañez, M., Gómez, I., Luna, I., Senent, L., Oltra, S., Sanz, M.A. & Sanz, G.F. (2011) Aberrant methylation of tumor suppressor genes in patients with refractory anemia with ring sideroblasts. *Leukemia research*, **35,** 479–83.

Valinluck, V. & Sowers, L.C. (2007) Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1. *Cancer research*, **67,** 946–50.

Valinluck, V., Tsai, H.-H.H., Rogstad, D.K., Burdzy, A., Bird, A. & Sowers, L.C. (2004) Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). *Nucleic Acids Research*, **32,** 4100–8.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. & Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, **39,** 44–84.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*, **3,** RESEARCH0034.

Vardiman, J.W., Brunning, R., Arber, D.A., Le Beau, M.M., Porwit, A., Tefferi, A., Bloomfield, C.D., & Thiele, J. (2008) Introduction and overview of the classification of the myeloid neoplams. In *WHO classification of tumours of haematopoietic and lymphoid tissues*,

Swerdlow, S.H., Campo, E., Harris, NL., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. & Vardiman J.W. (4th ed) pp 18–30. Lyon: International Agency for Research on Cancer.

Vardiman, J.W., Thiele, J., Arber, D.A., Brunning, R.D., Borowitz, M.J., Porwit, A., Harris, N.L., Le Beau, M.M., Hellström-Lindberg, E., Tefferi, A. & Bloomfield, C.D. (2009) The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*, **114,** 937–51.

Vatassery, G.T., Maynard, V.R. & Hagen, D.F. (1978) High-performance liquid chromatography of various tocopherols. *Journal of Chromatography A*, **161,** 299–302.

Vener, C., Novembrino, C., Catena, F.B., Fracchiolla, N.S., Gianelli, U., Savi, F., Radaelli, F., Fermo, E., Cortelezzi, A., Lonati, S., Menegatti, M. & Deliliers, G.L. (2010) Oxidative stress is increased in primary and post-polycythemia vera myelofibrosis. *Experimental hematology*, **38,** 1058–65.

Vineis, P., Veglia, F., Garte, S., Malaveille, C., Matullo, G., Dunning, A., Peluso, M., Airoldi, L., Overvad, K., Raaschou-Nielsen, O., Clavel-Chapelon, F., Linseisen, J.P., Kaaks, R., Boeing, H., Trichopoulou, A., Palli, D., Crosignani, P., Tumino, R., Panico, S., Bueno-De-Mesquita, H.B., et al (2007) Genetic susceptibility according to three metabolic pathways in cancers of the lung and bladder and in myeloid leukemias in nonsmokers. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, **18,** 1230–42.

Visconte, V., Tiu, R. V & Rogers, H.J. (2014) Pathogenesis of myelodysplastic syndromes : an overview of molecular and non-molecular aspects of the disease. **49,** 216-27.

Voukelatou, G., Thanopoulou, E., Dallas, K., Fertakis, V., Dimopoulou, A., Micheva, I., Lampropoulou, P., Kouraklis-Symeonidis, A., Symeonidis, A., Zoumbos, N. (2009) CD34+ marrow cells from MDS patients are characterized by higher levels of intracellular reactive oxygen species and inadequate antioxidant defences. *Leukemia Research*, **33,**S93–4.

Wadhwa, M. & Thorpe, R. (2008) Haematopoietic growth factors and their therapeutic use. *Thrombosis and Haemostasis*, **99,** 863–73.

Wajed, S.A., Laird, P.W. & DeMeester, T.R. (2001) DNA methylation: an alternative pathway to cancer. *Annals of surgery*, **234,** 10–20.

Walker, B.A., Wardell, C.P., Chiecchio, L., Smith, E.M., Boyd, K.D., Neri, A., Davies, F.E., Ross, F.M. & Morgan, G.J. (2011) Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood*, **117,** 553–62.

Walsh, W.J. (2010) Oxidative stress, undermethylation, and epigenetics – The bermuda triangle of autism. *The autism file USA*, **35**, 30-35.

Wang, L., Liu, S., Niu, T. & Xu, X. (2005) SNPHunter: a bioinformatic software for single nucleotide polymorphism data acquisition and management. *BMC bioinformatics*, **6,** 60.

Wang, S., Wang, F., Shi, X., Dai, J., Peng, Y., Guo, X., Wang, X., Shen, H. & Hu, Z. (2009) Association between manganese superoxide dismutase (MnSOD) Val-9Ala polymorphism and cancer risk - A meta-analysis. *European journal of cancer (Oxford, England : 1990)*, **45,** 2874–81.

Wang, S.S., Davis, S., Cerhan, J.R., Hartge, P., Severson, R.K., Cozen, W., Lan, Q., Welch, R., Chanock, S.J. & Rothman, N. (2006) Polymorphisms in oxidative stress genes and risk for non-Hodgkin lymphoma. *Carcinogenesis*, **27,** 1828–34.

Wasson, G.R., McGlynn, A.P., McNulty, H., O'Reilly, S.L., McKelvey-Martin, V.J., McKerr, G., Strain, J.J., Scott, J. & Downes, C.S. (2006) Global DNA and p53 region-specific hypomethylation in human colonic cells is induced by folate depletion and reversed by folate supplementation. *The Journal of nutrition*, **136,** 2748–53.

Weitzman, S.A., Turk, P.W., Milkowski, D.H. & Kozlowski, K. (1994) Free radical adducts induce alterations in DNA cytosine methylation. *Proceedings of the National Academy of Sciences of the United States of America*, **91,** 1261–4.

Wheeler, H.E., Maitland, M.L., Dolan, M.E., Cox, N.J. & Ratain, M.J. (2012) Cancer pharmacogenomics: strategies and challenges. *Nature Reviews Genetics*, **14,** 23–34.

William J. Walsh (2010) Oxidative stress, undermethylation, and epigenetics – The bermuda triangle of autism. *The autism file*, **35**, 30-35.

William, W.N., Heymach, J. V, Kim, E.S. & Lippman, S.M. (2009) Molecular targets for cancer chemoprevention. *Nature reviews. Drug discovery*, **8,** 213–25.

Wilson, A.S., Power, B.E. & Molloy, P.L. (2007) DNA hypomethylation and human diseases. *Biochimica et biophysica acta*, **1775,** 138–62.

Wiseman, D.H., Greystoke, B.F. & Somervaille, T.C.P. (2013) The variety of leukemic stem cells in myeloid malignancy. *Oncogene*, **33,** 3091–8.

Wongpaiboonwattana, W., Tosukhowong, P., Dissayabutra, T., Mutirangura, A. & Boonla, C. (2013) Oxidative stress induces hypomethylation of LINE-1 and hypermethylation of the RUNX3 promoter in a bladder cancer cell line. *Asian Pacific Journal of Cancer Prevention*, **14,** 3773–8.

Woods, B.A. & Levine, R.L. (2015) The role of mutations in epigenetic regulators in myeloid malignancies. *Immunological reviews*, **263,** 22–35.

Wu, Q. & Ni, X. (2015) ROS-Mediated DNA Methylation Pattern Alterations in Carcinogenesis. *Current drug targets*, **16,** 13–9.

Wu, X., Liu, W., Tian, Y., Xiao, M., Wu, Y. & Li, C. (2011) Aberrant methylation of death-associated protein kinase 1 CpG islands in myelodysplastic syndromes. *Acta haematologica*, **125,** 179–85.

Wulfert, M., Küpper, A.C., Tapprich, C., Bottomley, S.S., Bowen, D., Germing, U., Haas, R. & Gattermann, N. (2008) Analysis of mitochondrial DNA in 104 patients with myelodysplastic syndromes. *Experimental hematology*, **36,** 577–86.

Wyche, K.E., Wang, S.S., Griendling, K.K., Dikalov, S.I., Austin, H., Rao, S., Fink, B., Harrison, D.G. & Zafari, A.M. (2004) C242T CYBA polymorphism of the NADPH oxidase is associated with reduced respiratory burst in human neutrophils. *Hypertension*, **43,** 1246–51.

Xiang, G., Zhenkun, F., Shuang, C., Jie, Z., Hua, Z., Wei, J., Da, P. & Dianjun, L. (2010) Association of DNMT1 gene polymorphisms in exons with sporadic infiltrating ductal breast carcinoma among Chinese Han women in the Heilongjiang Province. *Clinical breast cancer*, **10,** 373–7.

Xie, M., Lu, C., Wang, J., McLellan, M.D., Johnson, K.J., Wendl, M.C., McMichael, J.F., Schmidt, H.K., Yellapantula, V., Miller, C.A., Ozenberger, B.A., Welch, J.S., Link, D.C., Walter, M.J., Mardis, E.R., Dipersio, J.F., Chen, F., Wilson, R.K., Ley, T.J. & Ding, L. (2014) Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nature medicine*, **20,** 1472–8.

Xu, Y., Diao, L., Chen, Y., Liu, Y., Wang, C., Ouyang, T., Li, J., Wang, T., Fan, Z., Fan, T., Lin, B., Deng, D., Narod, S.A. & Xie, Y. (2013) Promoter methylation of BRCA1 in triple-negative breast cancer predicts sensitivity to adjuvant chemotherapy. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, **24,** 1498–505.

Xu, Z. & Taylor, J.A. (2009) SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. *Nucleic Acids Research*, **37,** W600–5.

Yamazaki, J., Taby, R., Vasanthakumar, A., Macrae, T., Ostler, K.R., Shen, L., Kantarjian, H.M., Estecio, M.R., Jelinek, J., Godley, L. a. & Issa, J.-P.J. (2012) Effects of TET2 mutations on DNA methylation in chronic myelomonocytic leukemia. *Epigenetics*, **7,** 201–7.

Yan, X.-J., Xu, J., Gu, Z.-H., Pan, C.-M., Lu, G., Shen, Y., Shi, J.-Y., Zhu, Y.-M., Tang, L., Zhang, X.-W., Liang, W.-X., Mi, J.-Q., Song, H.-D., Li, K.-Q., Chen, Z. & Chen, S.-J. (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature genetics*, **43,** 309–15.

Yang, A.S., Estécio, M.R.H., Doshi, K., Kondo, Y., Tajara, E.H. & Issa, J.-P.J. (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic acids research*, **32,** e38.

Yang, J., Manolio, T. a, Pasquale, L.R., Boerwinkle, E., Caporaso, N., Cunningham, J.M., de Andrade, M., Feenstra, B., Feingold, E., Hayes, M.G., Hill, W.G., Landi, M.T., Alonso, A., Lettre, G., Lin, P., Ling, H., Lowe, W., Mathias, R. a, Melbye, M., Pugh, E., *et al*. (2011) Genome partitioning of genetic variation for complex traits using common SNPs. *Nature genetics*, **43,** 519–25.

Yang, X.-X.X., He, X.-Q.Q., Li, F.-X.X., Wu, Y.-S.S., Gao, Y. & Li, M. (2012) Risk-association of DNA methyltransferases polymorphisms with gastric cancer in the Southern Chinese population. *International Journal of Molecular Sciences*, **13,** 8364–78.

Yara, S., Lavoie, J.-C. & Levy, E. (2015) Oxidative stress and DNA methylation regulation in the metabolic syndrome. *Epigenomics*, **7,** 283–300.

Ye, Z.-W., Zhang, J., Townsend, D.M. & Tew, K.D. (2015) Oxidative stress, redox regulation and diseases of cellular differentiation. *Biochimica et biophysica acta*, **1850,** 1607–21.

Yee, S.W., Gong, L., Badagnani, I., Giacomini, K.M., Klein, T.E. & Altman, R.B. (2010) SLC19A1 pharmacogenomics summary. *Pharmacogenetics and genomics*, **20,** 708–15.

Yeh, K.-T., Chang, J.-G., Lin, T.-H., Wang, Y.-F., Tien, N., Chang, J.-Y., Chen, J.-C. & Shih, M.-C. (2003) Epigenetic changes of tumor suppressor genes, P15, P16, VHL and P53 in oral cancer. *Oncology reports*, **10,** 659–63.

Yong, W.P., Innocenti, F. & Ratain, M.J. (2006) The role of pharmacogenetics in cancer therapeutics. *British Journal of Clinical Pharmacology*, **62,** 35–46.

Yoo, N.J., Kim, H.R., Kim, Y.R., An, C.H. & Lee, S.H. (2012) Somatic mutations of the KEAP1 gene in common solid cancers. *Histopathology*, **60,** 943–52.

You, F.M., Huo, N., Gu, Y.Q., Luo, M.-C., Ma, Y., Hane, D., Lazo, G.R., Dvorak, J. & Anderson, O.D. (2008) BatchPrimer3: a high throughput web application for PCR and sequencing primer design. *BMC bioinformatics*, **9,** 253.

Yu, W., Gwinn, M., Clyne, M., Yesupriya, A. & Khoury, M.J. (2008) A navigator for human genome epidemiology. *Nature genetics*, **40,** 124–5.

Yuan, C.M., Douglas-Nikitin, V.K., Ahrens, K.P., Luchetta, G.R., Braylan, R.C. & Yang, L. (2004) DRAQ5-based DNA content analysis of hematolymphoid cell subpopulations discriminated by surface antigens and light scatter properties. *Cytometry. Part B, Clinical cytometry*, **58,** 47–52.

Yuan, H.Y., Chiou, J.J., Tseng, W.H., Liu, C.H., Liu, C.K., Lin, Y.J., Wang, H.H., Yao, A., Chen, Y.T. & Hsu, C.N. (2006) FASTSNP: An always up-to-date and extendable service for SNP function analysis and prioritization. *Nucleic Acids Research*, **34,** 635–41.

Zaidi, S.K., Young, D.W., Montecino, M. a, Lian, J.B., van Wijnen, A.J., Stein, J.L. & Stein, G.S. (2010) Mitotic bookmarking of genes: a novel dimension to epigenetic control. *Nature reviews. Genetics*, **11,** 583–9.

Zhang, D.D. (2010) The Nrf2-Keap1-ARE signaling pathway: The regulation and dual function of Nrf2 in cancer. *Antioxidants & redox signaling*, **13,** 1623–6.

Zhang, G.-L., Liang, Y., Zhu, J.-Y., Jia, Q., Gan, W.-Q., Sun, L.-M. & Hou, H.-M. (2015) Oxidative stress-mediated antiproliferative effects of furan-containing sulfur flavors in human leukemia Jurkat cells. *Food chemistry*, **180,** 1–8.

Zhang, S.-J., Rampal, R., Manshouri, T., Patel, J., Mensah, N., Kayserian, A., Hricik, T., Heguy, A., Hedvat, C., Gönen, M., Kantarjian, H., Levine, R.L., Abdel-Wahab, O. & Verstovsek, S. (2012) Genetic analysis of patients with leukemic transformation of myeloproliferative neoplasms shows recurrent SRSF2 mutations that are associated with adverse outcome. *Blood*, **119,** 4480–5.

Zhang, Y., Zhang, L., Sun, D., Li, Z., Wang, L. & Liu, P. (2011) Genetic polymorphisms of superoxide dismutases, catalase, and glutathione peroxidase in age-related cataract. *Molecular vision*, **17,** 2325–32.

Zhao, Y., Guo, J., Zhang, X., Zhang, Z., Gu, S., Fei, C., Li, X. & Chang, C. (2013) Downregulation of p21 in myelodysplastic syndrome is associated with p73 promoter hypermethylation and indicates poor prognosis. *American Journal of Clinical Pathology*, **140,** 819–27.

Zheng, Q., Zeng, T., Chen, J., Liu, H., Zhang, H. & Su, J. (2013) Association between DNA Methyltransferases 3B Gene Polymorphisms and the Susceptibility to Acute Myeloid Leukemia in Chinese Han Population. *PLoS ONE*, **8,** e74626.

Zhong, S., Tang, M.W., Yeo, W., Liu, C., Lo, Y.M.D. & Johnson, P.J. (2002) Silencing of GSTP1 gene by CpG island DNA hypermethylation in HBV-associated hepatocellular carcinomas. *Clinical cancer research : an official journal of the American Association for Cancer Research*, **8,** 1087–92.

Zhou, F.-L., Zhang, W.-G., Wei, Y.-C., Meng, S., Bai, G.-G., Wang, B.-Y., Yang, H.-Y., Tian, W., Meng, X., Zhang, H. & Chen, S.-P. (2010) Involvement of Oxidative Stress in the Relapse of Acute Myeloid Leukemia. *Journal of Biological Chemistry*, **285,** 15010–5.

Zhou, F., Shen, Q. & Claret, F.X. (2013) Novel roles of reactive oxygen species in the pathogenesis of acute myeloid leukemia. *Journal of leukocyte biology*, **94,** 423–9.

Zhou, T., Chen, P., Gu, J., Bishop, A., Scott, L., Hasty, P. & Rebel, V. (2015) Potential Relationship between Inadequate Response to DNA Damage and Development of Myelodysplastic Syndrome. *International Journal of Molecular Sciences*, **16,** 966–89.

Zhu, J., Du, S., Zhang, J., Wang, Y., Wu, Q. & Ni, J. (2015) Polymorphism of DNA methyltransferase 3B -149C/T and cancer risk: a meta-analysis. *Medical oncology*, **32,** 399.

Ziech, D., Franco, R., Pappa, A. & Panayiotidis, M.I. (2011) Reactive oxygen species (ROS)-induced genetic and epigenetic alterations in human carcinogenesis. *Mutation research*, **711,** 167–73.

Zielonka, J., Vasquez-Vivar, J. & Kalyanaraman, B. (2008) Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nature protocols*, **3,** 8–21.

Supplementary Data

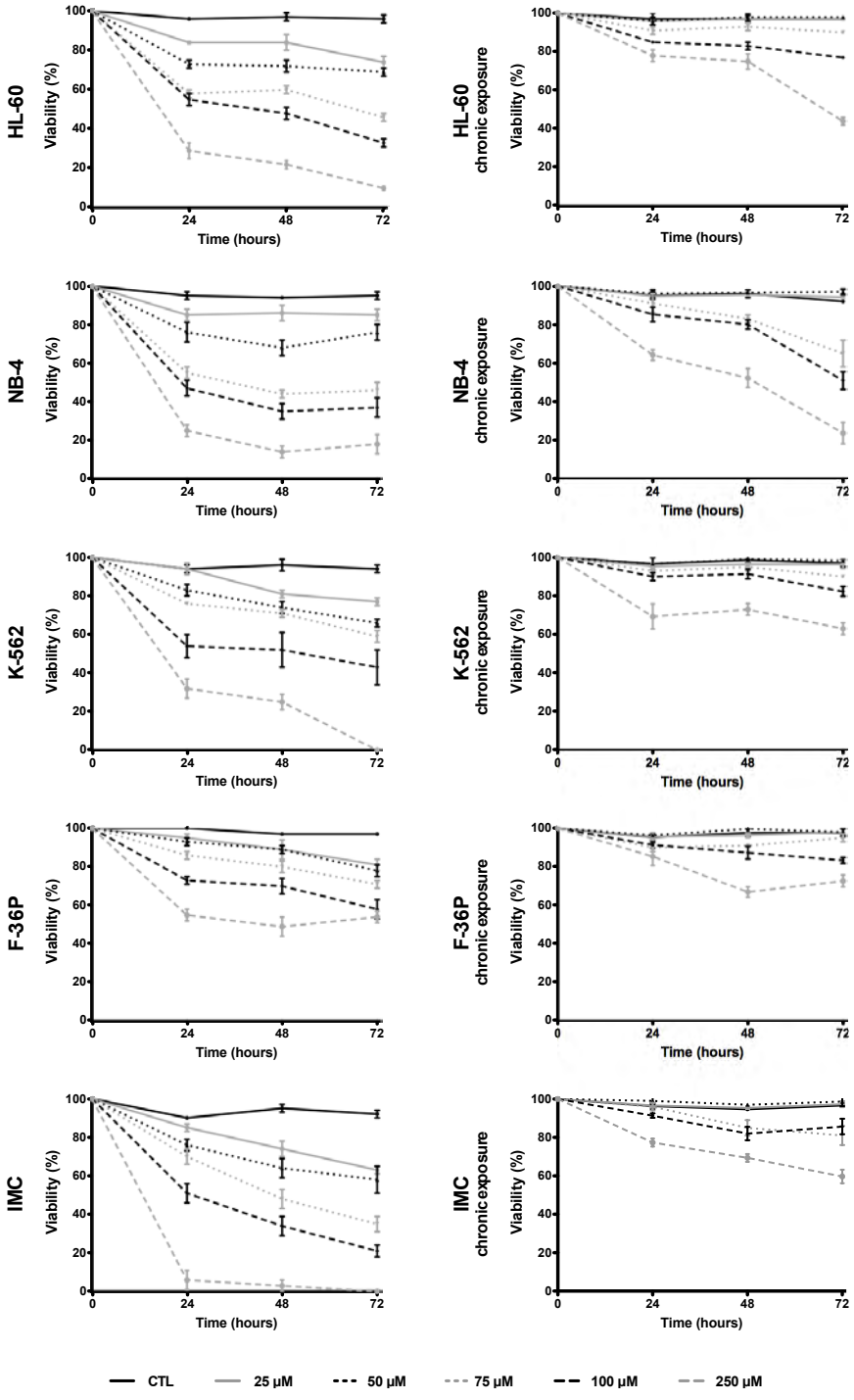
Referent to **Chapter VI:** *Genetic variants involved in oxidative stress, base excision repair, DNA methylation, and folate metabolism pathways influence myeloid neoplasms susceptibility and prognosis*

Table S1. Tetra-primer-AMRS-PCR general conditions.

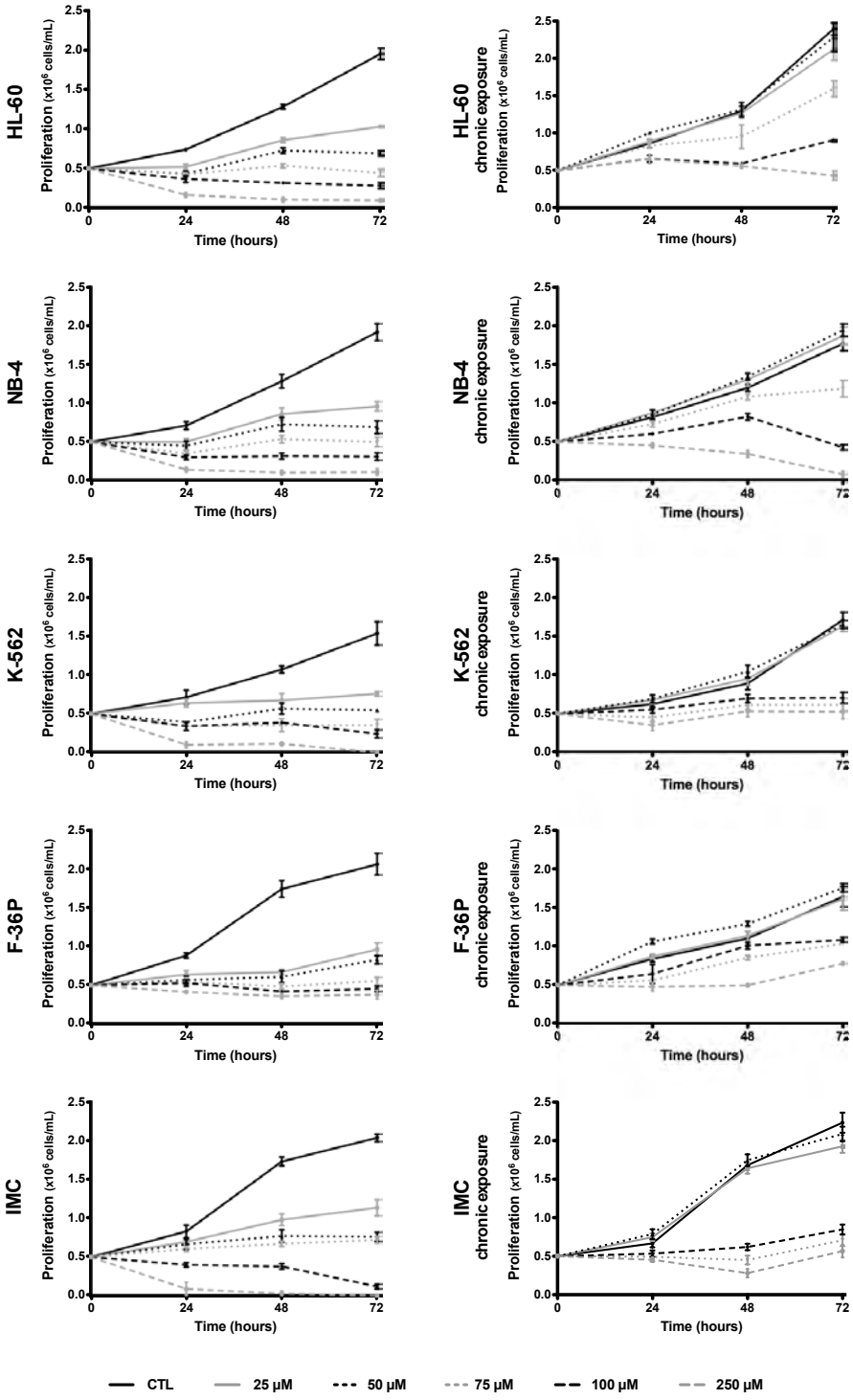
Gene: dbSNP	Primers (5′ →3′)	Ta (PCR cycles)	PCR products
DNMT1: rs759920	FO: TCCTGTGTGGAGGGAAGGAAGAACAAAA	58˚C (10 cycles)	Control: 448 bp
	RO: CTCAACCTCCTGGGCTTAAGCGATCCTC	63˚C (30 cycles)	Allele G: 277 bp
	FI: ATTAATCCCCACCAACCCCAAAACACAG	55˚C (15 cycles)	Allele A: 226 bp
	RI: GTGTGCCTGCCTACTTCTCGGACCTTCT		
DNMT3A: rs2289195	FO: GCACGGGAAGACAGGGTCATCGGGAATA	72˚C[a] (10 cycles)	Control: 189 bp
	RO: CATTCGATCTGGTGATTGGGGGCAGTCC	63˚C (25 cycles)	Allele A: 128 bp
	FI: CCTATGTGCGGAAGCACCAGCTGAGCAA		Allele G: 116 bp
	RI: CGGTAGGTACCATCCTGTCCCCTCCCCC		
DNMT3B: rs2424908	FO: CAAGGTTGAGGGGGTCTCTTTCCAGAAT	58˚C (10 cycles)	Control: 388 bp
	RO: ACTCAGATGTTTGTCAACGCACTGGAAT	63˚C (30 cycles)	Allele T: 259 bp
	FI: CTGCTCCAATGCTGCCCCTCATTATT	55˚C (15 cycles)	Allele C: 183 bp
	RI: GCCCCAAGGAGTGGTCAATGGTAACTAAG		
KEAP1: rs11085735	FO: ACTCTCCAAGGAGCTTAGCTTCATCCTG	68˚C[a] (12 cycles)	Control: 169 bp
	RO: ACCACAACAGTGTGGAGAGGTGAGTG	63˚C (25 cycles)	Allele A: 119 bp
	FI: GGAAGAGGAAACAGCCTCAGGAAGAGTA		Allele C: 103 bp
	RI: AAGTCCCAAGACACTGAGATCCGAGG		
MTRR: rs162036	FO: CAGCGTGATCTGCCCTAACAGTGATTCT	58˚C (10 cycles)	Control: 285 bp
	RO: TACCAATACCAGCGTATGCCTGTGTTCC	63˚C (30 cycles)	Allele A: 204 bp
	FI: CGTCCTTTTGAAAATAAAGGCAGACACCAA	55˚C (15 cycles)	Allele G: 136 bp
	RI: AGCATCAGGGCTGTTACCTTTCTGCC		
NEIL1: rs4462560	FO: CCGATTTGGAAGTTTGTAGCCCTAGCTG	63˚C (15 cycles)	Control: 399 bp
	RO: GCAGCTTGGAGGAAACACTGTTTTTTTG	58˚C (20 cycles)	Allele C: 205 bp
	FI: AATGCAAAAAATTAGCCAGGTGTGGTTGT		Allele G: 250 bp
	RI: CCGAGTAGCTGAGATTACAGGTGCACG		
NFE2L2: rs13001694	FO: TTTAGTTTATCCTTTTGTTACCAATTCC	55˚C (10 cycles)	Control: 294 bp
	RO: GAAATGGCAGAATATTACTTTCTTGTTT	50˚C (30 cycles)	Allele A: 191 bp
	FI: GATCTGGACAAGTCACTCTACCTTCA	47˚C (15 cycles)	Allele G: 157 bp
	RI: AAGGTAGAGTCTGGGTATATTTTATTGAC		
XRCC1: rs1799782	FO: TGTACCTGTCACTCCCCATGGCCTTCTC	63˚C (15 cycles)	Control: 405 bp
	RO: AGGAGTCCCAGCCTCCAGACCTCTCAAC	58˚C (20 cycles)	Allele T: 251 bp
	FI: TGAGGCCGGGGGCTCTCTTCTTCATCT		Allele C: 208 bp
	RI: GCTCACCTGGGGATGTCTTGTTGATACG		

Ta, Annealing temperature; FO, forward outer primer; RO, reverse outer primer; FI, Forward inner primer; RI, reverse inner primer. [a]Touchdown cycling with decrease of 0.5 °C in each cycle

Referent to **Chapter VII:** *Acute and chronic exposure to hydrogen peroxide modulates the methylome of normal and malignant hematological cells*



Supplementary Figure S1. Viability dose-response curves. HL-60, NB-4, K-562, F-36P and IMC cells were treated with 50 µM of hydrogen peroxide (H_2O_2) during 6 months in order to obtain cell lines chronically exposed to H_2O_2. Then, parental and chronic exposure cells were incubated at an initial density of 0.5 $\times 10^6$ cells/ml, during 72 h, in the absence or in the presence of different concentrations of H_2O_2, as indicated in figure. Data are expressed as mean ± SEM obtained from 5 independent experiments.



Supplementary Figure S2. Proliferation dose-response curves. HL-60, NB-4, K-562, F-36P and IMC cells were treated with 50 µM of hydrogen peroxide (H_2O_2) during 6 months in order to obtain cell lines chronically exposed to H_2O_2. Then, parental and chronic exposure cells were incubated at an initial density of 0.5 $\times 10^6$ cells/ml, during 72 h, in the absence or in the presence of different concentrations of H_2O_2, as indicated in figure. Data are expressed as mean ± SEM obtained from 5 independent experiments.

Table S2
Real-time PCR primers

Gene	GenBank Accession	Primer sequences
DNMT1	NM_001130823.1	Forward: 5'-ACCATCAGGCATTCTACCA-3' Reverse: 5'-TCTCCTTGTCTTCTCTGTCAT-3'
DNMT3A	NM_022552.4	Forward: 5'-CGCTAATAACCACGACCAG-3' Reverse: 5'-CGATTCCATCAAAGAGAGACA-3'
DNMT3B	NM_001207055.1	Forward: 5'-ACTTGGTGATTGGCGGAAG-3' Reverse: 5'-GTGAGTAATTCAGCAGGTGGTAA-3'
EZH2	NM_001203247.1	Forward: 5'-CCAAGAGAGCCATCCAGACT-3' Reverse: 5'-GGAGGAGGTAGCAGATGTCAA-3'
MECP2	NM_004992.3	Forward: 5'-AGACATTGTTTCATCCTCCAT-3' Reverse: 5'-AATCCGCTCCGTGTAAAG-3'
MBD1	NM_001204136.1	Forward: 5'-ACGCTCAGACACCTATTAC-3' Reverse: 5'-AAGATGCCTTGTTTGAAGTC-3'
EP300	NM_001429.3	Forward: 5'-AGGAGGAAGACCAGCCAAGTA-3' Reverse: 5'-CATCAGTGCCTGTCGTAGTTCT-3'
TET2	NM_001127208.2	Forward: 5'-AGGTAACTAAGCAAGAGAATCCA-3' Reverse: 5'-GAGAGTAAGAGCCTTATGGTCAA-3'
HDAC1	NM_004964.2	Forward: 5'-GGAAATCTATCGCCCTCACA-3' Reverse: 5'-AACAGGCCATCGAATACTGG-3'
GAPDH	NM_002046.5	Forward: 5'-AGCCACATCGCTCAGACAC-3' Reverse: 5'-GAGGCATTGCTGATGATCTTG-3'
GUSB	NM_000181.3	Forward: 5'-GAAAATACGTGGTTGGAGAGCTCATT-3' Reverse: 5'-CCGAGTGAAGATCCCCTTTTTA-3'

DNMT1, DNA (cytosine-5-)-methyltransferase 1; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3 alpha; DNMT3B, DNA (cytosine-5-)-methyltransferase 3 beta; *EZH2*, enhancer of zeste 2 polycomb repressive complex 2 subunit; *MECP2*, methyl CpG binding protein 2; *MBD1*, methyl-CpG binding domain protein 1; *EP300*, E1A binding protein p300; *TET2*, tet methylcytosine dioxygenase 2; *HDAC1*, histone deacetylase 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GUSB*, glucuronidase, beta.

Table S3
Function and chromosomal localization of studied genes

Symbol	Name	Function	Localization
TP73	Tumor protein p73	Cell cycle control; Apoptosis regulation	1p36.32
CASP8	Caspase 8, apoptosis-related cysteine peptidase	Apoptosis regulation	2q33.1
VHL	von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase	Transcription factor binding; Apoptosis regulation; Cell cycle control	3p25.3
RARB	Retinoic acid receptor, beta	Transcription regulation; Signal transduction	3p24.2
MLH1	MutL protein homolog 1	Cell cycle control; Mismatch repair	3p22.2
RASSF1	RAS association (RalGDS/AF-6) domain family member 1	Cell cycle control; RAS signaling	3p21.31
FHIT	Fragile histidine triad gene	Nucleotide metabolism	3p14.2
APC	Adenomatosis polyposis coli	WNT signaling; Cell adhesion	5q22.2
ESR1	Estrogen receptor 1	Transcription regulation; Signal transduction; Cell growth regulation	6q25.1
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cell cycle control; Apoptosis regulation	9p21.3
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Cell cycle control	9p21.3
DAPK1	Death associated protein kinase 1	Apoptosis regulation	9q21.33
KLLN	Killin, p53-regulated DNA replication inhibitor	Transcription factor	10q23.31
CD44	CD44 molecule	Cell adhesion	11p13
GSTP1	Glutathione S-transferase pi	Apoptosis regulation	11q13.2
ATM	ATM serine/threonine kinase	DNA repair; Cell cycle control	11q22.3
CADM1	Cell adhesion molecule 1	Cell cycle control	11q23.3
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	Cell cycle control	12p13.1
CHFR	Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase	Cell cycle control; Ubiquitination	12q24.33
BRCA2	Breast cancer 2, early onset	DNA repair; Cell cycle control	13q13.1
CDH13	Cadherin 13	Cell adhesion	16q23.3
HIC1	Hypermethylated in cancer 1	Cell cycle control; Transcription regulation	17p13.3
BRCA1	Breast cancer 1, early onset	DNA repair; Cell cycle control	17q21.31
TIMP3	Tissue inhibitor of metalloproteinase 3	Apoptosis regulation; Tyrosine kinase signaling	22q12.3
CTNNB1	Catenin (cadherin-associated protein), beta 1	WNT signaling; Cell adhesion	3p22.1
CASR	Calcium-sensing receptor	Cellular calcium homeostasis	3q21.1
PARK2	Parkin RBR E3 ubiquitin protein ligase	Ubiquitination	6q26
CDK6	Cyclin-dependent kinase 6	Cell cycle control	7q21.2
CELF2	CUGBP, Elav-like family member 2	RNA processing	10p14
CREM	cAMP responsive element modulator	Transcription factor binding	10p11.21
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Apoptosis regulation	12p13.31
CD27	CD27 molecule	Apoptosis regulation	12p13.31
PAH	Phenylalanine hydroxylase	L-phenylalanine catabolism; Amino acid biosynthesis	12q23.2
MLH3	MutL homolog 3	Cell cycle control; Mismatch repair	14q24.3
TSC2	Tuberous sclerosis 2	Cell cycle control	16p13.3
CDH1	Cadherin 1, type 1	Cell cycle control; Cell adhesion	16q22.1
BCL2	B-cell CLL/lymphoma 2	Apoptosis regulation	18q21.33
KLK3	Kallikrein-related peptidase 3	Angiogenesis regulation	19q13.33

Table S4
Cell proliferation and oxidative stress levels of HL-60, NB-4, K-562, F-36P and IMC cells at low (15 – 25) and high passages (6 months cultures)

	Doubling time (h)	ROS (RFU)	GSH (RFU)	ROS/GSH
HL-60				
Low	36 ± 1	25,167 ± 113	215 ± 5	113 ± 2
High	37 ± 3	25,825 ± 209	230 ± 12	111 ± 3
NB-4				
Low	36 ± 2	15,929 ± 610	308 ± 10	53 ± 3
High	35 ± 2	15,002 ± 345	310 ± 14	48 ± 2
K-562				
Low	45 ± 1	54,004 ± 266	757 ± 8	71 ± 1
High	45 ± 3	53,153 ± 291	777 ± 11	69 ± 3
F-36P				
Low	37 ± 2	19,173 ± 358	199 ± 25	112 ± 8
High	38 ± 1	19,992 ± 243	210 ± 11	109 ± 4
IMC				
Low	37 ± 2	9,111 ± 163	392 ± 12	23 ± 1
High	35 ± 1	9,769 ± 115	401 ± 12	23 ± 2

ROS, reactive oxygen species; RFU, relative fluorescence units; GSH, reduced glutathione.