Mariana Sousa Ribeiro

EVALUATION OF THE INTERPLAY BETWEEN POLYMORPHISMS IN ENZYMES FROM ONE-CARBON METABOLISM AND MYELODYSPLASTIC SYNDROMES

Dissertação para a obtenção do grau de Mestre em Biotecnologia Farmacêutica, orientada pela Professora Doutora Ana Bela Sarmento Ribeiro e pelo Professor Doutor João Nuno Moreira e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro de 2015

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UNIVERSIDADE DE COIMBRA

Faculdade de Farmácia

Universidade de Coimbra

Evaluation of the interplay between polymorphisms in enzymes from one-carbon metabolism and Myelodysplastic Syndromes

Avaliação da relação entre polimorfismos em enzimas envolvidas no ciclo do carbono e a Síndrome Mielodisplásica

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pela Professora Doutora Ana Bela Sarmento Ribeiro (Universidade de Coimbra) e pelo Professor Doutor João Nuno Moreira (Universidade de Coimbra) e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Mariana Sousa Ribeiro, 2015

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"When you affirm big, believe big, and pray big, big things happen."

Norman Vincent Peale

Agradecimentos

A realização deste trabalho não seria possível sem o apoio e de várias pessoas a quem gostaria de deixar o meu agradecimento.

Em primeiro lugar, à Professora Doutora Ana Bela Sarmento Ribeiro, pela oportunidade de poder integrar o seu grupo de trabalho, pela orientação neste projeto, pelo apoio e em especial pela confiança.

Ao Professor Doutor João Nuno Moreira, na qualidade de orientador interno pela sua disponibilidade e apoio institucional.

À Doutora Ana Cristina Gonçalves, pelo incentivo à realização deste trabalho, pela disponibilidade e partilha de conhecimentos, pela ajuda, pela paciência infindável e pela motivação durante esta caminhada que nem sempre foi fácil.

Ao Serviço de Hematologia do Centro Hospitalar da Universidade de Coimbra e do Hospital da Figueira da Foz, por generosamente cederem todas as amostras de doentes e informações necessárias para realizar este trabalho.

Às minhas colegas do Laboratório de Biologia Molecular Aplicada da Faculdade de Medicina da Universidade de Coimbra, por toda a ajuda e disponibilidade, pela partilha de conhecimentos, pela vossa amizade, pelo incentivo que sempre souberam dar e por me terem recebido de braços abertos. Obrigada ainda pelos momentos de risadas, pelos cafés mais demorados que o suposto, pelas tardes menos produtivas pelas parvoíces e por terem tornado este percurso mais leve e divertido.

Ao meu Fil, a pessoa mais incrível que Coimbra me deu a conhecer. Por ter sido o meu companheiro de viagem e nunca ter me ter deixado perder o ânimo. Por acreditar incondicionalmente em mim, mesmo quando eu não o faço. Por se multiplicar em quantos sejam precisos para me ajudar. Obrigada pelo teu carinho e amor infindáveis, contigo ao meu lado este caminho foi mais bem fácil.

À Quantunna, por me ter dado uma segunda família em Coimbra. Pelos momentos incríveis que me proporcionou e pelas amizades improváveis que me trouxe. Pelas noites mal dormidas, pelos ensaios fora de horas e pelas noitadas de boémia sem conta. Por ser a prova de que até mesmo as pessoas mais diferentes podem partilhar as mesmas paixões. A todos

aqueles que partilharam comigo esta experiência, obrigada pelo companheirismo e por me fazerem acreditar que, quando todos reunimos forças no mesmo sentido, somos capazes de coisas incríveis.

A Coimbra, a cidade dos Estudantes, que nestes 5 anos foi a minha casa e me viu crescer. A partir de agora, será sempre a minha casa, porque amores assim não acontecem facilmente. Obrigada Coimbra, por cada dia me lembrares daquilo que eu sempre disse: não podia ter sido noutro sítio.

Por fim, um agradecimento especial à minha família,

Aos meus pais, os meus heróis de todos os dias, por terem feito tudo quanto podiam para que eu pudesse chegar aqui. Pelo amor sem medida, pelo apoio constante, por acreditarem sempre em mim e nunca deixarem que eu perca a força. Por estarem sempre presentes, mesmo quando a distância assim não o permite. Por serem o meu exemplo de vida e por tornarem isto possível. Nunca haverão palavras para agradecer o suficiente.

Aos meus irmãos, os meus pequeninos, pelo abraço apertado quando chego a casa, pelas brincadeiras e até pelas birrinhas, pela cumplicidade e pelo amor incondicional. Por todas as vezes que me deixam o coração apertadinho quando me perguntam "Quando vens?". Porque isto sem irmãos não tinha piada nenhuma.

À minha avó, pelo carinho, pelo sorriso que mostra sempre que estou por perto, por ter sempre tomado conta de mim e ter contribuído grandemente para que eu, hoje, seja a pessoa que sou. À minha madrinha, pela amizade e apoio sem fim e por celebrar as minhas conquistas como se dela fossem. À Rosita e à Raquel, por terem sido uma presença constante no meu crescimento, pela amizade e pelos bons momentos em família.

A todos, o meu muito obrigada!

Resumo

O cancro é uma das maiores causas de morte a nível mundial, prevendo-se que o número de novos casos supere os 24 milhões nas próximas duas décadas. O processo pelo qual as células adquirem propriedades cancerígenas é atualmente considerado como sendo dependente da aquisição de mutações, cujas consequências se traduzem em danos no genoma.

O tipo de mutação mais comum é o que envolve a substituição de um nucleótido que, quando tem apresenta uma frequência de pelo menos 1% na população, é denominado polimorfismo de nucleótido único (SNP, do inglês *single nucleotide polymorphism*). Dependendo da região em que ocorre e da alteração funcional ao qual pode estar associado, o SNP pode conferir tanto um efeito protector como um factor de risco aumentado para o desenvolvimento de doença.

A síndrome mielodisplásica (SMD) é um conjunto de doenças relacionadas com o sistema hematopoiético, caracterizada por uma hematopoiese ineficaz, displasias, e ainda uma susceptibilidade acrescida para o desenvolvimento de leucemia mieloide aguda (LMA).

A transcrição de genes supressores tumorais, como é o caso do *P15* e do *P16*, pode ser inactivada pela metilação aberrante da região promotora, nomeadamente das ilhas CpG, o que tem adquirido especial interesse em neoplasias hematológicas, em particular na SMD. Nutrientes provenientes da dieta, como é o caso do folato e da vitamina B_{12} podem influenciar a produção de grupos metil e, consequentemente, o processo de metilação. Para além disso, a SMD parece estar relacionada com SNPs em enzimas que regulam o ciclo do carbono, o mecanismo responsável tanto pela metilação do DNA como pela síntese de nucleótidos.

Uma vez que a hipometilação global e a hipermetilação localizada do DNA são características de tumores humanos, incluindo a SMD, e o suprimento de folato está associado a estas características, foi colocada a hipótese de que polimorfismos em genes que intervêm neste ciclo, nomeadamente os genes *MTR*, *SCL19A1*, *MTRR* e *CBS*, podem afectar o estado de metilação do DNA e, assim, influenciar não só o desenvolvimento de SMD, como a progressão da doença para LMA.

Neste sentido, o principal objectivo deste trabalho foi a avaliação da relação entre os polimorfismos genéticos associados aos genes *MTR* (rs1805087 e rs2229276), *SLC19A1* (rs1051266), *MTRR* (rs162036) e *CBS* (844ins68), que regulam o ciclo do carbono, e o risco associado ao desenvolvimento de SMD. Analisamos ainda a influência das variantes

polimórficas nos níveis de folato e vitamina B_{12}, bem como o seu papel no grau de metilação das sequências LINE-1 e dos genes *P15* e *P16*.

Para isto, foram genotipadas as amostras de 77 doentes e 80 controlos não neoplásicos, para cada um dos genes em estudo. Os genótipos associados aos genes *MTR* e *MTRR* foram obtidos através da técnica ARMS-PCR, tendo sido utilizada a técnica de RFLP-PCR para a análise do gene *SLC19A1* e o PCR convencional para o gene *CBS*. O grau de metilação da sequência LINE-1 foi avaliado por ensaios COBRA e a frequência de metilação dos genes *P15* e *P16* foi obtida pela realização da técnica MS-PCR.

Posteriormente, foi avaliada a força de associação entre as variantes polimórficas e o risco de desenvolvimento de SMD, através do cálculo do risco relativo por análise de regressão logística. Avaliou-se ainda a relação entre os polimorfismos genéticos, a progressão da doença para LMA e a sobrevivência global dos doentes, com recurso a regressão logística e análise de Kaplan-Meier. De seguida, analisou-se a possibilidade de o risco para o desenvolvimento de SMD ser influenciado pelos perfis génicos ou haplotipos. Por fim, o teste exacto de Fisher foi usado ara avaliar relação entre as variantes polimórficas e o grau de metilação dos genes *P15* e *P16* e a sequência LINE-1.

Da avaliação dos polimorfismos no risco de desenvolvimento da doença, observámos que o genótipo GG do variante rs2229276 associada ao gene *MTR* pode constituir um factor protector para o desenvolvimento de SMD (OR=0.29) e que a presença do alelo G parece estar relacionada com uma menor predisposição para a doença (OR=0.33). No que diz respeito à associação entre as variantes polimórficas e a presença ou ausência de alterações citogenéticas, o genótipo heterozigótico (WT-Ins) do gene CBS (844ins68) mostrou-se relevante em indivíduos com cariótipo normal para as alterações mais comuns na SMD (OR=0.22). Pela análise das curvas de Kaplan-Meier, observou-se que os indivíduos portadores do genótipo AG para o polimorfismo rs162036 do gene *MTRR* apresentam um aumento da progressão da doença para LMA, bem como uma tendência, embora sem significado estatístico, para uma diminuição da sobrevivência dos doentes. Para além disso, a avaliação dos níveis de vitamina B12 parece estar relacionada com o genótipo GG do gene *MTR* (rs2229276). Em relação ao polimorfismo rs1051266 relativo ao transportador *SLC19A1*, este parece influenciar o estado de metilação do gene *P16* nos doentes com SMD (OR=3.80).

Os resultados deste estudo permitem-nos concluir que a presença de variantes polimórficas desempenha um papel importante na SMD e realçam a importância do estudo de genes envolvidos no ciclo do carbono na patogénese desta doença.

Palavras chave: Sindrome Mielodisplásica; Polimorfismos; Metabolismo de um carbono; Epigenética; Metilação; Vitamina B12

Abstract

Cancer is a leading cause of death worldwide, predicted to rise more than 24 million new cases globally in the next two decades. The process by which normal cells become progressively transformed into malignant derivatives is now known to require the acquisition of mutations, which arise as a consequence of damage to the genome.

The most common mutational change is associated with the substitution of a single nucleotide, called single nucleotide polymorphism (SNP) when its frequency on the population is, at least, of 1%. Depending on the region where it occurs and the functional changes that it may cause, a SNP may confer a protective effect or modulate an increased susceptibility to disease development.

Myelodysplastic Syndrome (MDS) is a family of clonal hematopoietic stem cells disorders that involves a multistep pathogenesis characterized by dysplasia, innefective hematopoiesis and susceptibility to transformation into Acute Myeloid Leukemia (AML).

Transcriptional inactivation of tumor suppressor genes, such as *P15* and *P16,* by promoter CpG island hypermethylation has been subject of interest in haematological malignancies, namely in MDS. Dietary factors such as folate and vitamin B_{12} could influence the supply of methyl groups and, therefore the biochemical pathways of methylation processes. Moreover, MDS is thought to be associated with SNPs in enzymes from one-carbon metabolism, the pathway that is responsible for DNA methylation and nucleotide synthesis.

Since global hypermethylation and targeted hypomethylation are considered defining characteristics of human tumours, including MDS, and folate depletion in certain human cells lines can result in global hypomethylation and also targeted hypermethylation, we hypothesized that genetic variability in 1-carbon metabolism, namely in *MTR*, *SLC19A1*, *MTRR* and *CBS* genes, can affect the methylation status and contribute, not only to MDS development, but also to progression to AML.

In this context, our major goal was to evaluate the interplay between genetic polymorphisms in enzymes involved in the folate mechanism, namely *MTR* rs1805087 and rs2229276, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68, and the risk of developing MDS. Furthermore, we assessed the influence of these genetic polymorphisms in folate and vitamin B_{12} levels in MDS patients, as well as the role of gene variants in the degree of *P15*, *P16* and LINE-1 methylation. To this end, we genotyped 77 MDS patients and 80 healthy

controls, assessing ARMS-PCR for *MTR* and *MTRR* genes, RFLP-PCR for determining the variants for *SLC19A1*, and conventional PCR for detecting *CBS* variants. *P15* and *P16* methylation status were obtained by MS-PCR, while LINE-1 level of methylation was performed by COBRA assay.

Posteriorly, we evaluated the strength of association between polymorphic variants and the development of MDS by calculating the associated odds ratio, through logistic regression analysis. We also evaluated the relation between genetic profiles, evolution to AML and overall survival in MDS patients, by logistic regression and Kaplan-Meier curves analysis. Next, we investigated the influence of genetic profiles and haplotypes on the risk of MDS development. The influence of genetic polymorphisms on *P15*, *P16* and LINE-1 methylation was performed using Fisher's exact test.

Concerning odds ratio for risk disease, our main results revealed that AG genotype from *MTR* rs2229276 is a protector factor for the development of MDS, presenting a 0.29-fold decreased associated risk. Adittionally, the G allele also proved to have a protector effect on MDS, with a 0.33-fold decreased risk on disease development. In relation to cytogenetic alterations, the *CBS* 844ins68 WT-Ins genotype was found to be significantly associated with the absence of the most common cytogenetic abnormalities in MDS (OR=0.22). Subsequently, the analysis of Kaplan-Meier curves showed that heterozygous (AG) individuals for *MTRR* rs162036 not only presented a significant increase to AML evolution (p=0.049), but also show a tendency to be associated with a worse overall survival. In relation to folate and vitamin B_{12} levels, only vitamin B_{12} proved to be influenced by polymorphisms, in this case, by GG genotype from *MTR* rs2229276 variant (p=0.039). The odds ratio relating *P15* and *P16* methylation and genetic variants showed that GG genotype from *SLC19A1* rs1051266 negatively influences *P16* methylation in MDS patients (OR=3.80).

The results of this study allow us to accomplish that polymorphisms play important roles in MDS and highlight the importance of the study of one-carbon metabolism in the pathogenesis of the disease.

Keywords: Myelodysplastic Syndromes; Polymorphisms; One-carbon metabolism enzymes; Epigenetics; Methylation; Vitamin B_{12}

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Abbreviations

5-methyl THF - 5-methyltetrahydrofolate

A - Adenine

ABC - Adenosine Triphosphate Binding Cassette

a-KG - a-Ketoglutarate

ALL - Acute Lymphocytic Leukemia

AML - Acute Myeloid Leukemia

ARMS-PCR - Amplification-Refractory Mutation System-Polymerase Chain Reaction

BM - Bone Marrow

C- Cytosine

CBS - Cystathionine β-synthase

CGH - Comparative Genomic Hybridization

CLP - Common Lymphoid Progenitor

CML - Common Myeloid Progenitor

COBRA - Combined Bisulfite Restriction Analysis

CpG - Cytosine-phosphate-Guanine Dinucleotide

CSC - Cancer Stem Cell

DNA - Deoxyribonucleic Acid

DNMT - DNA Methyl Transferase

dTMP - Deoxythymidine Monophosphate

dUMP - Deoxyuridine Monophosphate

FAB - French-American-British Cooperative Group

G - Guanine

HAT - Histone Acetyltransferase

HCS - Hematopoietic Stem Cell

HDAC - Histone Acetyl Deacetylases

IDH - Isocitrate Dehydrogenase

IGF2 - Insulin Growth Factor-2

IPSS - International Prognostic Scoring System

LINE-1 - Long-Interspersed Elements

MAF - Minor Allele Frequency

MDAS - M.D. Anderson Scoring System

MDR - Multiple Drug Resistance

MDS - Myelodysplastic Syndromes

MDS-U - Myelodysplastic Syndrome Unclassifiable

miRNA - micro Ribonucleic Acid

MMP - Multipotent Progenitor

mRNA - Messenger Ribonucleic Acid

MS-PCR - Methylation-Specific Polymerase Chain Reaction

MTHFR - Methylenetetrahydrofolate Reductase

MTR - Methionine Synthase

MTRR - Methionine Synthase Reductase

NK - Natural Killer cells

OR - Odds Ratio

OS - Overall Survival

PTEN - Phosphatase and Tensin Homolog

RA - Refractory Anemia

RAEB-1- Refractory Anemia with Excess Blasts type-1

RAEB-2 - Refractory Anemia with Excess Blasts type-2

RARS - Refractory Anemia with Ringed Sideroblasts

RCMD - Refractory Cytopenia with Multilineage Dysplasia

RCUD - Refractory Cytopenia with Unilineage Dysplasia

RFC; SLC19A1 - Reduced Folate Carrier

RFLP-PCR - Restriction Fragment Length Polymorphism-Polymerase Chain Reaction

R-IPSS - Revised International Prognostic Scoring System

RN - Refractory Neutropenia

RNA - Ribonucleic Acid

RT - Refractory Thrombocytopenia

SAH - S-adenosylhomocysteine

SAM - S-adenosylmethionine

SLC - Solute Carrier

SNP - Single Nucleotide Polymorphism

SNP-A - Single-Nucleotide Polymorphism Microarrays

T - Thymine

TET2 - Tet Methylcytosine Dioxygenase 2

THF - Tetrahydrofolate

TNFα - Tumor Necrosis Factor α

UTR - Untranslated Region

WHO - World Health Organization

WPSS - WHO-based Prognostic Scoring System

INTRODUCTION

1.1. Cancer

The human body is regulated by highly controlled mechanisms that assure the cells' homeostasis, defined as the internal state necessary for establishing systemic equilibrium and maintaining stability (Sokolosky & Wargovich, 2012). The maintenance of cell number homeostasis in normal tissues reflects a strictly adjusted balance between the rates of cell proliferation and cell death. Under pathologic conditions such as exposure to cytotoxic, genotoxic, or nongenotoxic agents, an imbalance in these rates may indicate subsequent risk of carcinogenesis and is, therefore, an underlying factor in the pathogenesis of numerous human disease states, including cancer (James *et al.*, 1998).

Cancer is a complex progressive multistep disorder that results from the accumulation of genetic and epigenetic abnormalities that drive the transformation of normal cells into malignant derivatives. This process shapes each tumor in such a dynamic and unique way that it is extremely difficult to determine the alterations that cause, maintain, and spread the disease (Pavet *et al.*, 2011).

Over the past years, cancer is becoming more recognized as a heterogeneous disease with hierarchies of cellular populations that demonstrate a range of differentiation phenotypes, resulting in intraclonal diversity. The majority of cells in bulk tumors may be non-tumorigenic mature cells, and only a small subpopulation of cells within tumors is responsible for tumor initiation, growth, and recurrence. These are called "cancer stem cells" (CSCs) (Wang *et al.*, 2013) and the model that proposes them to be in the origin, progression, metastasis, and recurrence of cancer is known as CSC theory (Figure 1). It suggests that the heterogeneity and hierarchy between all of the cells within a tumor result from the asymmetric division of CSCs and that tumors are highly hierarchical with a unique self-renewing population of cells at the top of the hierarchy. All other cells comprising the tumor bulk are derived from differentiated CSCs (Chen, Huang & Chen, 2013).

Contrary to normal stem cells, which are remarkable for their highly controlled proliferation and maintenance of their genomic integrity, CSCs are often distinguished by their lack of control of such processes. However, their properties are similar to a normal stem cell, and in terms of self-renewal (which enables a stem cell to produce another stem cell with essentially the same development and replication potential) and multipotency for differentiation (which evolves the production of daughter cells that become tissue-specific

specialized cells), CSCs are very much analogous to normal adult stem cells (Allegra *et al.*, 2014).

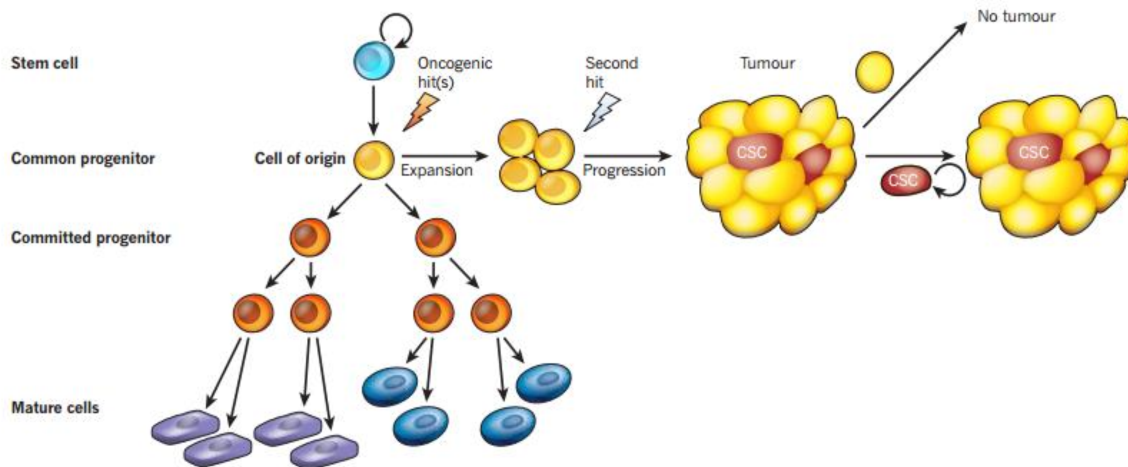

Figure 1. The cell of origin and evolution of a cancer stem cell. Normal cellular hierarchy comprising stem cells that progressively generate common and more restricted progenitor cells, yielding all the mature cell types that constitute a particular tissue. Although the cell of origin for a particular tumour could be an early precursor cell such as a common progenitor, the accumulation of further epigenetic mutations by a cell within the aberrant population (in this case expanded) during neoplastic progression may result in the emergence of a CSC (adapted from Visvader, 2011).

Several signalling pathways are involved in the self-renewal behavior of CSCs, including Wnt/-catenin, Notch, and Hedgehog signalling, which mediate the resistances against radiotherapy and chemotherapy. Despite the moderate success of currently available therapeutic approaches to tumors, they have several limitations. One of the main therapy drawbacks is that there is insufficient elimination of CSCs, which are later capable of driving tumor recurrence and regrowth. Further, frequently there is multiple drug resistance (MDR) with advanced tumors. Surviving CSCs will lead to tumor recurrence (Wang *et al.*, 2013).

The CSC model suggests that inhibiting CSC renewal or promoting their differentiation should induce tumor regression. Drugs could impair CSC self-renewal, induce specific cell death, their differentiation, or target their niche. All of these strategies would lead to the depletion of the pool of CSCs and subsequent tumor regression. However, if the CSC potential is reversible, or if newly acquired mutations confer resistance to therapy, then tumor regression would only be transient, leading to cancer relapse (Beck & Blanpain, 2013).

By prospectively identifying and characterizing cancer stem cells it might be possible to identify more effective therapies (Figure 2). The identification and characterization of cancer

stem cells should therefore also lead to diagnostic methods that can distinguish between disseminated tumorigenic and non-tumorigenic cells, as well as provide a better understanding of the mechanisms that regulate migration of cancer stem cells (Pardal, Clarke & Morrison, 2003).

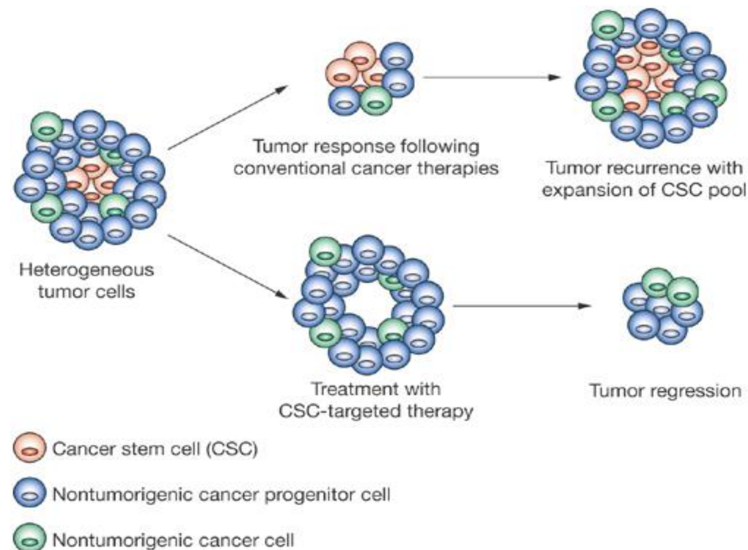

Figure 2. Therapeutic implications of CSCs. Inherent within the CSCs hypothesis is the assumption that current treatments for cancer can considerably diminish tumor burden, but have a decreased effect on CSCs,. Nontumorigenic cancer progenitor cells are capable of cell division, but their capability to divide is limited, and they are unable to match the rates of tumor cell apoptosis and senescence. Therefore, to achieve cancer remission or cure, it will be necessary to develop novel therapies that are cytotoxic to CSCs (adapted from Das, Srikanth & Kessler, 2008).

1.2. The hematopoietic system

Blood cells are produced throughout life. In mammals, hematopoiesis is defined by the production of hematopoietic blood cells through the expansion and differentiation of hematopoietic stem cells (HSCs) and pogenitors. This process occurs mainly in the bone marrow (BM) in the abluminal side of sinusoids that invade the bone cavity made by osteoclasts shortly after calcium deposits in the cartilaginous matrix (Cumano & Godin, 2009). During prenatal life the major phases of hematopoiesis occur in the yolk sac, liver, bone marrow, and thymus. The major postnatal hematopoietic organs are red bone marrow, lymph nodes, spleen, and thymus (Fenaux *et al.*, 2014a).

It has been long known that BM cells can regenerate the blood compartment. Thus, BM transplantation was among the first cell replacement therapeutic approaches attempted. Blood cells comprise multiple cell types and have accordingly variable life spans that go from

a few days to several years (Cumano & Godin, 2009). Those that are short-lived, need to be replenished continuously; the average human requires approximately one hundred billion of new hematopoietic cells each day. The continued production of these cells depends directly on the presence of HSCs, the ultimate, and only, source of all blood cells (Domen, Wagers & Weissman, 2006).

Hematopoietic stem cells are generally quiescent and mediate ongoing blood cell generation over the lifetime of the organism through their sustained ability to self-renew, allowing the maintenance of the HSC pool, and the capacity to differentiate and give rise to a differentiated progeny comprising different cell types (Cumano & Godin, 2009 & Beerman *et al.*, 2010) (Figure 3). Hematopoiesis begins with the HSC giving rise to another HSC by self-renewing and to multipotent progenitors (MPPs), which lose self-renewal potential but remain fully differentiate into all multilineages. MPPs further give rise to oligopotent progenitors that are common lymphoid and myeloid progenitors (CLPs and CMPs, respectively). All these oligopotent progenitors differentiate into their restricted lineage commitment: CLPs give rise to T and B cell progenitors, and Natural Killer cells (NK) progenitors, while CMPs advance to megakaryocyte cells (which develop to trombocytes), erythrocytes, and myeloblast progenitors (which origin monocytes and granulocytes) (Mosaad, 2014).

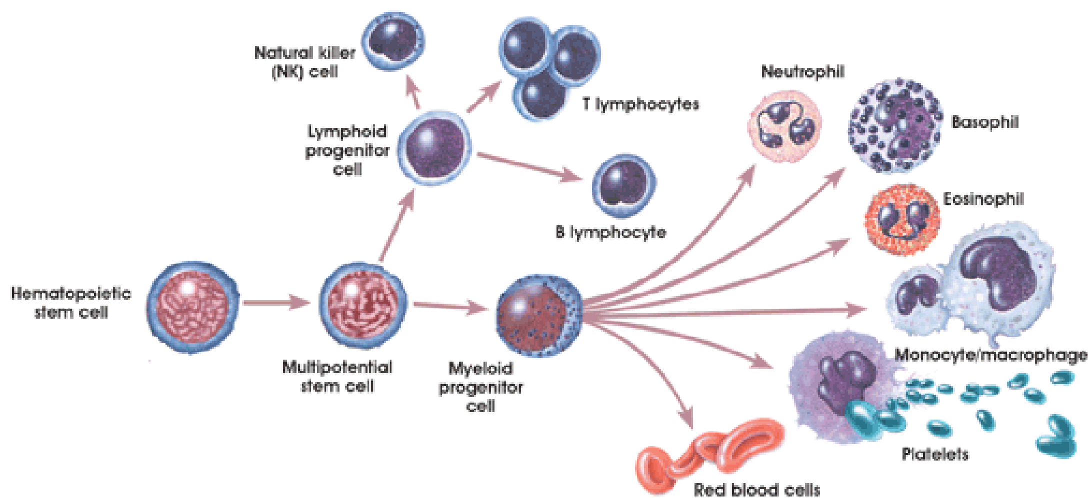

Figure 3. Hematopoietic Cell Differentiation. HSCs can self-renewal and also give rise to different progenitor cells, which differentiate in all the types of blood cells (adapted from http://stemcells.nih.gov/info/basics/pages/basics4.aspx).

The hematopoietic stem cell (HSC) niche commonly refers to the pairing of hematopoietic and mesenchymal cell populations regulating HSC self-renewal, differentiation and proliferation. Anatomical localization of the niche is a dynamic unit from

the developmental stage, allowing proliferating HSCs to expand before they reach the bone marrow where they adopt a quiescent phenotype that protects HSCs' integrity and functions (Boulais e Frenette, 2015).

1.3. Myelodysplastic Syndromes

1.3.1. Pathophisiology and Clinical Presentation

Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders characterized by ineffective hematopoiesis, leading to impaired peripheral blood cell production (cytopenias), and most commonly a hypercellular, dysplastic-appearing bone marrow, which is often accompanied by hyperplasia and enhanced apoptosis (Nimer, 2008; Nikoloski, Van Der Reijden & Jansen, 2012).

The pathophysiology of MDS is a multistep process involving cytogenetic changes and/or gene mutations and wide spread gene hypermethylation at advanced stages, progressing to acute myeloid leukemia (AML) in one third of the cases. (Fenaux *et al.*, 2014b)**.** Signs and symptoms of anemia, accompanied by infectious or bleeding complications, predominate in MDS, with some patients having systemic symptoms or features of autoimmunity, perhaps indicative of the pathogenesis of their disease (Steensma, 2012).

Several reports had described an MDS-like syndrome in the first half of the 20th century, usually considering them as a "refractory anemia" that preceded the onset of AML and by the 1970s the term "preleukemia" was often applied to most patients with this disorder. The 1976 French-American-British Cooperative Group (FAB) emphasized that not all patients with MDS progress to AML but often succumb to complications of ineffective hematopoiesis and bone marrow failure. The "preleukemia" terminology then faded away, and the term "myelodysplastic syndromes" became widely accepted for this disorder in 1982 (Vardiman, 2012).

MDS can present varying degrees of peripheral blood abnormalities. The most common is anemia (typically macrocytic), occurring in approximately 80% to 85% of patients. Thrombocytopenia occurs in around 30% to 45% of MDS cases, with approximately 40% of patients found to have neutropenia at diagnosis. Up to 53% of patients with MDS experience bleeding and up to 25% experience serious bleeding events during the course of disease. The

clinical manifestation of MDS is nonspecific and highly variable, depending on the MDS subtype, and it ranges from indolent to life threatening (Foran & Shammo, 2012).

There is increasing evidence that haploinsufficiency, epigenetic changes, and abnormalities in cytokines, the immune system, and bone marrow stroma all contribute to the development of the myelodysplastic syndromes (Tefferi & Vardiman, 2009). This heterogeneity of MDS supports the idea that different therapeutic approaches should take into account the diverse morphologic and clinical presentations of MDS patients rather than a restricted therapeutic strategy (Visconte *et al.*, 2014).

1.3.2. Ethiology and Risk Factors

Human cancer incidence increases exponentially with advancing age, and thus the greatest risk factor for cancer is aging (Holmes *et al.*, 1991). MDS is no exception, as the existing data consistently suggest that MDS is predominantly a disease of the elderly (Figure 4). Approximately 86% of patients are aged ≥60 years at the time of their diagnosis (median age, 76 years), and only 6% of cases diagnosed are aged ≤50 years. Men have a higher incidence rate than women by a factor of approximately 1.8 (Tefferi & Vardiman, 2009), and white individuals have a higher incidence rate than other racial/ethnic groups. (Ma, 2012). In developed countries, the annual incidence of MDS is approximately 4-5 cases per 100,000 people (Strom, Vélez-Bravo & Estey, 2008), which increases to 30 or more cases per 100 000 in individuals older than 70 years.

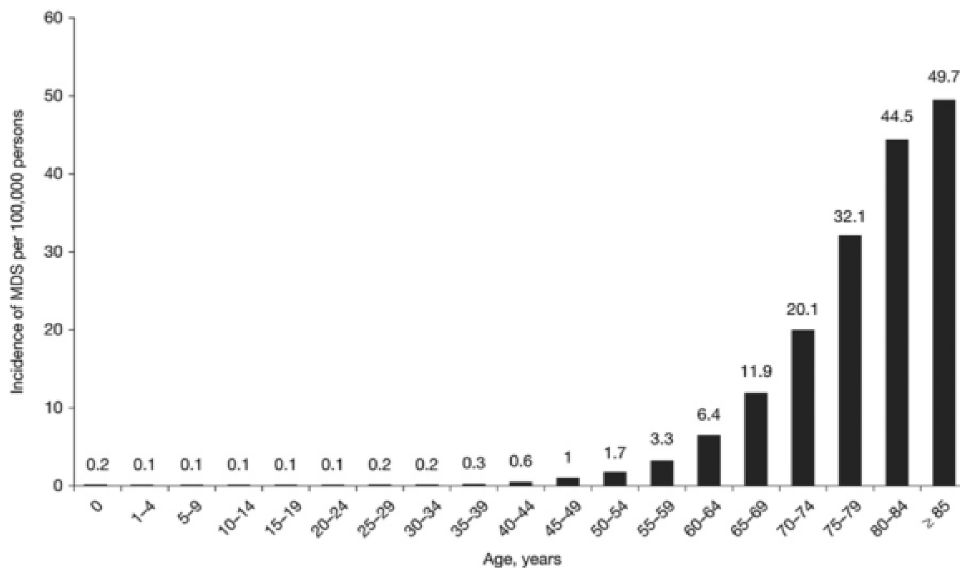

Figure 4. Incidence rate of myelodysplastic syndromes (MDS) in different age groups in the United States (2001 to 2008) (adapted from Ma, 2012).

Patients with MDS who have a history of cancer treatment are considered to have "secondary" or "therapy- related" MDS, and they tend to have a much poorer prognosis (Ma, 2012). The chemotherapy associated with the development of MDS includes particularly alkylating agents or topoisomerase inhibitors, and is followed by radiation therapy. The typical latency period for secondary MDS after exposure to alkylating agents or radiation therapy is 5 to 10 years, and the risk appears to be dose dependent. MDS following exposure to topoisomerase inhibitors is much less common, with a latency period of approximately 2 years (Sekeres, 2010). The combination of chemotherapy and radiotherapy increases the risk of secondary leukemia, which is also influenced by the dose and the schedules of the drug administration (Leone *et al.*, 2007).

One of the more common established risk factors to *de novo* MDS, in addition to age and male gender, is environmental and occupational exposure to organic solvents, such as benzene and its derivatives (Sekeres, 2010), which is related with the smoke from cigarettes. (Ma, 2012). Aspects related with lifestyle, like the diet or the use of hair dyers, may also represent a risk factor for the development of MDS. (Leone *et al.*, 2007)

The relationship between alcohol intake and MDS development has also been studied by a variety of investigation groups, but the results are not consentient and its role in this context is not clearly defined. (Ma, 2012). Moreover, the risk of both MDS and AML is also increased in certain genetic syndromes: the Diamond–Blackfan syndrome, the Shwachman–

Diamond syndrome, dyskeratosis congenita, Fanconi's anemia and severe congenital neutropenia (Tefferi & Vardiman, 2009). In this field, more research has to be done, to contribute to the development of preventive measures to reduce disease occurrence and to treat patients more effectively in order to reduce mortality and improve quality of life.

1.3.3. Classification

Throughout the years, various classification systems have been developed to describe MDS. Thus, the terminology to classify it has similarly evolved to adjust to the increasing knowledge. As a result, several classification systems are currently used for the diagnosis of MDS (Foran & Shammo, 2012).

The FAB classification was the classification of the disease based on observations and understanding of the disease at that time. In order to address some of its limitations, the World Health Organization (WHO) proposed, in 1999, a newer classification system. The principle of the WHO classification was to use all available information – clinical, morphologic, and other biologically significant information – to define specific disease entities of clinical significance (Vardiman, 2012).

In 2008, a new edition of the WHO classification of MDS was published, including some changes from the previous edition. As important aspects, this new classification recognized seven distinct pathologic subtypes of MDS that were based on these morphologic features, the percentage of bone marrow cells that are blasts, and the number of affected hematopoietic lineages (Lindsley & Ebert, 2013). A comparison between the different subtypes is presented in Table 1.

Table 1. Criteria for myelodysplastic syndrome according to the World Health Organization (WHO) classification (adapted from Vardiman, 2012).

Disease entity	Blood findings	Bone marrow findings
Refractory cytopenia with unilineage dysplasia (RCUD): *Refractory anemia (RA),* *Refractory neutropenia (RN),* *Refractory thrombocytopenia (RT)*	• Unicytopenia or bicytopeniaa • No or rare blasts (<1%)	• Unilineage dysplasia: ≥10% of the cells in one myeloid lineage • <5% blasts • 15% of erythroid precursors are ring sideroblasts
Refractory anemia with ringed sideroblasts (RARS)	• Anemia • No blasts	• ≥15% of the erythroid precursors are ring sideroblasts • Dyserythropoiesis only • <5% blasts
Refractory anemia with multilineage dysplasia (RCMD)	• Cytopenia(s), no or rare blasts • No Auer rods • $<1\times10^9$/L monocytes	• Dysplasia in >10% of cells in 2 or more lineages • <5% blasts in marrow • No Auer rods • $<1\times10^9$/L monocytes
Refractory anemia with excess blasts-1 (RAEB-1)	• Cytopenia(s) • <5% blasts • No Auer rods • $<1\times10^9$/L monocytes	• Unilineage or multilineage dysplasia • 5–9% blasts • No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	• Cytopenia(s) • 5–19% blasts • Auer rods ± • $<1\times10^9$/L monocytes	• Unilineage or multilineage dysplasia • 0–19% blasts • Auer rods ±
MDS, unclassifiable (MDS-U)	• Cytopenias • <1% blasts	• Unequivocal dysplasia in less than 10% of cells in one or more myeloid lines when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS • <5% blasts
MDS associated with isolated del(5q)	• Anemia • Usually normal to elevated platelets No or rare blasts	• Normal to increased megakaryocytes with hypolobated nuclei <5% blasts del(5q) is the sole cytogenetic abnormality • No Auer rods

1.3.4. Prognosis

The current paradigm of MDS's management begins by assessment of disease risk to direct the goals of treatment. Risk stratification has emerged as an indispensable step in management decision-making. It provides prognostic information for the patients and allows physicians to tailor therapy according to the risk of the disease (Komrokji *et al.*, 2013).

Prognostic stratification is, like in other diseases, crucial in MDS. The most widely used prognostic classification system used for MDS is the International Prognostic Scoring System (IPSS), which was developed in the mid-1990s by Greenberg *et al.* and allowed a unified approach to the care of patients with MDS (Table 2). Using this system, patients are classified

into one of 4 soubgroups: low-risk, intermediate-1 (int-1), intermediate-2 (int-2), and high-risk. The stratification in one of the categories above is calculated based on three characteristics: bone marrow blast proportion, number of peripheral cytopenias, and cytogenetic abnormalities (Garcia-Manero, 2010). The two lower-risk subgroups (low-risk and int-1) are characterized by prolonged survival and comorbidities accounting for 50% of deaths, and treatments aiming to improve cytopenias (growth factors and transfusions). The two higher-risk subgroups (int-2 and high-risk) have a higher rate of leukemic progression and shorter survival. The treatment aims at slowing disease progression through chemotherapy and demethylating agents and even cures MDS after bone marrow transplantation (Triantafyllidis *et al.*, 2012).

The strengths of the IPSS model include its simplicity and the fact that it does not require any testing beyond the routine diagnostic evaluation (Bejar, 2013). Despite the facilities in its use, the IPSS study did not include enough patients to determine the prognosis of less common cytogenetic abnormalities (Seiter, 2013). Additionally, because the IPSS weights only the number of cytopenias present, it appeared to underestimate disease risk in a subset of patients with severe cytopenias but few other risk factors (Bejar, 2013).

Thus, as a refinement to adjust for these deficiencies, a revised model of the IPSS (R-IPSS) was proposed in 2012 (Komrokji *et al.*, 2013). Additionally, the WHO also attempted to refine the original IPSS score, developing the WHO-based prognostic scoring system (WPSS) (Duong *et al.*, 2014), followed by the development of a new classification system, the Global M.D. Anderson Scoring System (MDAS) (Garcia-Manero, 2014). In summary, the different prognostic models described above are currently available for MDS risk stratification, and offer potential advantages. A simple, unified and comprehensive model is necessary, following the growing knowledge about the biology of the disease, thus allowing the tailoring of treatments towards more homogenous groups of patients.

Table 2. Current risk models in myelodysplastic syndromes.

	IPSS Prognostic Score Value				
Prognostic Category	**0**	**0.5**	**1**	**1.5**	**2**
Cytogenetics	Good	Intermediate	Poor		
BM basts, %	<5	5-10		11-20	21-30
Cytopenias	0/1	2/3			

Cytogenetic Groups: Good, normal; -Y; del(5q), del(20q). **Intermediate**, any not considered good or poor. **Poor**, complex (≥3 abnormalities), chromosome 7 abnormalities
Cytopenias definitions: Hemoglobin, <10 gm/dL; **Neutrophils**, $<1800^{9}$/L; **Platelets**, $<100 \times 10^{9}$/L

1.3.5. Pathogenesis

It is commonly accepted that MDS develops through a multi-step process encompassing alterations within the hematopoietic stem cell, the bone marrow microenvironment, and the complex interactions between both. However, the generally accepted primary hypothesizes involves an initial deleterious genetic event within a hematopoietic stem cell, subsequent development of excessive cytokines/inflammatory response leading to a pro-apoptotic/proliferative state, and resultant peripheral cytopenias despite a hypercellular bone marrow. (Warlick & Smith, 2007).

The predominant common pathway underlying the inherent marrow derangement causing ineffective hematopoiesis in MDS has been the varying degrees of apoptosis of the hemopoietic precursors and their progeny. This apoptotic process in hematopoietic precursors is increased early in the disease with associated peripheral cytopenias and diminishes with disease progression, then permitting the expansion of the abnormal clone (Greenberg, 2012). The subsequent generations of this transformed clone have an unequal tendency towards apoptotic death in the presence of increased amounts of a cascade of pro-inflammatory cytokines. One of the key systems for this switching appears to be tumor necrosis factor α (TNFα) and its receptors (Kitagawa *et al.*, 2011). Furthermore, accumulating evidence has shown that marrow failure in some MDS is associated with autoimmunity, T-cell mediated myelosuppression and cytokine-induced cytopenias (Barrett & Sloand, 2009) (Figure 5). Therefore, MDS should be considered as a disease of not only hematopoietic cells, but also of the bone marrow microenvironment, a concept that becomes critical when developing therapeutic strategies. Thus, cell proliferation followed by marked apoptosis was the most significant biological characteristic present in all subtypes of MDS (Kitagawa *et al.*, 2011).

Recent technological advances have enabled the identification of many new mutations, which have implicated novel pathways in MDS pathogenesis. With recent technical and scientific advances, molecular abnormalities, including copy number abnormalities and point mutations, can now be identified in the vast majority of MDS cases. Metaphase cytogenetic analysis detects chromosomal abnormalities in approximately 50% of cases, although sensitive techniques such as single-nucleotide polymorphism microarrays (SNP-A) and array comparative genomic hybridization (CGH) can identify abnormalities in nearly 80% of cases. Recurrent mutations that alter many essential cellular processes have been identified, including RNA (ribonucleic acid) splicing, epigenetic regulation of gene expression, DNA (deoxyribonucleic acid)-damage response, and tyrosine kinase signalling. Specific mutations in

these pathways are, in some cases, strictly associated with distinct morphologic features or clinical phenotypes. Combinations of point mutations and chromosomal abnormalities may be on the basis of the clinicopathologic heterogeneity of MDS (Lindsley & Ebert, 2013; Seiter, 2013).

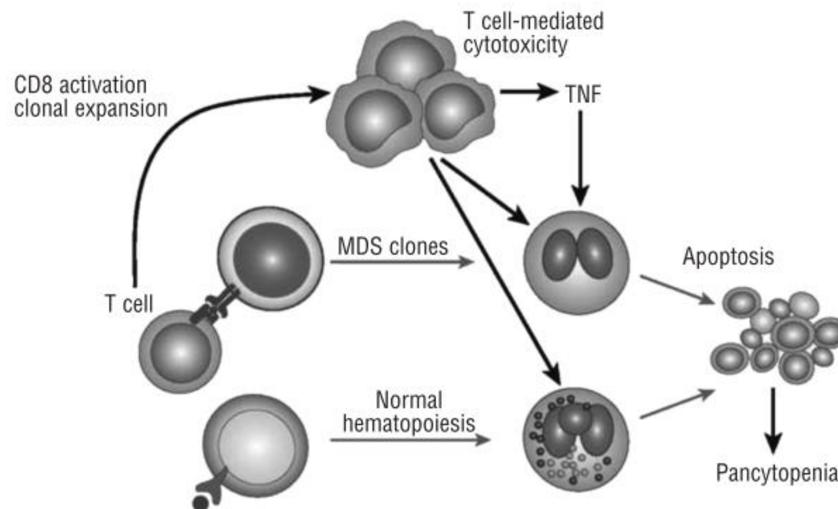

Figure 5. Model of immune interactions with myelodysplastic syndromes and the response to treatment. Myelodysplastic syndromes clones express a neoantigen or overexpress an antigen. This triggers expansion of T cell cytotoxic clones for the myelodysplastic syndromes cell. Activated T-cells secrete cytokines, tumor necrosis factor α and interferon γ, which promote apoptosis of normal progenitor cells suppressing hematopoiesis (adapted from Barrett & Sloand, 2009).

The mechanisms by which MDS progresses to AML are continuously being studied, even though there are different ways to approach the problem. One is to consider those MDS-specific changes associated with more rapid progression to AML, including some chromosomal subsets, specific mutations (e.g., DNMT3a), and a high degree of CpG island methylation abnormalities. Another way is to consider those changes that are exclusive to very advanced MDS, or AML derived from MDS. Nevertheless, it was advanced a speculative model whereby MDS pathogenesis, in some cases, is largely attributable to aberrant epigenetic regulation, and MDS progression is a consequence of acquired abnormalities both in growth and apoptosis controlling genes (Issa, J. P. J., 2013) (Figure 6).

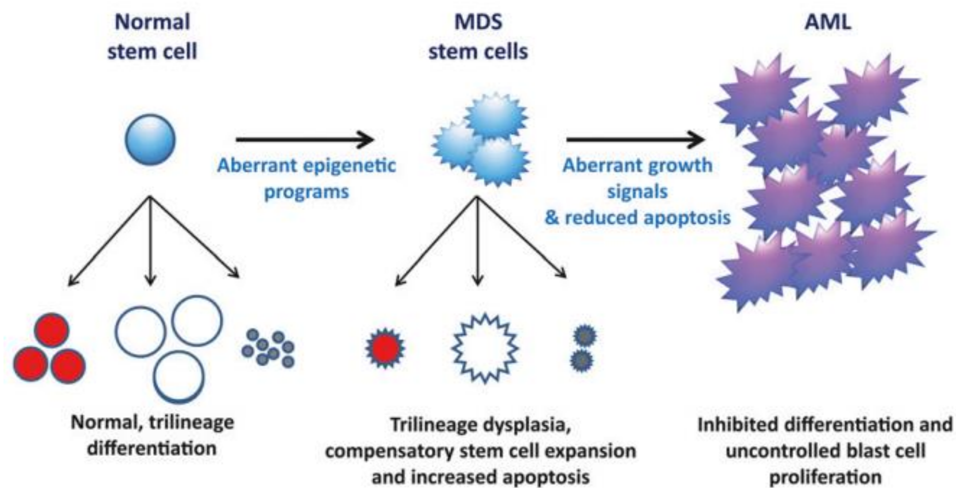

Figure 6. A model of MDS formation and progression. It is hypothesized that the altered differentiation programs and dysplasias pathognomonic of MDS are due to aberrant epigenetic regulation. These differentiation defects signal compensatory stem cell growth but also result in increased apoptosis, which explains the paradox of hypercellular marrows but peripheral cytopenias in MDS. With time, MDS cells acquire mutations that confer uncontrolled growth signals and/or inhibited apoptosis. These mutations (and, possibly additional epigenetic defects) lead to the blast expansion and inhibit differentiation, characteristic of the transition of MDS to AML (adapted from Issa, J. P. J., 2013).

1.4. Epigenetics

1.4.1. Mechanisms

Cancer has traditionally been viewed as a set of diseases that are driven by the accumulation of genetic mutations that have been considered the major causes of neoplasia. However, this paradigm has now been expanded to incorporate the disruption of epigenetic regulatory mechanisms. (Soo You & A. Jones, 2013).

The genetic path to cancer is relatively straightforward: mutation of tumor suppressors and/or oncogenes causes either loss or gain of function and abnormal expression. Epigenetic pathway, on the other hand, is not as simple (Soo You & A. Jones, 2013); it is defined as the study of heritable changes in gene expression that are not mediated by changes in the genomic DNA sequence (Bejar, Levine & Ebert, 2011). Most of these heritable changes are established during differentiation and are stably maintained through multiple cycles of cell division, enabling cells to have distinct identities while containing the same genetic information. This heritability of gene expression patterns is mediated by epigenetic modifications, which include methylation of cytosine bases in DNA, post-translational modifications of histone proteins and non-coding RNAs including microRNAs (miRNAs) (Sharma, Kelly & Jones, 2009).

These epigenetic regulatory mechanisms can contribute to regulation of the major molecular processes in the nucleous including transcription, replication, repair, and RNA processing (Crider *et al.*, 2012). In MDS, the most relevant molecular mediators of the epigenetic state are gene expression patterns maintained by methylation of DNA and covalent modification of histones. (Bejar, Levine & Ebert, 2011).

1.4.1.1. DNA methylation

DNA methylation is one of the most well-characterized epigenetic mechanisms. It consists in a covalent modification of genomic DNA that changes gene expression, occurring mainly at cytosines within a CpG dinucleotide (regions preferentially found at the 5' ends of genes, in which a cytosine and a guanine are linked by a phosphate) when a methyl group from S- adenosylmethionine (SAM) is enzymatically transferred to the 5 position of cytosine to generate 5-methylcytosine in genomic DNA (Crider *et al.*, 2012) (Figure 7).

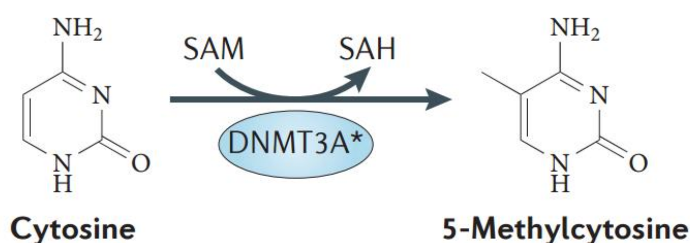

Figure 7. Methylation of Cytosine. The DNA cytosine residues are converted to 5-methylcytosine by the action of the DNA methyltransferases (DNMTs). The donor of methyl groups, SAM, is converted to S-Adenosyl-L-homocysteine (SAH) (adapted from Shih *et al.*, 2012).

The enzymes that catalyse this transfer are known as DNA Methyltransferases (DNMTs). Mammals have three types of DNMTs: DNMT1, DNMT3A and DNMT3B, which generate and maintain the precise DNA methylation patterns found in mammalian genome. The first one is believed to act as the primary maintenance methyltransferase to methylate hemimethylated DNA after DNA replication and preserve parental DNA methylation patterns in daughter cells. In contrast, DNMT3A/B function as *de novo* methyltransferases to methylate fully unmethylated CpG sites (Sharma *et al.*, 2009; Crider *et al.*, 2012).

Typically, methylated DNA is associated with transcriptional repression and hypomethylation is associated with active chromatin (Karagiannis & Maulik, 2012). As so, in

normal cells, the majority of CpG islands remain unmethylated, and the associated genes are actively transcribed. However, when located in repetitive regions of genome, DNA methylation prevents genomic instability and spurious transcription initiation (Rodríguez-Paredes & Esteller, 2011) (Figure 8).

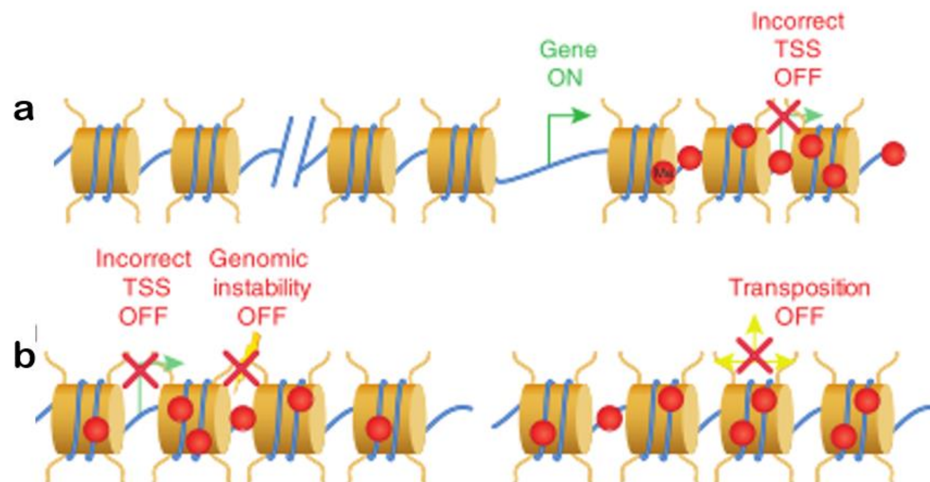

Figure 8. DNA methylation patterns in normal cells. DNA methylation takes place along the whole genome, and its disruption is a typical hallmark of cancer. a) CpG islands and CpG island shores are commonly unmethylated, allowing gene transcription. Additionally, DNA methylation within the gene bodies avoids spurious transcription initiations. b) Methylation of repetitive sequences prevents genomic instability and, again, spurious transcription initiations. Moreover, transposable elements cannot be activated in a methylated environment. (adapted from Rodríguez-Paredes & Esteller, 2011).

1.4.1.2. Histone modifications

Histones are small proteins that form a core around which DNA is wrapped, forming nucleosomes, the basic in-vivo structural unit of DNA. Nucleosomes consist of eight histone molecules (two each of Histones H2A, H2B, H3 and H4) around which a loop of DNA is wrapped. While H2A and H2B are thought to play primarily a structural role, it has become apparent that H3 and H4 are key integrators of a variety of signals that regulate gene transcription (Issa, J.-P., 2011).

Thus, post-translational modifications of histones such as acetylation, methylation, phosphorylation and ubiquitination, are also an important part of epigenetic regulation. (Stein, 2014). The histone modification patterns are regulated by enzymes such as histone acetyltransferases (HATs) and deacetylases (HDACs), which introduce and remove acetyl groups, respectively (Soo You & A. Jones, 2013).

Epigenetic processes work together to establish and maintain the global and local condensed or descondensed chromatin states that eventually determine gene expression (Rodriguez-Paredes & Esteller, 2011). DNMTs, for example, may regulate gene silencing accompanying promoter DNA methylation by recruiting histone deacetylases HDACs and other chromatin-binding proteins to promoter sites to maintain histone deacetylation, and eventually lead to a closed chromatin configuration. This closed chromatin configuration results in exclusion of transcription factors, thus insuring allele-specific inactivation, providing a link between methylation and chromatin remodelling and modification (Klose & Bird, 2006). An important aspect is that methylation is dominant to histone deacetylation, so transcription does not occur without first inhibiting methylation (Baylin, 2005; Issa, J.-P., 2011). On the other hand, even though DNA methylation can control gene activity, it is not enough to completely inhibit transcription. The local chromatin structure also plays a role in determining whether genes are transcribed or repressed (Baylin, 2005) (Figure 9).

Overall, the interactions between DNA methylation machinery and histone modifying enzymes further enhance the complexity of epigenetic regulation of gene expression, which determines and maintains cellular identity and function (Sharma *et al.*, 2009).

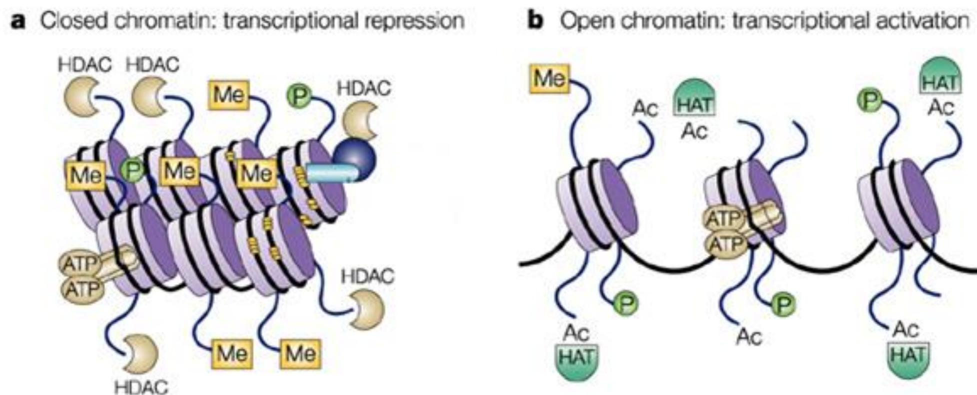

Figure 9. Chromatin structure regulates transcriptional activity.DNA methylation and histone deacetylation induce a closed-chromatin configuration and transcriptional repression. b) Histone acetylation and demethylation of DNA relaxes chromatin, and allows transcriptional activation (adapted from Johnstone, 2002).

1.4.1.3. MicroRNAs

Besides these two major mechanisms, miRNAs also play an important role in DNA epigenetic processes. Defined as small non-coding RNAs, miRNA are short molecules that mediate gene expression through post-transcriptional silencing of target genes (Sharma et al., 2009). The target gene specificity of each miRNA is dictated by sequence-dependent

interaction between approximately 22-nt-long mature miRNAs, especially their 6- or 7-nucleotide “seed” sequences at the 5′ end, and the 3′ untranslated regions (3’UTR) of mRNAs (Bartel, 2009). However, the relationship between miRNAs and components of the epigenetic machinery is twofold. First, miRNAs can be deregulated by aberrant epigenetic silencing, as many of them are located in introns of protein coding genes and are regulated by the promoter of the host gene (Singh & Campbell, 2013). Conversely, miRNAs can regulate the epigenetic machinery: e.g. overexpression of miR-29b in AML induces downregulation of DNMT3s and thus hypomethylation (Itzykson & Fenaux, 2014).

1.4.2. DNA methylation and cancer

Epigenetic regulation and inheritance are now recognized as important determinants in the context of the developmental origins of disease and is now clear that epigenetic aberrations are involved in numerous disease states and these are particularly well-characterized in cancer. (Karagiannis & Maulik, 2012).

In fact, change of DNA methylation patterns in CpG islands was the first and most significant abnormal epigenetic change identified in cancerous cells (Ghavifekr Fakhr *et al.*, 2013). In recent years, it has become apparent that this pattern observed in cancer generally show a dramatic shift with that of normal tissue. Contrary to normal cells, cancer cells show genome-wide hypomethylation and site-specific CpG island promoter hypermethylation (Rodríguez-Paredes & Esteller, 2011) (Figure 10).

As so, targeted hypermethylation of CpG islands promotes the progression of tumorigenesis by silencing tumor suppressor genes. For example, PTEN, a protein that prevents rapid proliferation, is commonly hypermethylated in brain and thyroid cancers, whereas APC, a protein involved in cell-cycle regulation, cell–cell adhesion, and cell mobility, is inactivated by hypermethylation in many lung, breast, and colorectal cancers. In addition to tumor suppressorgenes, hypermethylation of DNA repair genes and transcription factors can indirectly lead to tumorigenesis through silencing of further downstream targets or accumulation of genetic errors (Virani *et al.*, 2012).

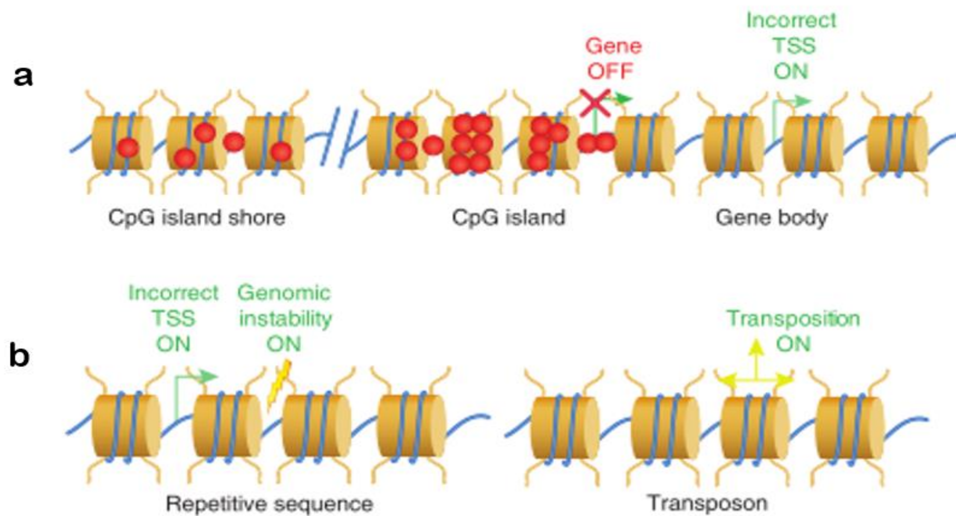

Figure 10. DNA methylation patterns in cancer cells. Even though both CpG islands and CpG island shores may be strongly methylated, gene bodies lack this modification. As a result, transcription of many genes gets blocked, and aberrant transcription may occur from incorrect transcription start sites (TSS). (b) Global hypomethylation triggers genomic instability and leads to aberrant transcription. Concomitant activation of transposons may lead to gene disruption (adapted from Rodríguez-Paredes & Esteller, 2011).

In patients with MDS, the hypermethylation may occur early in the disease and, as mentioned above, is found to be associated with more rapid progression to AML (Bravo *et al.*, 2014). In both MDS and AML, several genes have been described as targets of DNA hypermethylation. It has been proposed that repression of genes that are crucial for cell cycle arrest and induction of differentiation might contribute to the malignant transformation of normal hematopoietic cells (Cortesão, 2010; Nikoloski *et al.*, 2012).

Among the DNA methylation regulators, mutations in tet methylcytosine dioxygenase 2 (*TET2*), which catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine, lead to a loss of function in about 19-26% of MDS patients, whereas mutations in DNMT3A have been reported in 3-8 % of patients with MDS, leading to a reduction in methyltransferase catalytic activity (Seiter, 2013; Bravo *et al.*, 2014). Mutations in isocitrate dehydrogenase 1 and 2 (*IDH1*/*IDH2*) lead to a decrease in a-ketoglutarate (a-KG), *TET* inhibition and widespread promoter hypermethylation of DNA. (Bravo *et al.*, 2014).

The discovery of mutations in these epigenetic regulators might be in line with observations describing both deregulated DNA methylation patterns in MDS, which correlated with overall survival, and clinical response to hypomethylating agents, which was associated with a decrease in DNA methylation (Nikoloski *et al.*, 2012). Mutations in cell cycle regulators *CDKN2A* (*P14* and *P16*) and *CDKN2B* (*P15*), *CTNNA1*, E-cadherin (*CDH1*), among

others, had been found in MDS cases. Genome-wide increases in promoter hypermethylation predict survival, even after taking into consideration age, sex, and IPSS risk group, and are seen during progression to AML (Bejar *et al.*, 2011).

Along with CpG hypermethylation as a determinant event in tumorigenesis, hypomethylation of distal regulatory regions and repetitive elements is also an important part of epigenetic regulation. These repetitive elements make up about half of the genome and are usually heavily methylated. Centromeric tandem repeats, adjacent-centromeric tandem repeats, and short- (Alu) and long- interspersed elements (LINE-1) are the most frequently studied repetitive elements in cancer that are found to be hypomethylated. (Virani et al., 2012). LINE-1 retrotransposition is, particularly, a prominent source of inter-individual genetic variation and an important cause of human genetic disease. In cancer, LINE-1 hypomethylation can result in its activation and consequent retrotransposition throughout the genome leading to disruption of genes and widespread chromosomal instability. Hypomethylation of LINEs has been observed in colon cancer and chronic lymphocytic leukemia, and contributed to the development of malignat phenotype of these cells (Luczak & Jagodziński, 2006). Moreover, an activated LINE-1 promoter can initiate sense or antisense transcription through other genes (Pavicic *et al.*, 2012).

Furthermore, global methylation of genomic DNA can also be responsible for the activation of some proto-oncogenes, as well as genes encoding proteins involved in genomic instability. Aditionally, in some cases it can be associated with malignant cell proliferation, invasion and metastasis, and lead to loss of imprinting, as in the case of the *IGF2* gene (encoding insulin-like growth factor-2), resulting in an increased biallelic expression of *IGF2* that efficiently stimulates proliferation of cancer cells (Rodríguez-Paredes & Esteller, 2011; Luczak & Jagodziński, 2006).

1.5. Genetic Variability

The human diversity source of all genetic variation lies in the mutational process, which occurs at different rates in different parts of the genome. Thus, those genes and genomic regions that have high mutation rates are more likely to generate disease mutations, and hence be associated with a disease (Eyre-Walker & Eyre-Walker, 2014).

Human genetic variants are typically referred as either common or rare, to denote the frequency of the minor allele in the human population. Common variants include polymorphisms, defined as genetic variants with a minor allele frequency (MAF) of at least one percent in the population, whereas rare variants have a MAF of less than 1%. Genetic variants are also discussed in terms of their nucleotide composition. In the broadest sense, variants in the human genome can be divided into two different nucleotide composition classes: single nucleotide variants and structural variants (Figure 11) (Frazer *et al.*, 2009).

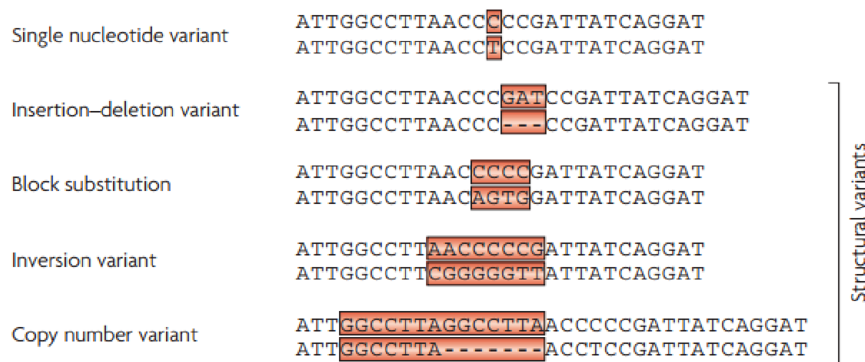

Figure 11. Classes of human genetic variants. Single nucleotide variants occur when a single nucleotide (A, T, G or C) is altered. Insertion–deletion variants are DNA sequence variations in which one or more base pairs are present in some genomes but absent in others. Block substitutions describe cases in which a string of adjacent nucleotides varies between two genomes. An inversion variant results from a reversion of the order of the base pairs, in a defined section of a chromosome (adapted from Frazer *et al.*, 2009).

Single nucleotide polymorphisms (SNPs) are the most prevalent class of genetic variation among individuals (Frazer *et al.*, 2009). It has been estimated that the human genome contains 12 to 16 million SNPs, which are thought to be responsible for most of the inter-individual human genetic variability (e.g., memory, creativity, motor coordination or individual responses to pharmacological therapies) (Regateiro, 2010). It is estimated that 50% of SNPs occur in noncoding regions, 25% lead to nonsynonymous mutations, and the remaining 25% are silent mutations (Shastry, 2009). Nonsynonymous mutations comprise missense and nonsense mutations. The first one occurs when the resulting codon codifies a different aminoacid and the degree of consequences depends on the way the polymorphism affect the functional activity of the codified protein. Nonsense mutations are the result of point DNA mutations that convert a codifying codon in a “stop” codon in RNA, which generates a truncated protein. On the other hand, silent SNPs are also called “synonymous SNPs” because the alteration of a nucleotide in the codon does not change the encoded amino acids (Regateiro, 2010).

Because polymorphisms can influence promoter activity (gene expression), messenger RNA (mRNA) conformation (stability), and translational efficiency, they can therefore be responsible for the susceptibility of an individual to many common diseases, and genome evolution (Shastry, 2009).

1.5.1. Genetic variability in enzymes from one-carbon metabolism

Nutrition is a major environmental exposure that influences all aspects of health and lifespan. Folate, an essential nutrient, plays an important role not only in DNA synthesis but also in the maintenance of methylation reactions in the cells (Nazki, Sameer & Ganaie, 2014), important mechanisms in the pathogenesis of MDS (Figure 12). It is a fundamental water-soluble vitamin occurring naturally in select foods as well as in the synthetic form (folic acid) used in supplements and in food fortification programs. Besides DNA, RNA and protein methylation, DNA synthesis and maintenance are also critical cellular pathways dependent on folate as a one-carbon source (Crider *et al.*, 2012).

Under normal dietary conditions, absorbed folate is metabolized to 5-methyltetrahydrofolate (5-methyl THF) in the intestine and/or liver. The 5-Methyl THF is the primary folate constituent taken up by nonhepatic tissues, which then is converted, via the methionine synthase reaction, to tetrahydrofolate (THF), the most effective substrate for polyglutamate synthetase. Folic acid is normally first reduced to dihydrofolate by dihydrofolate reductase and subsequently to THF to enter the folate pool. In some cases, the capacity of dihydrofolate reductase is exceeded and folic acid may appear in the circulation in the oxidized form. Once the THF coenzyme is formed from either folic acid or dietary folate, it is first converted to 5,10-methylene THF by the vitamin B-6–dependent enzyme serine hydroxymethyltransferase and subsequently irreversibly reduced to 5-methyl THF by methylenetetrahydrofolate reductase (*MTHFR*). This reaction is key to maintaining the flux of methyl groups for the remethylation of homocysteine to methionine via the vitamin B_{12}–dependent methionine synthase (*MTR*) reaction (Crider *et al.*, 2012). At this stage, methionine synthase reductase (*MTRR*) is also an important enzyme, acting in the maintenance of adequate levels of activated vitamin B_{12} and thus essential in the conversion of homocysteine to methionine (Botto *et al.*, 2003). On the other hand, adequate levels of homocysteine are regulated by cystathionine β-synthase (*CBS*), which irreversibly metabolizes this amino acid to cystathionine, playing a central role in the transsulfuration pathway (Summers *et al.*, 2008).

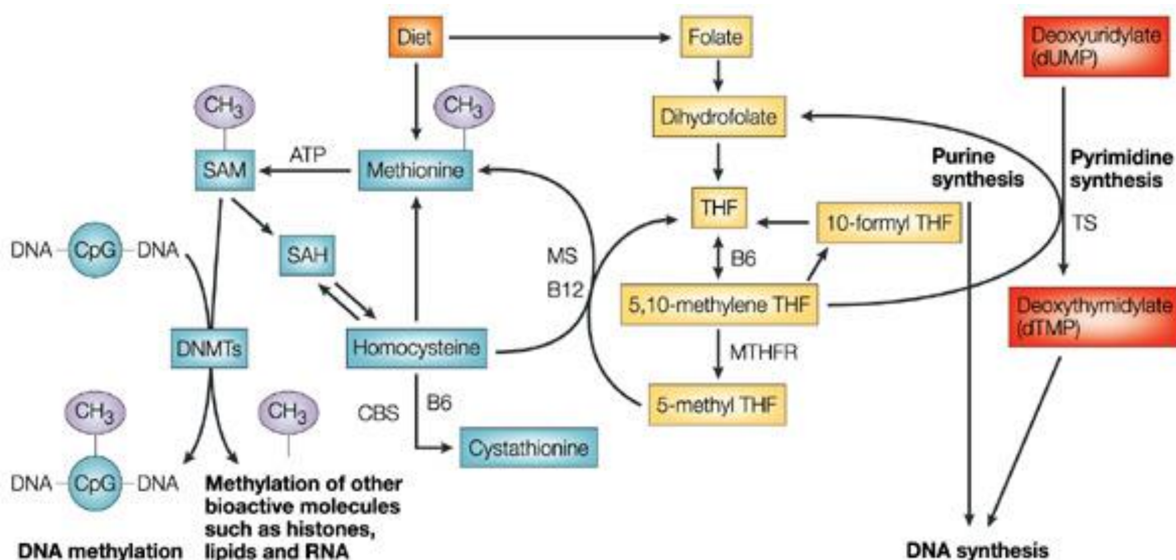

Figure 12. Overview of folate-mediated one-carbon metabolism links to methylation reactions and nucleotide synthesis. Abbreviations: *MS*, methionine synthase; *CBS*, Cystathionine-β-synthase; ATP, adenosine triphosphate; *TS*, thymidylate synthase; *MTHFR*, methylenetetrahydrofolate reductase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; *THF*, tetrahydrofolate; 10-Formyl THF, 10-formyl tetrahydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; 5,10-methylene THF, 5,10-methyltetrahydrofolate; B6, vitamin B6; B12, vitamin B_{12} (adapted from Lamprecht & Lipkin, 2003).

When synthesized (or either provenient by diet), methionine is the substrate for SAM, a cofactor and methyl group donor for numerous methylation reactions (Crider *et al.*, 2012). After donating its methyl group, SAM is converted to S-adenosylhomocysteine (SAH), a competitive inhibitor of numerous methyltransferases. SAM itself is a potent inhibitor of *MTHFR*. When SAM is present in high concentration, *MTHFR* is inhibited, which decreases the synthesis of 5-methyl-THF and hence remethylation of homocysteine. Conversely, when SAM concentrations are low, it favors the remethylation of homocysteine. 5-methyl-THF formation can also be modified by common *MTHFR* gene polymorphisms (Nazki *et al.*, 2014).

Besides the phase of DNA methylation, the one-carbon metabolism is also responsible for purine and pyrimidine synthesis. This process is mediated by the formation of 10-formyl THF which can donate one-carbon groups for purines biosynthesis, whereas 5,10-methylenetetrahydrofolate can be used as a cofactor for the conversion of deoxyuridine monophosphate (dUMP) into dTMP (deoxythymidine monophosphate) (Blom & Smulders, 2011).

Folates are hydrophilic molecules that are anions at physiological pH and thus cross biological membranes poorly by diffusion. Reflecting this, mammalian cells have evolved sophisticated uptake systems for facilitating cellular uptake of folate cofactors. One of the

major facilitative folate transporters include reduced folate carrier (RFC; *SLC19A1*), a transmembrane protein that belongs to solute carrier (SLC) group and uses a bi-directional anion-exchange mechanism to transfer folate molecules across the membrane (Liu & Ward, 2010; Hou & Matherly, 2014). Evidencing its physiological importance, low levels of RFC could result in a number of pathophysiological states associated with folate deficiency, ranging from cardiovascular disease, fetal abnormalities, neurological disorders, and possibly to cancer (Hou & Matherly, 2014).

In addition to folate, a number of other dietary nutrients are required to maintain one-carbon flux, ensuring normal homocysteine remethylation, SAM formation, as well as DNA synthesis and methylation. These nutrients include vitamin B_6 (serine hydroxymethyltransferase activity), riboflavin (*MTHFR* stability), vitamin B_{12} (methionine synthase function), and choline (betaine precursor as a hepatic methyl source via betaine: homocysteine methyltransferase) (Crider *et al.*, 2012). Modifying the intake of these nutrients alters DNA methylation (Park *et al.*, 2012).

The fact that folate metabolism can affect both DNA synthesis and methylation has made gene-environmental variants that impact on this pathway attractive candidates as cancer susceptibility factors. These include dietary intakes of folate and folic acid, and functional polymorphisms in the genes coding for folate metabolism enzymes (Nazki *et al.*, 2014).

In fact, different polymorphisms in the enzymes that catalyse one-carbon metabolism have been associated with susceptibility to various types of cancer. In hematology malignancies, particularly, studies show that the *MTHFR* 677TT genotype is associated with an increased risk for ALL (acute lymphocytic leukemia) and the *MTRR* 66AG genotype is associated with and increase risk for MDS based on the DNA methylation status. (Kim et al., 2009). Adittionally, the *MTR* 2756AG polymorphism is thought to reduce enzyme activity with the GG genotype and to increase homocysteine levels and DNA hypomethylation with the AA genotype (Kim *et al.*, 2009).

Despite the current progresses, MDS is still poorly characterized and the development of new strategies tailoring different targets that affect the pathogenesis of the disease are continuously being investigated.

AIMS

Our major goal is to evaluate the interplay between genetic polymorphisms and the risk of developing MDS, by investigating in what way genetic variability of enzymes of one-carbon metabolism affect methylation status in MDS patients, as well as its involvement in nutrient levels that could influence the supply of methyl groups.

In particular, the aims of the study are:

- To analyse the effect of the polymorphic variants of *MTR* (rs1805087 and rs2229276), *SLC19A1* (rs1051266), *CBS* (844ins68) and *MTRR* (rs162936) genes in the development of myelodysplastic syndromes;

- To evaluate the influence of polymorphic variants of *MTR*, *SLC19A1*, *CBS* and *MTR* in patients' evolution to acute myeloid leukemia and overall survival;

- To evaluate the relation between polymorphisms in *MTR*, *SLC19A1*, *CBS* and *MTR* and the presence or absence of cytogenetic abnormalities in MDS patients;

- To assess the interplay between folate and vitamin B_{12} levels and genetic variability of enzymes from one-carbon metabolism;

- To investigate the relation between the degree of *P15*, *P16* and LINE-1 methylation and the polymorphic variants of *MTR*, *SLC19A1*, *CBS* and *MTRR* genes;

MATERIALS AND METHODS

3.1. Selection and characterization of controls and MDS patients

This study enrolled 157 individuals, among 77 MDS patients and 80 non-neoplastic controls. Patients and controls were selected in the department of Clinical Hematology of the Centro Hospitalar Universitário de Coimbra/University Hospitals of Coimbra (CHUC/HUC) and Hospital Distrital da Figueira da Foz (HDFF) according to international criteria of MDS. The present study was conducted in accordance with the Declaration of Helsinki. 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 enrolment.

MDS patients were diagnosed and classified into the different WHO subtypes, according with the diagnosis criteria of this organization.

3.2. Extraction and Quantification of genomic DNA

Genomic DNA extraction (peripheral blood and bone marrow) from MDS patients and controls was performed following Bartlett and White's protocol (Bartlett *et al.*, 2003) for extraction of DNA from whole blood, after its optimization.

At first, 500 µL of peripheral blood or bone barrow were placed into a 10 mL falcon-tube, to which was added a red blood cell lyses solution containing 0.01 M Tris HCl (Tris Base, ULTROL® Grade, Calbioquem®), 320 mM Sucrose (D(+)Sucrose, AppliChem), 5 mM de $MgCl_2$ and 1% Triton X100 (MpBiomedicals). Then, it was mixed on a rotating blood mixer for 4 min at room temperature, followed by a 3000 g centrifugation, for 5min. The supernatant was discarded and 160µL of a cellular lysis solution containing 4 M Tris HCl, 150 mM NaCl, 0.06 M EDTA (EDTA Na_2, MpBiomedicals) and 1% SDS (SDS pure, AppliChem) were added to the pellet. The mixture was transferred to a 1.5 mL eppendorf, followed by the addition of 42 µL of 5 M sodium perchlorate. It was mixed, by inverting the tube several times. The eppendorf was then placed in water bath for 15 to 20 min, at 65°C, followed by cooling down to room temperature. After temperature reduction, 350 µL of ice-cold chloroform were added and placed on the rotator for 30 to 60 min. Subsequently, it was centrifuged at 2400 g for 2 min, after which the upper phase was transferred into a new 1.5 mL eppendorf tube and the pellet was discarded. After that, 420 µL of ice-cold ethanol were added, inverting then the tube gently to allow DNA to precipitate. Using a sterile pipet, the DNA was transferred to new 1.5

mL eppendorf tube and left to air dry, allowing the evaporation of ethanol. Finally, the dried DNA was resuspended in 100 µL of elution buffer.

After obtaining genomic DNA samples, their degree of purity and concentration, by absorption spectroscopy, was quantified using NanoDrop® 1000 Spectrophometer. The 260 nm/280 nm ratio was used to evaluate the DNA purity. Concerning DNA concentration, only the samples with concentration values above 100ng/µL were considered. For quantification and establishment of DNA degree of purity, it was used an elution buffer (previously used to dissolve DNA in the extraction process), which was designated as blank. The samples were then stored at -20°C for later use.

3.3. Analysis of polymorphisms of the *MTR* (rs1805087 and rs2229276), *SLC19A1* (rs1051266), *MTRR* (rs162036) and *CBS* (844ins68) genes

3.3.1. Analysis of polymorphisms of the genes *MTR* (rs1805087 and rs2229276) and *MTRR* (rs162036) by Tetra-Primer ARMS-PCR (Amplification-Refractory Mutation System-Polymerase Chain Reaction)

In order to genotype *MTR* and *MTRR* gene variants, Tetra-Primer ARMS-PCR assays were performed. This technique requires four primers that are used to generate three potential PCR products: Forward outer (FO) and reverse outer (RO) primers amplify a larger outer DNA amplicon, giving a control band, while forward inner (FI) and reverse inner (RI) primers amplify specific alleles, generating products with different sizes. This allele specificity is granted by a mismatch between the 3'-terminal oligonucleotide of one inner primer and the target DNA, and enhanced by a second mismatch at position -2 from the other inner primer (Ye *et al.*, 2001) (Figure 13).

As so, to the DNA sample were added 0.2 mM of dNTPs mix (nucleotide triphosphates containing deoxyribose), 1x buffer (Reaction Buffer for NZYTaq DNA Polymerase), 2mM $MgCl_2$ (NZYtech), 10% DMSO (Dimethyl sulfoxide, ChemCruz™), 1U Taq Polymerase (NZYTaq DNA polymerase, NZYtech) and each one of the designed primers, described in Table 3.

Amplified products were resolved on 4% agarose gels, stained with Midori Green Advance (Nippon Genetics Europe GmbH) and visualized under UV illumination.

In relation to the first polymorphic variant, results from electrophoresis established three different bands of 190 bp, 202 bp and 328 bp corresponding to G allele, A allele and total gene present in all samples, respectively. As so, homozygous individuals for A allele show a 328 bp and a 202 bp bands, while GG individuals for present two bands, at 328 bp and at 190 bp. Heterozygous genotype is defined when the three bands are present simultaneously for one sample. When analyzing variant rs2229276 of *MTR* gene, the gene band was observed at 539 bp, whereas AA and GG genotype bands were noted at 350 bp and 286 bp, respectively. Analysis of *MTRR* rs162036 variant genotypes reported the presence of a 136 bp band corresponding to AA genotype, a 204 bp band associated with GG genotype, and a 285 bp gene band. The reaction conditions and the established PCR protocol are detailed in

Table **4**.

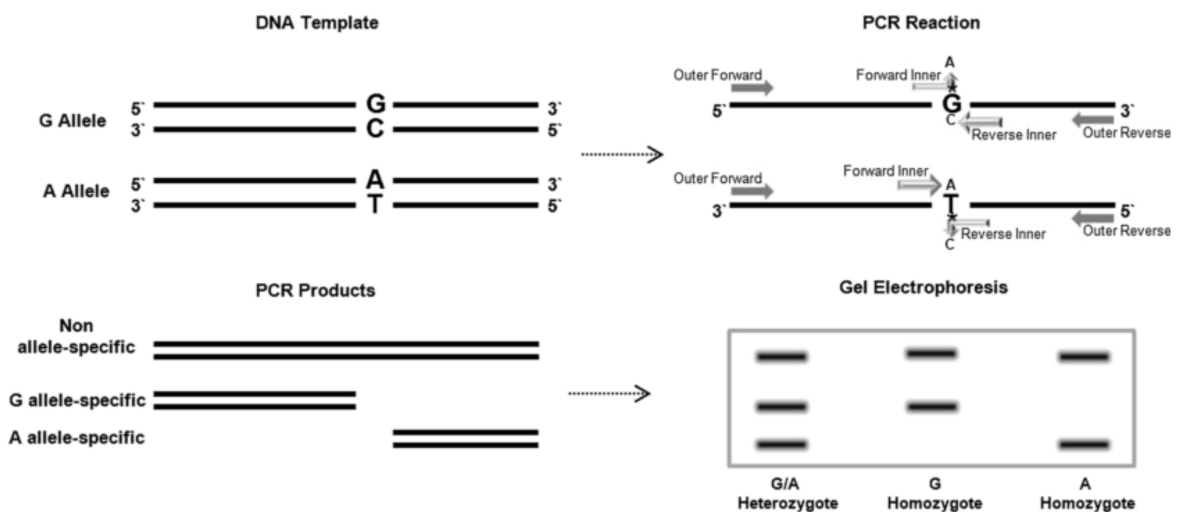

Figure 13. Schematic presentation of the tetra-primer ARMS-PCR method, using C/T substitution as an example. Two allele-specific amplicons are generated using two pairs of primers, one pair producing an amplicon representing the C allele and the other pair producing an amplicon representing the T allele. b) By positioning the two outer primers at different distances from the polymorphic nucleotide, the two allele-specific amplicons differ in length, allowing them to be discriminated by gel electrophoresis (adapted from Medrano & Oliveira, 2014).

3.3.2. Analysis of polymorphism of the gene *CBS* (844ins68) by PCR (Polymorphism-Polymerase Chain Reaction)

The amplification of 844ins68 of *CBS* gene was carried out using conventional PCR, which requires two primers, one of which attaches to the top strand at one end of the segment of interest, while the other attaches to the bottom strand at the other end.

In this study, we used two specific primers described in Table 3, at a final concentration of 0.2 μM, and mixed them with 1x buffer (Reaction Buffer for NZYTaq DNA Polymerase), 0.2mM dNTPs mix, 1.5mM $MgCl_2$ (NZYtech) and 1U Taq Polymerase (NZYTaq DNA polymerase, NZYtech). The reaction conditions and the established PCR protocol are detailed in Table 4.

Amplified products were resolved on 4% agarose gels, stained with Midori Green Advance (Nippon Genetics Europe GmbH) and visualized under UV illumination.

In this case, the presence of the insertion was detected with a single band at 350 bp, which corresponds to the homozygous genotype Ins-Ins (insertion). When none of the alleles presented the 68 bp insertion, the genotype was defined as WT-WT (wild type) and a 282 bp band was observed. In case of heterozygosity, the two bands were identified, and the genotype was defined as WT-Ins (wild type-insertion).

3.3.3. Analysis of polymorphism of the gene *SLC19A1* (rs1051266) by RFLP-PCR (Restriction Fragment Length Polymorphism-Polymerase Chain Reaction)

The amplification of the *SLC19A1* polymorphism was performed using RFLP-PCR, a two-step genotyping technique. Firstly, DNA fragments containing the variant are amplified using specific primers that are complementary to the target sequence; then, the amplified fragments are incubated with an appropriate restriction enzyme.

The presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allowing allele identification (Figure 14).

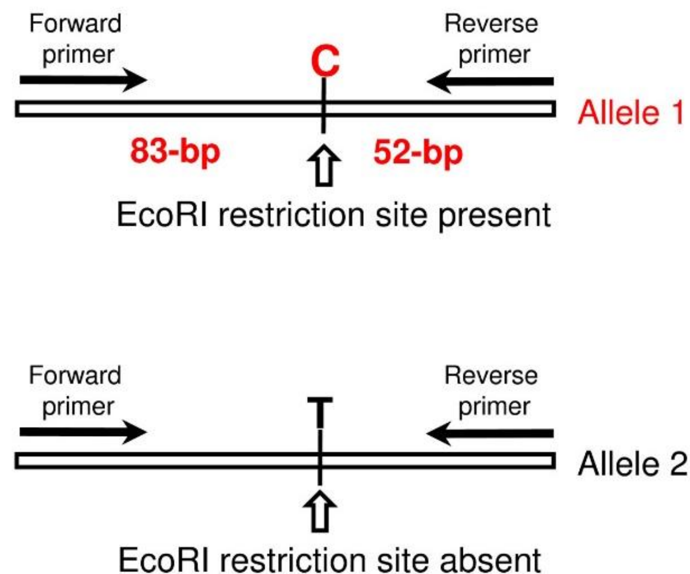

Figure 14. Schematic representation of Restriction Fragment Length Polymorphism-Polymerase Chain Reaction technique for single nucleotide polymorphism genotyping with EcoRI digestion. In this example, a 135-bp fragment encompassing the SNP is amplified by PCR and subsequently digested with the *EcoRI* restriction endonuclease. The C allele creates the *EcoRI* restriction site, generating two restriction fragments (83 and 52-bp). The T allele removes the restriction site and cannot be cleaved by EcoRI (adapted from Mitsouras & Faulhaber, 2009**).**

To this end, specific primers (Table 3) were added to the DNA template, followed by the addition of 1x buffer (Reaction Buffer for NZYTaq DNA Polymerase), 0.2mM dNTPs mix, 1.5mM $MgCl_2$ (NZYtech) and 1U Taq Polymerase (NZYTaq DNA polymerase, NZYtech).

After amplification, the products were digested with a mix containing restriction enzyme (New England BioLabs) at a final concentration of 1U/µL and a specific buffer (New England BioLabs).

Amplified products were resolved on 4% agarose gels, stained with Midori Green Advance (Nippon Genetics Europe GmbH) and visualized under UV illumination.

According to the patterns observed in electrophoresis, five band were identified: three for GG genotype at 125, 68 and 67 bp, whereas AA genotype was characterized by two bands, a first one at 62 bp and a second one at 162 bp.

Table 3. Primers sequences, amplicons size and annealing temperatures used

Technique	Gene	SNP	Primer Sequence (5'→3')	T_a°C	Product length	Reference
Tetra Primer ARMS-PCR	*MTR*	rs1805087	FO:GTGTTATCAGCATTGACCATTACTAC OR: GAAGACCTCTGATTTGAACTAGAAGA FI: TGGAAGAATATGAAGATATTAGACATGA RI: ACTTACCTTAGAGACTCATAATTGC	52°C	Gene: 328 bp G allele: 190 bp A allele: 202 bp	You *et al.,* 2008
	MTR	rs2229276	FO: CGTTAGCAGGAGCCGCAAATCC RO: CTCTGACACCCATGGTGGCG FI: CAAGACGACATTCACCTGTACTCG RI: CTGGGGCACAGCAGCCGCT	61,4°C	Gene: 593 bp G allele: 286 bp A allele: 350 bp	You *et al.,* 2008
	MTRR	rs16036	OF: CAGCGTGATCTGCCCTAACAGTGATTCT OR: TACCAATACCAGCGTATGCCTGTGTTCC IF: CGTCCTTTTGAAAATAAAGGCAGACACCAA IR: AGCATCAGGGCTGTTACCTTTCTGCC	58°C 63°C 55°C	Gene: 285 bp G allele: 204 bp A allele: 136 bp	You *et al.,* 2008
PCR	*CBS*	844ins68	F: ATAGAATATCGAGGCATGTCCGGCG R: TGGGGCCCAGGGTCAGCCAGGCTCC	63°C	No ins: 282 bp Ins: 350 bp	Griffioen *et al.* 2005
RFLP-PCR	*SLC19A1*	rs1051266	F: AGTGTCACCTTCGTCCCCTC R: CTCCCGCGTGAAGTTCTT	63°C	G allele: 125; 68; 67 bp A allele: 162;62 bp	Dervieux et al., 2004
MS-PCR	*P15*	-	F: TAGTGAGGATTTCGCGACGC R: ACGACCGATCGTTAACTCCG	60°C	Methylated: 148 bp	Herman et al., 1996
	P16	-	F: TTATTAGAGGGTGGGGCGGATCGC R: GACCCCGAACCGCGACCGTAA	65°C	Methylated: 34 bp	
COBRA	LINE-1	-	F: TTGAGTTGTGGTGGGTTTTATTTAG R: TCATCTCACTAAAAAATACCAAACA	60°C	Unmethylated: 413 bp Methylated; 287; 247; 166; 128; 38 bp	

T_a, annealing temperature; pb, base pairs

Table 4. Reaction Conditions for PCR, Tetra Primer ARMS-PCR, RFLP-PCR and MS-PCR

Gene	SNP	Initial Denaturation	Denaturation	Annealing	Extension	No. Cycles	Final Extention	Restriction Enzyme	Digestion
MTR	rs1805087	95°C, 5'	95°C, 30"	52°C, 30"	72°C, 30"	x30	72°C, 10'	-	-
	rs2229276		95°C, 30"	61,4°C, 30"	72°C, 30"	x25		-	-
MTRR	rs162036		95°C, 30" 95°C, 30" 95°C, 30"	58°C, 30" 63°C, 30" 55°C, 30"	72°C, 30" 72°C, 30" 72°C, 30"	x10 x30 x15		-	-
CBS	844ins68		95°C, 45"	63°C, 45"	72°C, 60"	x35		-	-
SLC19A1	rs1051266		95°C, 45"	63°C, 45"	72°C, 60"	x35		*CfoI*	
P15	-	95°C, 15'	95°C, 30"	60°C, 30"	72°C, 30"	x35		-	-
P16	-	95°C, 15'	95°C, 30"	65°C, 30"	72°C, 30"	x35		-	-
LINE-1	-	95°C, 5'	95°C, 30"	60°C, 30"	72°C, 30"	x50	72°C, 5'	*Hinf-1*	37°C, overnight

3.4. Analysis of methylation patterns of the genes *P15*, *P16* and LINE-1 in MDS patients

3.4.1. DNA modification by bisulfite

Treatment of DNA with bisulfite is used to determine the locations of unmethylated cytosines and 5-methylcytosines at single-nucleotide resolution. It converts cytosines to uracil while leaving 5-methylcytosines intact, allowing for single-nucleotide resolution information about the methylated areas of DNA.

Ir order to analyse the methylation status of the genes *P15*, *P16* and LINE-1 in MDS patients, the genomic DNA was firstly modified using the Zymo Research Kit EZ DNA Methylation-Gold™ TM Kit. Briefly, 20 µL of genomic DNA were placed into a PCR tube and mixed with 130 µL of the CT Conversion Reagent. Then, the PCR tube was placed in a thermocycler BioRad1000 and submitted to a DNA denaturation cycle, at 98°C for 10 minutes, followed by 2.5 hours at 64°C for the conversion by bisulfite.

After the temperature cycles, the modified DNA was placed into a purification column Zymo-Spin™ IC Column, ensued by the addition of wash and desulphonation buffers, each one followed by centrifugation. The modified and purified DNA was then stored at -4°C for later use.

3.4.2. Analysis of *P15* and *P16* methylation by MS-PCR (Methylation-Specific Polymerase Chain Reaction)

Methylation status of *P15* and *P16* was performed using MS-PCR (Methylation-Specific Polymerase Chain Reaction), after genomic modification of the DNA samples from MDS patients, following the protocol established by Herman *et al.* (Herman *et al.*, 1996), with some modifications

As so, 0.2mM dNTPs mix, 2mM $MgCl_2$ (NZYtech), 1U/µL polymerase (Supreme NZYTaq DNA Polymerase, NZYtech) and two primers at a final concentration of 0.25 µM (Table 3) were added to 100 ng of modified DNA. The reaction occurred in a thermocycler BioRad1000, following the established protocol detailed in Table 4. Beyond the DNA samples from MDS patients and healthy controls, it were also amplified three control samples, one of which without DNA (named "blank"), and both the methylated and unmethylated controls (EpiTect PCR Control DNA Set, Qiagen). After the PCR reaction, the amplified products were run on 2% agarose gels (NZYtech), stained with Midori Green Advance (Nippon Genetics Europe GmbH) and visualized under UV illumination.

3.4.3. Analysis of LINE-1 methylation by COBRA (Combined Bisulfite Restriction Analysis)

Combined bisulfite restriction analysis is a quantitative technique used to determine DNA methylation levels at specific gene loci in small amounts of genomic DNA. It associates bisulfite conversion based-PCR with restriction enzymatic digestion (Xiong e Laird, 1997). In this technique, genomic DNA samples are firstly treated with sodium bisulfite, allowing the introduction of methylation-dependent sequence differences into DNA and the conversion on unmethylated cytosines to uracyles. Subsequent PCR amplification replaces the uracil residues with thymines and the 5-methylcytosine residues with cytosines. This change results in the generation of a restriction site only if the cytosine in the GpG nucleotide is methylated. If the cytosine is unmethylated, the restriction site is not created and the methylation sensitive restriction enzyme does not cleave the sample. The PCR product is then digested and separated by gel electrophoresis.

In this case, the PCR mix contained 1x buffer (Reaction Buffer for NZYTaq DNA Polymerase), 0.2 mM dNTPs mix, 1.5 mM $MgCl_2$ (NZYtech), 2.5 U/µL polymerase (NZYTaq DNA Polymerase, NZYtech), 10% DMSO (ChemCruz™) and 0.25 µM of each one of the primers (Table 3). The PCR protocol is detailed in

Table **4**.

The digested fragments were resolved on 4% agarose gel, stained with Midori Green Advance (Nippon Genetics Europe GmbH) and visualized under UV illumination. The percentage of fully methylated sites were be calculated from the ratio between the enzyme-cleaved PCR products and the total amount of PCR products.

3.5. Statistical Analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) version 22, GraphPad Prism version 6 and Arlequin version 3.5.2. Allele and genotype frequencies were determined and the expected frequency of control genotypes was evaluated by the Hardy–Weinberg equilibrium test, which states that the amount of genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. To test the hypothesis of association between genetic polymorphisms and MDS, methods based on logistic regression analyses were used. Odds ratios and 95% confidence

intervals (95%CI) were calculated for each genotype compared with the homozygous for the major allele (the allele with greater frequency among controls), which were set as the reference genotype. Analysis were done under codominant, dominant and recessive inheritance models. The association between *P15* and *P16* methylation status and patient genotypes was carried out by Chi-Square test and Fisher's exact test. The association between *LINE-1* methylation degree and patient genotypes was performed using Kruskal-Wallis non-parametric test. The influence of polymorphisms in overall survival (OS) and evolution to AML was analysed by Kaplan Meier curves and verified by log-rank tests. One-way ANOVA test and was used to compare Vitamin B_{12} and folate values across the genotypes. For all statistical analysis, a $p<0.05$ was considered statistically significant.

RESULTS

4.1. Characterization of MDS patients and controls

This study was conducted in 77 MDS patients, with a median age of 73 years, (22 to 89 years), being 44 females (57.1%) and 33 males (42.9 %), as well as in 80 non-neoplastic controls being 37 females (46.2 %) and 43 males (53.8 %) with a median age of 61 years (23 to 92 years).

Accordingly to available data, patients were grouped according to the World Health Organization classification (2008). Three (3.9 %) patients were diagnosed with 5q-, five (6.5 %) with refractory anemia with excess blasts type 1, four (5.2 %) with refractory anemia with excess blasts type 2, five (6.5 %) with refractory anemia with ringed sideroblasts, forty-two (54.5 %) with refractory cytopenia with multilineage dysplasia, eight (10.4 %) with refractory cytopenia with unilineage dysplasia and ten (13.0 %) with chronic myelomonocytic leukemia. Distribution of patients in different MDS subtypes is graphically represented in Figure 15.

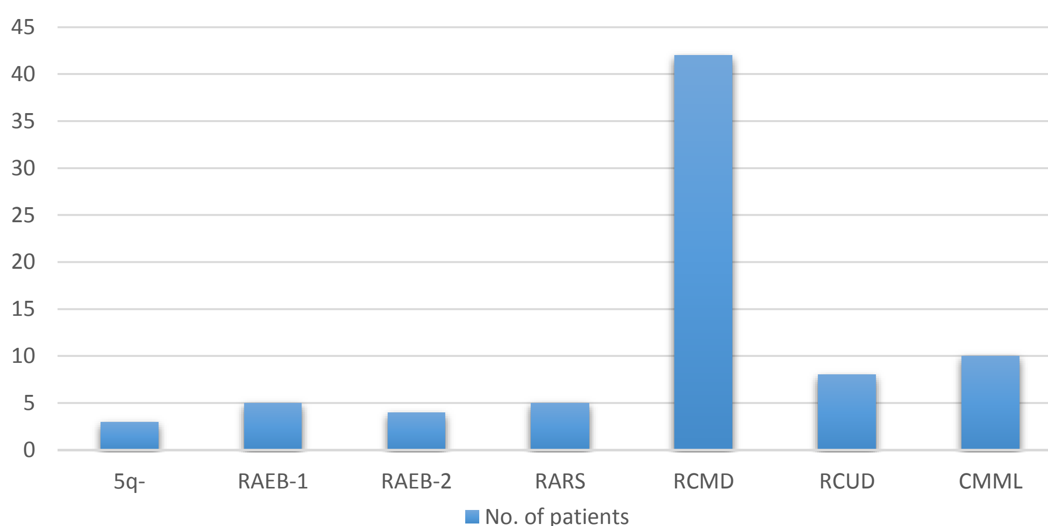

Figure 15. Graphic representation of distribution of patients into different MDS subtypes, according with WHO. 5q-, 5q Syndrome; RAEB-1, Refractory Anemia with Excess Blasts type 1; RAEB-2, Refractory Anemia with Excess Blasts type 2; RARS, Refractory Anemia with Ringed Sideroblasts; RCMD, Refractory Cytopenia with Multilineage Dysplasia; RCUD, Refractory Cytopenia with Unilineage Dysplasia; CMML, Chronic Myelomonocytic Leukemia.

Additionally, patients were characterized according to International Prognostic Scoring System (IPSS). As so, 28 patients were classified with low-risk risk (low), 28 with intermediate-1 risk (int-1) and 8 patients were included in the high-risk level group. The high-risk group comprises 7 individuals classified with intermediate-2 risk (int-2) and one high-risk patient.

Available clinical data with respect to 13 patients was insufficient to properly classification, and so only 64 patients were considered for this analysis.

Distribution of patients in different IPSS groups is graphically represented in Figure 16.

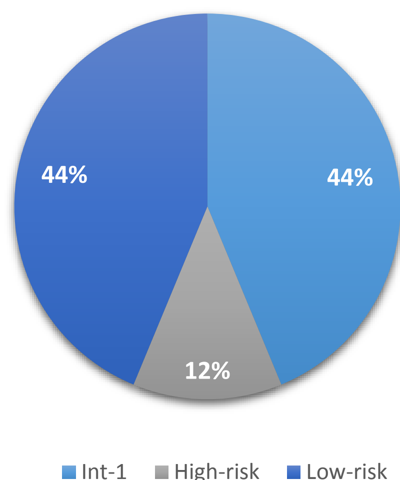

Figure 16. Graphic representation of distribution of patients into different IPSS categories. Int-1, Intermediate-1 risk.

4.2. Allelic and genotypic distribution of *MTR* (rs1805087 and rs2229276), *SLC19A1* (rs1051266), *CBS* (844ins68) and *MTRR* (rs162036) gene variants

In order to establish a relation between allele frequencies in patients when compared to those observed in controls, allele and genotypic frequencies were calculated, and presented in Table 5 and Table 6.

4.2.1. Allelic frequencies analysis

The results from the allele frequencies determinations for *MTR* rs1805087 evince a higher frequency of A allele in MDS patients (A, 83.1%) when compared to controls (A, 81.2%). Concerning to *MTR* rs2229276 polymorphic variant, allele frequency analysis denotes a higher prevalence of A allele (61.7%) in neoplastic individuals, in comparison to the control population (54.4%). Evaluation of allele frequencies for *SLC19A1* rs1051266 gene variant indicates that MDS patients have higher frequency of G allele (47.4%) and lower frequency for A allele (52.2%) when compared to those presented by controls (A, 63.8%; G, 36.2%). Analysis of *MTRR* rs162036 allele frequencies revealed that A allele is more prevalent in MDS patients (A, 85.7%; G, 14.3%) than it is in control group (A, 82.5%; G, 17.5%). Lastly, allele frequencies for

CBS 844ins68 showed a prevalence of the WT allele for both MDS patients (89.0%) and non-neoplastic individuals (89.4%).

All allele frequencies in MDS patients were in conform to Hardy-Weinberg equilibrium. The same was not observed in controls population, as allele frequencies for *MTR* rs2229276 and *CBS* 844ins68 are deviated from this equilibrium. However, our previous studies including this genetic variants in a larger population proved that the Hardy-Weinberg equilibrium is respected. Thus, this deviation can be overcome by enlarging the number of studied individuals.

Table 5. Allele frequencies for polymorphic variants of *MTR*, *SLC19A1*, *MTRR* and *CBS* genes, in patients and controls

	MTR rs1805087		***MTR*** rs2229276		***SLC19A1*** rs1051266		***MTRR*** rs162036		***CBS*** 844ins68	
	A (%)	G (%)	A (%)	G (%)	A (%)	G (%)	A (%)	G (%)	W (%)	Ins (%)
Reference (European P.)*	83.9	16.1	57.1	42.9	43.8	56.2	81.4	18.6	-	-
MDS	83.1	16.9	61.7	38.3	52.6	47.4	85.7	14.3	89.0	11.0
Controls	81.2	18.8	54.4	45.6	63.8	36.2	82.5	17.5	89.4	10.6

* Allele frequencies for European population were collected from Hap Map database, accordingly to dbSNP

4.2.2. Genotype frequencies analysis

Genotypic frequencies of *MTR* rs1805087, *MTR* rs2229276, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68 gene variants were calculated for patients and controls. Results are shown in Table 6.

In the polymorphic variant rs1805087 of *MTR* gene, a prevalence of AA genotype was observed, in higher frequency in MDS patients (68.8%) than in controls (65.0%). The homozygous genotype for the minor allele (GG) was found to be slightly higher in MDS patients (2.6%), while the AG genotype presented lower frequency (28.6%), compared to controls (2.5% and 32.5%, respectively).

Concerning *MTR* rs2229276 variant, AG genotype was found to be the most prevalent in patients (53.2%), even so, it was lower than in controls (78.8%). As to AA genotype, patients showed superior frequencies (35.1%) compared to those observed in control group (15.0%), and also higher prevalence of GG genotype (11.7%) when confronted with data for controls (6.25%). In relation to *SLC19A1* rs1051266 polymorphic variant, the most frequent genotype in patients was AG (45.5%), which was present in controls in a frequency of 40%. In contrast, the AA genotype in control group was found to be the most prevalent (43.8%), also occurring in 29.9% of MDS patients. The homozygous GG genotype was the less prevalent in both cases, even though it was more frequent in patients (24.7%) that in controls (16.2%).

Evaluation of genotype frequencies referring to *MTRR* rs162036 variant showed that AA genotype was present at higher frequencies in patients (75.3%) than in controls (70.0%), while AG (25.0%) and GG (5.0%) genotypes were found to be more frequent in controls, compared to MDS patients (28.8% and 3.9%, respectively).

Lastly, analysis of *CBS* 844ins68 polymorphic variant determined that the wild-type (WT) genotype was the most frequent, either in patients or in controls, showing higher frequencies in controls (83.8%) than in MDS patients (80.5%). In addition, accordingly to results, 16.9% of MDS patients showed a 68bp insertion in one of the alleles (WT-Ins), what was also reported in controls, albeit not so prevalent (11.2%). Finally, the 68bp insertion in both alleles (Ins-Ins) was observed with more prevalence in controls (5.0%), when compared to MDS patients (2.6%).

4.2.3. Association between *MTR*, *SLC19A1*, *CBS* and *MTR* gene variants individual genotypes and the risk of MDS development

In order to determine whether polymorphic variants from one-carbon metabolism enzymes are defined as risk or protector factors for the development of the disease, odds ratios (OR) were estimated by using conditional logistic regression. Codominant (DD vs Dd; DD vs dd), dominant (DD vs Dd + dd) and recessive (dd vs DD + Dd) models were used to assess OR (CI95%) and *p* values, where DD is the reference homozygous genotype for the ancestral allele.

The genotype frequencies distributions and odds ratio results are summarized in Table 6. There were no significant differences in *MTR* rs1805087, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68 genotypes between MDS patients and controls, and so the risk on MDS development may not be associated with this polymorphic variants.

On the other hand, the codominant model for *MTR* rs2229276 predicted that the heterozygous genotype AG has a 0.29-fold decreased risk of MDS (95%CI 0.13 – 0.63; p=0.002). Additionally, the presence of the G allele has a protector effect on the development of MDS, conferring a 0.33-fold reduction in risk of MDS (95%CI 0.15 – 0.72; p=0.005).

Table 6. Genotype frequencies and odds ratio for myelodysplastic syndromes

Model		Control group (N=80)		Myelodysplastic Syndromes (N=77)			
***MTR* rs1805087**		n	%	n	%	OR (CI95%)	*p*
CDM	AA	52	65.0	53	68.8	Ref.	
	AG (ref. AA)	26	32.5	22	28.6	0.83 (0.42-1.65)	0.594
	GG (ref. AA)	2	2.5	2	2.6	0.98 (0.13-7.23)	0.985
DM	AG+GG (ref. AA)					0.84 (0.43-1.64)	0.610
RM	GG (ref. AA + AG)					1.04 (0.14-7.57)	0.969
***MTR* rs2229276**							
CDM	AA	12	15.0	27	35.1	Ref.	
	AG (ref. AA)	63	78.75	41	53.2	**0.29 (0.13-0.63)**	**0.002**
	GG (ref. AA)	5	6.25	9	11.7	0.80 (0.22-2.90)	0.734
DM	AG+GG (ref. AA)					**0.33 (0.15-0.71)**	**0.005**
RM	GG (ref. AA + AG)					1.99 (0.64-6.22)	0.239
***SLC1A1*, rs1051266**							
CDM	GG	13	16.25	19	24.7	Ref.	
	AG (ref. GG)	32	40.0	35	45.4	0.75 (0.32-1.76)	0.505
	AA (ref. GG)	35	43.75	23	29.9	0.45 (0.19-1.08)	0.075
DM	AG+AA (ref. GG)					0.59 (0.27-1.30)	0.193
RM	AA (ref. GG + AG)					0.55 (0.28-1.06)	0.073
***MTRR*, rs162036**							
CDM	AA	56	70.0	58	75.3	Ref.	
	AG (ref. AA)	20	25.0	16	20.8	0.77 (0.36-1.64)	0.501
	GG (ref. AA)	4	5.0	3	3.9	0.72 (0.15-3.38)	0.681
DM	AG+GG (ref. AA)					0.76 (0.38-1.55)	0.455
RM	GG (ref. AA + AG)					0.77 (0.17-3.56)	0.738
***CBS*, 844ins68**							
CDM	WT	67	83.75	62	80.5	Ref.	
	WT-Ins (Ref. WT)	9	11.25	13	16.9	1.56 (0.62-3.91)	0.341
	Ins (Ref. WT)	4	5.0	2	2.6	0.54 (0.10-3.05)	0.486
DM	WT-Ins + Ins-Ins (Ref. WT)					1.25 (0.55-2.83)	0.597
RM	Ins-Ins (Ref. WT-WT + WT-ins)					0.51 (0.09-2.85)	0.440

Statistically significant values are highlighted in bold.
Ref., reference; OR, odds ratio; CI, confidence interval; CDM, codominant model; DM, dominant model; RM, recessive model

4.2.4. Association between *MTR*, *SLC19A1*, *CBS* and *MTR* gene variants genetic profiles and the risk of MDS development

Determination of the relative risk of disease development associated with different genetic profiles in *MTR* rs1805087/rs2229276, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68, was performed using Fisher's exact test.

Data was analysed employing Arlequin, from where genetic profiles were described according to our population, resulting a total of 49 profiles. Odds ratio was then estimated using Graphpad. Results are presented in Table 7.

According to our results, no statistically significant associations between gene profiles and the associated risk for MDS were observed. Even though two gene profiles were

associated with lower *p* values (AG AA GG AA WT-WT and AA AG AA AG WT-WT for *MTR* rs1805087, *MTR* rs2229276, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68, respectively) these results must be confirmed in larger and prospective studies.

Table 7. Relative risk for MDS associated with genetic profiles

	Profile	Odds ratio (CI95%)	*p*
1.	AA AA AA AA WT-ins	5.33 (0.25-112.90)	0.239
2.	AA AA AA AA Wt-Wt	7.56 (0.38-149.00)	0.116
3.	AA AA AA AG Ins-WT	1.04 (0.06-16.96)	1.000
4.	AA AA AA GG Wt-Wt	3.16 (0.13-78.75)	0.490
5.	AA AA AG AA Ins-WT	0.34 (0.01-8.53)	1.000
6.	AA AA AG AA WT-WT	3.20 (0.33-31.50)	0.361
7.	AA AA AG AG WT-WT	0.34 (0.01-8.53)	1.000
8.	AA AA GG AA WT-WT	2.11 (0.19-23.73)	0.615
9.	AA AG AA AA Ins-Ins	0.20 (0.01-4.29)	0.497
10.	AA AG AA AA WT-Ins	3.16 (0.13-78.7)	0.490
11.	AA AG AA AA WT-WT	0.43 (0.16-1.21)	0.142
12.	AA AG AA AG WT-WT	1.04 (0.14-7.58)	1.000
13.	AA AG AA GG WT-WT	0.143 (0.01-2.81)	0.245
14.	AA AG AG AA Ins-WT	0.51 (0.04-5.78)	1.000
15.	AA AG AG AA WT-WT	0.91 (0.33-2.51)	1.000
16.	AA AG AG AG Ins-WT	1.04 (0.06-16.93)	1.000
17.	AA AG AG AG WT-WT	0.82 (0.21-3.18)	1.000
18.	AA AG AG GG WT-Ins	3.16 (0.13-78.75)	0.490
19.	AA AG GG AA WT-Ins	5.33 (0.25-112.90)	0.239
20.	AA AG GG AA WT-WT	0.77 (0.17-3.56)	1.000
21.	AA AG GG AG WT-Ins	3.16 (0.13-78.75)	0.490
22.	AA AG GG AG WT-WT	1.04 (0.14-7.58)	1.000
23.	AA GG AA AA WT-WT	3.16 (0.13-78.75)	0.490
24.	AA GG AG AA WT-WT	2.14 (0.38-12.02)	0.437
25.	AA GG AG AG WT-WT	5.33 (0.25-112.90)	0.239
26.	AA GG GG AA WT-WT	5.33 (0.25-112.90)	0.239
27.	AA GG GG AG Ins-WT	0.35 (0.01-8.53)	1.000
28.	AA GG GG AG WT-WT	0.35 (0.01-8.53)	1.000
29.	AG AA AA AA WT-WT	2.14 (0.38-12.02)	0.437
30.	AG AA AG AA Ins-Ins	3.16 (0.13-78.75)	0.490
31.	AG AA AG AA Ins-WT	1.04 (0.06-16.93)	1.000
32.	AG AA AG AA WT-WT	1.04 (0.14-7.58)	1.000
33.	AG AA AG AG WT-WT	3.16 (0.13-78.75)	0.490
34.	AG AA GG AA WT-WT	9.86 (0.52-186.40)	0.056
35.	AG AG AA AA Ins-Ins	0.34 (0.01-8.26)	1.000
36.	AG AG AA AA Ins-WT	0.34 (0.013-8.26)	1.000
37.	AG AG AA AA WT-WT	0.20 (0.022-1.73)	0.210
38.	AG AG AA AG WT-WT	0.09 (0.01-1.63)	0.059
39.	AG AG AA GG WT-WT	3.157 (0.13-78.75)	0.490
40.	AG AG AG AA Ins-Ins	0.34 (0.01-8.26)	1.000
41.	AG AG AG AA WT-WT	1.04 (0.25-4.32)	1.000
42.	AG AG AG AG WT-Ins	3.16 (0.13-78.75)	0.490
43.	AG AG GG AA WT-Ins	3.16 (0.13-78.75)	0.490
44.	AG AG GG AA WT-WT	0.34 (0.034-3.32)	0.620
45.	AG GG AG GG WT-WT	0.34 (0.01-8.26)	1.000
46.	GG AA AG AA WT-WT	3.16 (0.13-78.75)	0.4904
47.	GG AA AG AG WT-WT	0.34 (0.01-8.26)	1.000
48.	GG AA GG AA Ins-Wt	0.34 (0.01-8.26)	1.000
49.	GG AA GG AG Ins-Ins	3.16 (0.13-78.75)	0.4904

The order of genes in which profiles are presented is *MTR* rs1805087, *MTR* rs2229276, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68, from the left to the right.

4.2.5. Association between *MTR* rs1805087 and *MTR* rs2229276 haplotypes and the risk of MDS development

In order to evaluate if *MTR* rs1805087 and rs2229276 influence together the risk for developing MDS, we performed haplotype analysis, using the software Arlequin.

Our results showed that haplotype analysis did not provide additional evidence for these genes in MDS development, as no statistical significance was obtained. Results are presented in Table 8.

Table 8. Relative risk for MDS associated with *MTR* haplotypes

Haplotype	OR (CI95%)	*p*
1. AA	1.32 (0.96-2.49)	0.42
2. AG	0.82 (0.43-1.5)	0.63
3. GA	0.96 (0.42-2.19)	1.00
4. GG	0.34 (0.01-8.5)	1.00

4.3. Evaluation of the influence of *MTR*, *SLC19A1*, *CBS* and *MTR* gene variants genotypes on the subtype of myelodysplastic syndromes, cytogenetic alterations and IPSS classification

In order to estimate if different genotypes were associated with different subtypes of disease, conditional logistic regression analysis was performed. Odds ratios were estimated with 95 percent confidence intervals.

As we can see in Table 9 and Table 10, none of the studied genotypic conditions referring to polymorphic variants from one-carbon metabolisms enzymes manifested any statistically relevant association with different MDS subtypes.

When analysing if the presence or absence of cytogenetic alterations was influenced by polymorphisms in enzymes from one-carbon metabolism, we distinguished three different groups among MDS patients: those who presented normal karyotype, those whose FISH was normal for the most common cytogenetic alterations in MDS, and a third group including these two characteristics.

As we can see in Table 11, the codominant model for *CBS* 844ins68 appeared to be statistically significant for those who presented a normal FISH test (OR=0.22; CI95% 0.05-0.96, p=0.044), and so the WT-Ins genotype can be associated with the absence of the less common cytogenetic alterations in MDS, compared to the reference genotype (WT).

Adittionally, with the intent to understand if there is a relation between polymorphic variants from *MTR*, *SLC19A1*, *MTRR* and *CBS* genes and the IPSS category into which patients were diagnosed, odds ratios were estimated by using conditional logistic regression. Results from statistical analysis are presented in Table 12.

The MDS patients risk groups (low, int-1 and high risk) do not appear be to influenced by *MTR* rs1805087, *MTR* rs2229276, *SLC19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68 polymorphic variants genotypes.

Table 9. Association between genotypes and International Prognostic Scoring System risk categories in myelodysplastic syndromes patients classified with 5q syndrome refractory and anemia with ringed sideroblasts and refractory anemia with excess blasts type 1 and 2.

Gene	Model		RARS		RAEB-1		RAEB-2	
			OR (CI95%)	*p*	OR (CI95%)	*p*	OR (CI95%)	*p*
***MTR* rs1805087**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	0.58 (0.06-5.53)	0.639	0.79 (0.08-8.07)	0.845	-	-
		GG (ref. AA)	-	-	16.67 (0.82-377.01)	0.067	-	-
	DM	AG+GG (ref. AA)	0.53 (0.06-5.04)	0.583	1.51 (0.24-9.71)	0.661	-	-
	RM	GG (ref. AA + AG)	-	-	17.75 (0.93-338.84)	0.056	-	-
***MTR* rs2229276**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	2.05 (0.20-20.83)	0.543	0.64 (0.08-4.85)	0.667	20.53 (0.20-20.83)	
		GG (ref. AA)	3.25 (0.18-58.06)	0.423	1.56 (0.12-19.60)	0.729	-	-
	DM	AG+GG (ref. AA)	2.26 (0.24-21.31)	0.476	0.80 (0.12-5.09)	0.811	1.66 (0.16-16.77)	0.668
	RM	GG (ref. AA + AG)	2.00 (0.20-20.17)	0.557	2.00 (0.20-20.17)	0.557	-	-
***SLC19A1* rs1051266**	CDM	GG	Ref.		Ref.		Ref.	
		AG (ref. GG)	1.69 (0.16-17.44)	0.661	0.51 (0.07-3.98)	0.525	-	-
		AA (ref. GG)	0.82 (0.05-14.02)	0.890	0.39 (0.03-4.62)	0.453	-	-
	DM	AG+AA (ref. GG)	1.33 (0.14-12.72)	0.803	0.46 (0.07-3.01)	0.420	-	-
	RM	AA (ref. GG + AG)	0.57 (0.06-5.38)	0.622	0.568 (0.06-5.38)	0.622	-	-
***MTRR* rs162036**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	-	-	0.64 (0.08-4.85)	0.667	4.00 (0.52-30.93)	0.184
		GG (ref. AA)	6.75 (0.50-91.47)	0.151	1.56 (0.12-19.60)	0.729	-	-
	DM	AG+GG (ref. AA)	0.75 (0.08-7.15)	0.803	0.80 (0.12-5.09)	0.811	3.29 (0.43-25.17)	0.251
	RM	GG (ref. AA + AG)	8.75 (0.65-118.20)	0.102	2.00 (0.20-20.17)		-	-
***CBS* 844ins68**	CDM	WT	Ref.		Ref.		Ref.	
		WT-Ins (Ref. WT)	1.21 (0.12-11.79)	0.871	1.21 (0.12-11.79)	0.871	1.64 (0.16-17.13)	0.680
		Ins (Ref: WT)	-	-	-	-	-	-
	DM	WT-Ins + Ins-Ins (Ref: WT)	1.04 (0.11-10.00)	0.976	1.04 (0.11-10.00)	0.976	1.40 (0.14-14.54)	0.776
	RM	Ins-Ins (Ref. WT + WT-Ins)	-	-	-	-	-	-

Ref., reference; OR, odds ratio; CI, confidence interval; CDM, codominant model; DM, dominant model; RM, recessive model; RARS, refractory anemia with ringed sideroblasts; RAEB-1, refractory anemia with excess blasts type 1; RAEB-2, refractory anemia with excess blasts type 2.

Table 10. Association between genotypes and International Prognostic Scoring System risk categories in Myelodysplastic Syndromes patients classified with refractory cytopenia with uniliniage dysplasia, refractory cytopenia with multilineage dysplasia, and chronic myelomonocytic leukemia

Gene	Model		RCUD		RCMD		CMML	
			OR (CI95%)	*p*	OR (CI95%)	*p*	OR (CI95%)	*p*
***MTR* rs1805087**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	2.72 (0.61-12.05)	0.187	0.93 (0.37-2.69)	0.989	1.74 (0.44-6.90)	0.430
		GG (ref. AA)	-	-	0.83 (0.05-13.94)	0.896	-	-
	DM	AG+GG (ref. AA)	2.45 (0.56-10.78)	0.235	0.98 (0.37-2.57)	0.964	1.57 (0.40-6.16)	0.520
	RM	GG (ref. AA + AG)	-	-	0.83 (0.05-13.76)	0.896	-	-
***MTR* rs2229276**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	0.62 (0.14-2.73)	0.529	0.72 (0.27-1.93)	0.515	1.37 (0.312-6.02)	0.676
		GG (ref. AA)	-	-	0.86 (0.19-3.94)	0.845	1.00 (0.09-11.03)	1.000
	DM	AG+GG (ref. AA)	0.50 (0.11-2.18)	0.357	0.74 (0.29-1.92)	0.542	1.30 (0.31-5.51)	0.720
	RM	GG (ref. AA + AG)	-	-	1.05 (0.26-4.24)	0.948	0.82 (0.09-7.35)	0.859
***SLC19A1* rs1051266**	CDM	GG	Ref.		Ref.		Ref.	
		AG (ref. GG)	3.00 (0.32-27.76)	0.333	0.61 (0.20-1.89)	0.394	0.35 (0.07-1.77)	0.205
		AA (ref. GG)	1.71 (0.14-20.50)	0.670	1.36 (0.39-4.77)	0.627	0.56 (0.11-2.90)	0.492
	DM	AG+AA (ref. GG)	2.47 (0.28-21.49)	0.412	0.83 (0.29-2.38)	0.736	0.43 (0.11-1.74)	0.237
	RM	AA (ref. GG + AG)	0.76 (0.14-4.09)	0.751	1.87 (0.68-5.15)	0.223	1.01 (0.24-4.29)	0.992
***MTRR* rs162036**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	1.24 (0.22-6.82)	0.8064	1.04 (0.34-3.19)	0.939	2.00 (0.44-9.08)	0.369
		GG (ref. AA)	-	-	0.41 (0.03-4.73)	0.472	4.33 (0.34-55.21)	0.259
	DM	AG+GG (ref. AA)	1.02 (0.19-5.53)	0.982	0.90 (0.32-2.55)	0.847	2.31 (0.58-9.27)	0.237
	RM	GG (ref. AA + AG)	-	-	0.40 (0.03-4.63)	0.465	3.61 (0.30-43.98)	0.314
***CBS* 844ins68**	CDM	WT	Ref.		Ref.		Ref.	
		WT-Ins (Ref. WT)	0.65 (0.07-5.83)	0.704	0.48 (0.14-1.64)	0.243	4.15 (0.97-17.64)	0.054
		Ins (Ref: WT)	-	-	-	-	-	-
	DM	WT-Ins + Ins-Ins (Ref: WT)	0.56 (0.06-4.94)	0.603	0.67 (0.22-2.09)	0.496	3.39 (0.82-14.05)	0.092
	RM	Ins-Ins (Ref. WT + WT-Ins)	-	-	0.67 (0.22-2.09)	0.496	-	-

Ref., reference; OR, odds ratio; CI, confidence interval; CDM, codominant model; DM, dominant model; RM, recessive model; RCUD, refractory cytopenia with uniliniage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; CMML, chronic myelomonocytic leukemia.

Table 11. Evaluation of the influence of polymorphisms in enzymes from one carbon metabolism and the presence or absence of cytogenetic alterations in myelodysplastic Syndromes patients

Gene	Model		Normal FISH		Normal Karyotype		Normal FISH + Normal Karyotype	
			OR (CI95%)	*p*	OR (IC95%)	*p*	OR (IC95%)	*p*
***MTR* rs1805087**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	1.60 (0.31-8.27)	0.575	1.30 (0.46-3.66)	0.614	1.69 (0.58-4.95)	0.340
		GG (ref. AA)	-	-	-	-	-	-
	DM	AG+GG (ref. AA)	1.69 (0.33-8.70)	0.531	1.19 (0.43-3.27)	0.742	1.56 (0.54-4.52)	0.414
	RM	GG (ref. AA + AG)	-	-	-	-	-	-
***MTR* rs2229276**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	0.72 (0.16-3.17)	0.661	0.48 (0.17-1.32)	0.155	0.35 (0.12-1.06)	0.063
		GG (ref. AA)	1.04 (0.09-11.52)	0.972	1.07 (0.23-4.92)	0.929	1.09 (0.24-5.03)	0.911
	DM	AG+GG (ref. AA)	0.76 (0.18-3.24)	0.715	0.56 (0.21-1.47)	0.241	0.45 (0.16-1.25)	0.128
	RM	GG (ref. AA + AG)	1.29 (0.14-11.54)	0.822	1.65 (0.41-6.67)	0.483	1.94 (0.47-8.01)	0.361
***SLC19A1* rs1051266**	CDM	GG	Ref.		Ref.		Ref.	
		AG (ref. GG)	2.58 (0.51-13.05)	0.252			3.20 (0.77-13.31)	0.110
		AA (ref. GG)	1.78 (0.34-9.16)	0.492	1.57 (0.45-5.43)	0.475	2.84 (0.63-12.78)	0.173
	DM	AG+AA (ref. GG)	2.18 (0.54-8.75)	0.273	1.54 (0.53-4.49)	0.432	3.05 (0.79-11.76)	0.106
	RM	AA (ref. GG + AG)	1.06 (0.25-4.53)	0.937	1.21 (0.45-3.25)	0.707	1.28 (0.45-3.65)	0.644
***MTRR* rs162036**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	0.42 (0.10-1.70)	0.224	0.73 (0.25-2.17)	0.576	0.36 (0.09-1.40)	0.139
		GG (ref. AA)	0.24 (0.02-3.06)	0.272	0.58 (0.05-6.73)	0.661	-	-
	DM	AG+GG (ref. AA)	0.42 (0.10-1.70)	0.224	0.73 (0.25-2.17)	0.576	0.36 (0.09-1.39)	0.139
	RM	GG (ref. AA + AG)	0.29 (0.02-3.54)	0.332	0.61 (0.05-7.03)	0.691	-	-
***CBS* 844ins68**	CDM	WT	Ref.		Ref.		Ref.	
		WT-Ins (Ref. WT)	**0.22 (0.05-0.96)**	**0.044**	1.83 (0.52-6.43)	0.345	0.67 (0.16-2.74)	0.574
		Ins (Ref: WT)	-	-	-	-	-	-
	DM	WT-Ins + Ins-Ins (Ref: WT)	0.28 (0.07-1.16)	0.080	1.31 (0.41-4.19)	0.652	0.54 (0.14-2.18)	0.391
	RM	Ins-Ins (Ref. WT + WT-Ins)	-	-	-	-	-	-

Ref., reference; OR, odds ratio; CI, confidence interval; CDM, codominant model; DM, dominant model; RM, recessive model
Statistically significant values are highlighted in bold.

Table 12. Analysis of the influence of polymorphisms in enzymes from one carbon metabolism and the International Prognostic Scoring System classification in myelodysplastic syndromes patients

Gene	Model		Low-risk		Intermediate-1 risk		High-risk	
			OR (CI95%)	*p*	OR (CI95%)	*p*	OR (CI95%)	*p*
***MTR* rs1805087**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	0,73 (0,240-2,22)	0,575	2,06 (0,68-6,12)	0,201	0,32 (0,04-2,80)	0,303
		GG (ref. AA)	-	-	-	-	-	-
	DM	AG+GG (ref. AA)	0,67 (0,22-2,00)	0,47	2,26 (0,76-6,75)	1,142	0,30 (0,03-2,64)	0,279
	RM	GG (ref. AA + AG)	-	-	-		-	-
***MTR* rs2229276**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	1,30 (0,44-3,82)	0,638	0,96 (0,33-2,81)	0,938	0,65 (0,15-2,94)	0,581
		GG (ref. AA)	1,56 (0,31-7,85)	0,593	1,30 (0,26-6,52)	0,75	-	-
	DM	AG+GG (ref. AA)	1,34 (0,48-3,79)	0,577	1,02 (0,36-2,85)	0,974	0,51 (0,11-2,28)	0,381
	RM	GG (ref. AA + AG)	1,33 (0,30-5,88)	0,704	1,33 (0,30-5,88)	0,704	-	-
***SLC19A1* rs1051266**	CDM	GG	Ref.		Ref.		Ref.	
		AG (ref. GG)	0,41 (0,11-1,46)	0,168	2,14 (0,59-7,84)	0,25	1,61 (0,15-17,02)	0,690
		AA (ref. GG)	0,44 (0,11-1,74)	0,245	1,33 (0,33-5,39)	0,687	3,50 (0,35-35,11)	0,287
	DM	AG+AA (ref. GG)	0,42 (0,130-1,38)	0,153	1,77 (0,52-5,94)	0,356	2,33 (0,26-20,66)	0,446
	RM	AA (ref. GG + AG)	0,80 (0,27-2,34)	0,684	0,80 (0,27-2,34)	0,684	2,50 (0,56-11,23)	0,232
***MTRR* rs162036**	CDM	AA	Ref.		Ref.		Ref.	
		AG (ref. AA)	0,64 (0,17-2,39)	0,503	0,84 (0,23-3,00)	0,787	3,00 (0,61-14,86)	0,178
		GG (ref. AA)	-	-	-	-	-	-
	DM	AG+GG (ref. AA)	0,95 (0,29-3,16)	0,939	0,65 (0,19-2,22)	0,459	2,45 (0,51-11,87)	0,264
	RM	GG (ref. AA + AG)	-	-	-	-	-	-
CBS 844ins68	CDM	WT	Ref.		Ref.		Ref.	
		WT-Ins (Ref. WT)	0,75 (0,16-3,46)	0,712	0,75 (0,16-3,46)	0,712	2,67 (0,44-16,32)	0,289
		Ins (Ref: WT)	1,25 (0,07-21,04)	0,877	1,25 (0,07-21,04)	0,877	-	-
	DM	WT-Ins + Ins-Ins (Ref: WT)	0,83 (0,21-3,29)	0,795	0,83 (0,21-3,29)	0,795	2,00 (0,34-11,70)	0,442
	RM	Ins-Ins (Ref. WT + WT-Ins)	1,30 (0,08-21,68)	0,857	1,30 (0,08-21,68)	0,857	-	-

Ref., reference; OR, odds ratio; CI, confidence interval; CDM, codominant model; DM, dominant model; RM, recessive model.

4.4. Association between genotypes from *MTR*, *SLC19A1*, *MTRR* and *CBS* gene variants and evolution to Acute Myeloid Leukemia in MDS patients

In order to analyze the effect of *MTR* (rs1805087 and rs2229276), *SLC19A1* (rs1051266), *MTRR* (rs162036) and *CBS* (844ins68) genetic variants in evolution to acute myeloid leukemia, Kaplan-Meier curves were obtained.

The results demonstrate that genetic polymorphisms from *MTR*, *SLC19A1* and *CBS* genes do not exert an interactive effect on the clinical evolution of patients to AML. In contrast, AG genotype from *MTRR* rs162036 variant proved to influence evolution to AML (p=0.049). Graphic representation is presented in Figure 17.

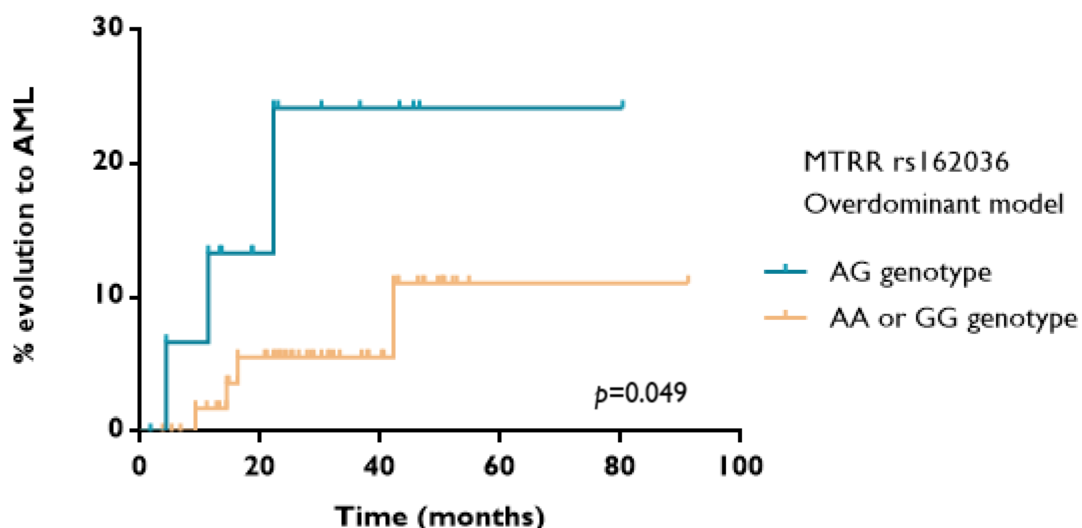

Figure 17. Representation of the influence of AA, AG and GG genotypes from *MTRR* rs162036 variant on evolution to AML, in MDS patients.

4.5. Analysis of the influence of *MTR*, *SLC19A1*, *MTRR* and *CBS* genes polymorphisms on overall survival of MDS patients

Evaluation of the effect of *MTR* (rs1805087 and rs2229276), *SLC19A1* (rs1051266), *MTRR* (rs162036) and *CBS* (844ins68) genetic variants was performed through Kaplan-Meier analysis. Statistical data revealed that none of the genotypes seemed to influence overall survival in MDS patients. However, even not statistically significant (p=0.071), we consider it important to highlight the influence of AG genotype from *MTRR* rs162036 polymorphic variant in worsening overall survival in MDS patients (Figure 18).

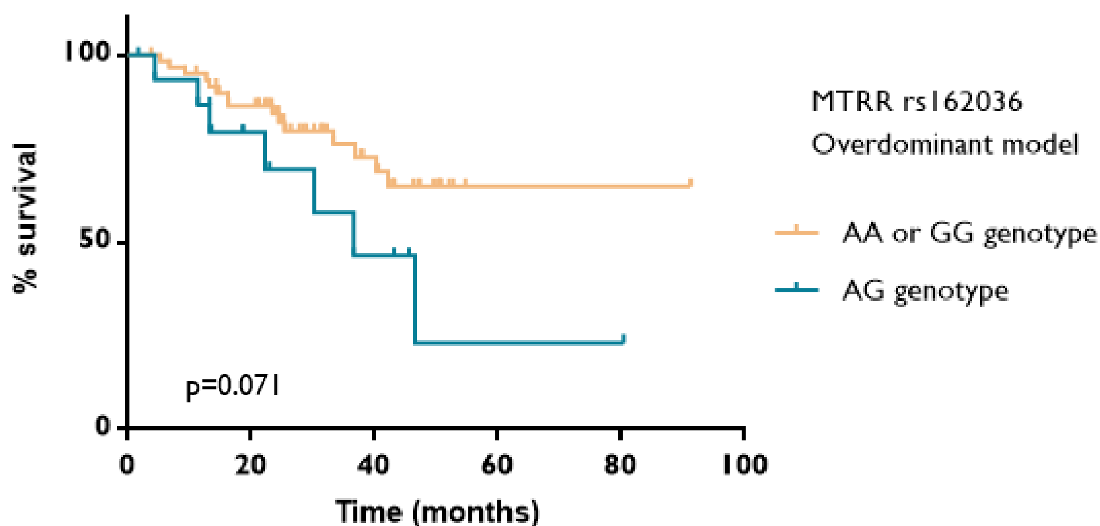

Figure 18. Representation of the influence of AA, AG and GG genotypes from *MTRR* rs162036 variant on overall survival, in MDS patients.

4.6. Analysis of the effect of *MTR* rs1805087 and rs2229276, *SCL19A1* rs1051266, *MTRR* rs162036 and *CBS* 844ins68 genotype variants on folate and vitamin B12 levels in MDS patients.

When testing whether the polymorphic variants of enzyme from one-carbon metabolism were associated with folate and vitamin B_{12} levels, Kruskal-Wallis and Mann-Whitney tests were used.

As shown in table 10, folate levels did not manifest any significant association with *MTR*, *SLC19A1*, *MTRR* and *CBS* variants genotypes in MDS patients. In contrast, we found that patients with GG genotype in the polymorphism rs2229276 of *MTR* gene had significantly higher levels of vitamin B_{12}, compared to those who had AG or AA genotypes, as observed in Figure 18 (p=0.0188).

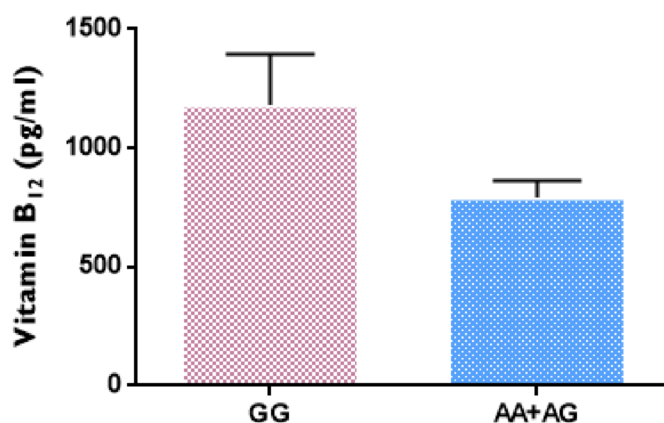

Figure 19. Analysis of vitamin B12 levels in MDS patients, according to *MTR* rs2229276 GG and AA+AG variant genotypes. The results are represented as mean ± SEM (standard error of the mean) of patients with GG and AA+AG genotypes.

Table 13. Correlation between folate and vitamin B12 levels and genotypes in polymorphisms of genes from one-carbon metabolism

	Folate (N=74)		**Vitamin B_{12} (N=74)**	
***MTR* rs1805087**	p	mean value	p	mean value
AA	0,212	12,39	0,7170	811,73
AG	0,168	16,38	0,3825	931,43
GG	0,802	14,50	0,1892	392,50
AA vs AG vs GG	0,380		0,2854	
***MTR* rs2229276**				
AA	0,203	12,86	0,490	814,52
AG	0,199	14,32	0,548	776,82
GG	0,935	12,40	**0,039**	**1181,88**
AA vs AG vs GG	0,400		0,119	
***SLC19A1* rs1051266**				
AA	0,540	12,95	0,089	697,86
AG	0,825	13,77	0,131	912,86
GG	0,707	13,93	0,983	841,00
AA vs AG vs GG	0,814		0,193	
***MTRR* rs162036**				
AA	0,404	13,33	0,157	778,63
AG	0,367	14,26	0,077	1073,63
GG	0,943	15,20	0,500	481,00
AA vs AG vs GG	0,660		0,175	
***CBS* 844ins68**				
WT	0,555	13,34	0,512	837,39
WT-ins	0,912	12,92	0,480	848,54
Ins	0,131	25,00	0,985	653,00
WT vs WT-ins vs Ins	0,214		0,773	

4.7. The interplay between genetic polymorphisms from *MTR*, *MTRR*, *SLC19A1* and *CBS* genes and the methylation patterns of *P15* and *P16* genes

According to data obtained from MS-PCR, methylation of *P15* was present in 69.6% of MDS patients, while *P16* was found to be methylated in 27.3% of patients. The influence of genetic variants in enzymes from one-carbon metabolism in methylation status of *P15* and *P16* was assessed by performing Fisher's exact test.

In Table 12, we observed that genetic variants in *MTR*, *SCL19A1*, *CBS* and *MTRR* genes did not associated significantly with *P15* methylation patterns (p>0.05 for all cases). However, *SLC19A1* rs1051266 polymorphic variant was found to be associated with *P16* methylation in MDS patients. Results show that GG genotype (recessive model) influences negatively *P16* methylation (p=0.032). In relation to *MTR*, *CBS* and *MTRR* genes, a statistically significance between polymorphic variants and *P16* methylation patterns was not observed.

Table 14. Methylation of *P15* and *P16* genes according to different genotypes

	P15		***P16***	
	CI95%	***p***	**CI95%**	***p***
***MTR* rs1805087**				
AA	0,98 (0,31-3,077)	1,000	1,18 (0,36-3,92)	1.000
AG	1,18 (0,36-3,92)	1,000	0,69 (0,19-2,48)	0,759
GG	0,422 (0,025-7,11)	0.517	2,76 (0,16-46,7)	0,474
***MTR* rs2229276**				
AG	0,70 (0,24-2,079)	0.593	2,20 (0,68-7,14)	0.263
AA	1,45 (0,44-4,75)	0.771	0,57 (0,16-2,02)	0,549
GG	1,45 (0,25-8,27)	1.000	0,42 (0,05-3,68)	0,664
***SLC19A1* rs1051266**				
AG	1,56 (0,53-4,63)	0.589	0,82 (0,27-2,47)	0,785
GG	0,65 (0,21-2,03)	0,557	**3,80 (1,19-12,09)**	**0.032**
AA	0,92 (0,29-2,91)	1.000	0,23 (0,047-1,11)	0,069
***MTRR* rs162036**				
AA	0,45 (0,11-1,79)	0,353	0,77 (0,22-2,650)	0,751
AG	3,18 (0,64-15,78)	0.197	1,67 (0,47-5,88)	0,503
GG	0,42 (0,025-7,11)	0.571	0,50 (0,02-11.00)	1,000
***CBS* 844ins68**				
WT	0,79 (0,219-2,88)	1.000	0,46 (0,14-1,56)	0,322
WT-ins	1,57 (0,38-6,47)	0,739	2,93 (0,82-10,39)	0,161
ins	0,42 (0,025-7,11)	0,517	0,503 (0,02-10,99)	1,000

We also analised the influence of the polymosphisms in patients who had both genes methylated, or none of them methylated. No statistical significance was obtained (data not shown).

4.8. Evaluation of the influence of genetic polymorphisms in global methylation of LINE-1

To explore the possible predictive value of in global methylation of LINE-1 retrotransposon, we performed Kruskal-Wallis and Mann-Whitney test and summarize obtained results in Table 13.

Although genotypes associated with polymorphic variants in *MTR*, *MTRR* and *CBS* genes were not significantly associated with LINE-1 methylation, there were modest differences in mean LINE-1 methylation and the variant rs1051266 from *SLC19A1* gene, in MDS patients (p=0.089) (Figure 19). Results are presented in Table 13.

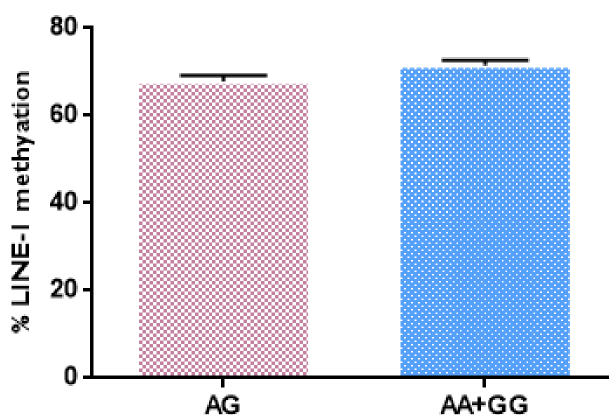

Figure 20. Analysis of the percentage of LINE-1 methylation in MDS patients, according to *SLC19A1* rs1051266 variant genotypes. The results are represented as mean ± SEM (standard error of the mean) of patients with AG and AA+GG genotypes.

Table 15. Influence of one-carbon metabolism genotypes on LINE-1 methylation in MDS patients.

	***MTR* rs1805087**		***MTR* rs2229276**		***SLC19A1* rs1051266**		***MTRR* rs162036**		***CBS* 844ins86**	
	p	% Met.	*p*	% Met.	*p*	% Met.	*p*	%Met.	*p*	% Met.
AA/WT	0.615	69.12	0.337	71.07	0.267	71.73	0.353	69.35	0.202	69.2
AG/WT-Ins	0.323	77.0	0.897	66.67	0.081	71.21	0.706	75.67	0.158	78.0
GG/Ins	0.256	70.82	0.226	69.69	0.412	67.82	0.225	70.47	0.477	71.4
AA vs AG vs GG	0.250	-	0.382	-	0.216	-	0.396	-	-	-
WT vs Ins Vs WT-Ins	-	-	-	-	-	-	-	-	0.229	-

% Met, percentage of methylation; AA, AG and GG genotypes are associated with *MTR*, *SLC19A1* and *MTRR* genes; WT, Ins and WT-Ins are related with *CBS* gene

DISCUSSION

Cancer is a leading cause of mortality worldwide, predicted to rise to more than 24 million new cases globally in 2035 (American Association for Cancer Research, 2014). The multi-step progress by which normal cells become progressively transformed to malignancy is called tumorigenesis , and it is now known to require the sequential acquisition of mutations which arise as a consequence of damage to the genome. The vast majority of mutations that give rise to cancer are not inherited, but arise spontaneously as a consequence of chemical damage to DNA, resulting in altered function of crucial genes (Bertram, 2000). Most of mutations leading to cancer affect cellular machinery that controls cell division, DNA damage, and signal transduction pathways (Lobo *et al.*, 2007).

The most common type of mutational change is the single nucleotide substitution, and when it has a population frequency of at least 1%, it is called a single nucleotide polymorphism (SNP). Most SNPs occur in noncoding regions of the genome, but an estimated 10 000–50 000 SNPs occur in coding regions and give rise to a change in amino acid in the protein product (Wright, Human & Unit, 2005). Genetic association studies with SNPs targeting cancer seek to determine prognostic information for survival, complications or response to pharmacological intervention, and thus, could be applied to clinical decision making. For instance, SNP can be protective in one case, whereas, in another, confer increased susceptibility. As so, genetic variants could alter the risk for metastatic or aggressive tumor (Erichsen & Chanock, 2004), and influence the development of cancer through numerous mechanisms, such as alterations in DNA repair, impairment in cell proliferation or modifications in the metabolism of agents that predispose to cancer (Braun & Anderson, 2007)

Myelodysplastic syndromes are a family of clonal disorders characterized by inefficient hematopoiesis, peripheral blood cytopenias, and risk of progression to AML. Much of its phenotypic heterogeneity is likely due to the variety of genetic lesions that contribute to disease pathogenesis. Furthermore, the majority of patients have mutations that alter the sequence and function of oncogenes or tumor suppressor genes (Bejar, Levine & Ebert, 2011).

Even though mutations have been shown to occur in MDS, a notable proportion of these affect genes involved in epigenetic maintenance, suggesting a dominant role of epigenomic deregulation in the pathogenesis of MDS. Aberrant DNA methylation is the dominant and most well-studied epigenetic alteration in MDS. Various genes, including cell cycle regulators, apoptotic genes, and DNA repair genes, are hypermethylated (epigenetically silenced) and have roles in pathogenesis and progression to leukemia (Khan *et al.*, 2013). In

contrast to increased DNA methylation, cancer-associated hypomethylation of DNA is frequently associated with repeat DNA elements, particularly long interspersed elements (LINE-1), which promotes carcinogenesis in multiple ways, including disruption of genes and genomic instability (Pavicic *et al.*, 2012).

One of the most frequently and best studied epigenetic events in MDS is the silencing of the cyclin-dependent kinase inhibitor gene *P15*, which controls the progression of cells from G1 to S phase. (Solomon *et al.*, 2008). Hypermethylation of *P15* promoter region occurs in approximately 50 per cent of MDS cases, and it has been reported to be acquired during disease progression and associated with leukemic transformation (Solomon *et al.*, 2008; Tien *et al.*, 2001). Hypermethylation of *P16* gene has also been found to be associated with myelodysplastic syndrome as well with the clonal progession in leukemia patients (Solomon *et al.*, 2008).

The network that is implicated in methylation reactions and nucleotide synthesis is known as one-carbon metabolism, in which folate acts as a methyl donor, thus playing a central role in both processes. As so, folate status can potentially be perturbed by polymorphisms in the genes involved in its metabolism (Liu & Ward, 2010). Over the past decades, dietary folate and genetic polymorphisms that alter folate metabolism have been linked to diseases such as Down's Syndrome (James *et al.*, 1999), colorectal cancer (Choi & Mason, 2002) , lymphoid malignancies (Matsuo *et al.*, 2001) and several other neoplasias.

In this context, we investigated the relevance of polymorphisms in enzymes from one-carbon metabolism and their possible correlation with the development of MDS, as well as their influence in the methylation status of tumor suppressor genes. Additionally, folate and vitamin B_{12} levels were evaluated according to polymorphisms, as well as LINE-1 methylation.

As previously mentioned, the 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*) gene encodes the methionine synthase enzyme, which plays a role in folate metabolism, catalyzing the remethylation of homocysteine to methionine, a reaction essential to adequately maintain normal methionine and intracellular homocysteine concentrations. An adenine to guanine transition at position 2756 of the *MTR* gene results in the substitution of aspartic acid, with glycine, and is related to alterations in the folate metabolic pathway, thus possibly influencing the risk of cancer (Galbiatti *et al.*, 2010). The functional role of this polymorphism has not yet been fully investigated and some results are controversial, but it is postulated to be a functional mutation as it occurs in a protein domain

involved in reductive methylation and regeneration of an active enzyme. (Ho, Massey & King, 2013).

Previous studies have demonstrated significant risk elevation with GG genotype for diverse pathologies, as follicular lymphoma (Matsuo *et al.*, 2001) or prostate cancer (Sharp & Little, 2004). Contrary, other investigations emphasize that *MTR* A2756G polymorphism may be a reduced risk factor for cancer, especially for ALL and colorectal cancer (Yu *et al.*, 2010), evidencing that that the biological role of this polymorphism is not quite clear now. In our study, we found no significant differences in *MTR* rs1805087 gene variants between MDS patients and controls, although recessive model for RAEB-2 showed a prevalence of GG genotype in this disease subtype (P=0.056). However, the confidence interval for odds ratio is too large to be considered significant, probably because the reduced number of patients comprised in this subtype, so this genotype may not be predictive of the increased risk for MDS and, in this particular case, for RAEB-1.

In relation to the effect of this polymorphism in methylation, available data is inconsistent. Initially, it was reported an association of the mutant 2756G allele with a decrease in enzyme activity, and thus an elevation in homocysteine levels, leading to DNA hypomethylation (Matsuo *et al.*, 2001). This results were consistent with those presented by Paz and her collaborators, who demonstrated that tumors occurring in homozygous carriers of the 2756G allele showed a lower number of hypermethylated CpG islands of tumor suppressor genes (Paz *et al.*, 2002). However, in subsequent investigations, some studies showed that subjects homozygous for G allele present significantly higher mean DNA methylation levels than the carriers of the wild-type *MTR* 2756AA genotype, indicating an increased enzymatic activity of the variant genotype (Weiner *et al.*, 2014). Despite this, our results show that methylation of *P15* and *P16* genes is not genotype-dependent for this polymorphic variant, as no statistically significance was obtained.

We also evaluated the effect of a second polymorphic variant in *MTR* (rs2229276), which encodes a missense mutation where a cytosine is replaced by a guanine at position 2758 of *MTR* gene. Concerning our attempt to investigate the association between this polymorphism with the risk for neoplasia, we observed that the AG genotype was associated with a decreased risk for neoplasia development, in a 0.29-fold (95%CI 0.13 – 0.63; p=0.002). In addition, we found that this protective effect may be due to the presence of G allele, as the dominant model (AA vs AG+GG) showed statistical significance (95%CI 0.15 – 0.72; p=0.005) and thus, one allele is sufficient to influence the risk of disease development. In this case, the

effect resulted in a 0.33-fold decrease in the risk of developing MDS. According to what we know so far, no studies have established a relation between this polymorphic variant and the risk of developing hematological malignancies.

Next, we analyzed the effect of polymorphisms in the levels of vitamin B_{12}, we found that homozygous individuals for the minor allele (GG) presented considerable higher levels of this metabolite (p=0.039). Vitamin B_{12} deficiency decrease the catabolism of homocysteine and causes elevation of intracellular homocysteine levels (Yasar *et al.*, 2012). In this context, we can assume that increased levels of vitamin B_{12} result in a potentiation of *MTR* enzyme and thus an increase of DNA methylation. However, we did not find any association between GG genotype and methylation of *P15* and *P16* genes.

According to literature, vitamin B_{12} and folic acid are major determinants of homocysteine levels, and a nutritional deficiency in either of these two vitamins results in hyperhomocysteinaemia (Yasar *et al.*, 2012). Nevertheless, our results did not show significant association between *MTR* rs2229276 polymorphism and folic acid levels. This may be explained by intermediate mechanisms in folate metabolism. As so, it would be important to evaluate the role of other enzymes that are involved in these mechanisms, such as *MTHFR* or *DHFR*. Additionally, several studies have evaluated the influence of this polymorphism in homocysteine levels (Sharp & Little, 2004), so we consider it would be an important next step in this work.

The principal mechanism by which folates are delivered to mammalian cells and tissues from the systemic circulation at neutral pH is the reduced folate carrier (RFC), which belongs to the solute carrier (SLC) group of transporters (Matherly, Hou & Deng, 2007). It has been reported that loss of *RFC* expression or function may have potentially profound pathophysiologic consequences including cancer (Matherly, Hou & Deng, 2007). Particularly in rs1051266, the G variant has been correlated with lower plasma folate and higher homocysteine levels in healthy individuals. On the other hand, individuals carriers of the A variant were reported to be associated with worst ALL (acute lymophoblastic leukemia) outcomes (Laverdie *et al.*, 2002). Our results, even not statistically significant, showed that individuals homozygous for the mutant variant (AA) may be associated with a decrease in the risk of MDS development. These results seemed to be concordant with the prevalence of GG individuals in statistically significant levels of *P16* methylation (P=0.0317), as *P16* is a tumor suppressor gene, and suppressor genes are frequently silenced by DNA methylation in cancers. In addition, we found that *P16* methylation was already been reported in

hematological malignancies such as non-Hodgkin's lymphoma (Herman *et al.*, 1997) and acute leukemia (Guo *et al.*, 2000).

Additionally, the AG genotype for this polymorphic variant was found to be associated, even with no statistical significance, with lower levels of LINE-1 methylation in MDS patients. This result may reveal the role of A allele in this polymorphism as a protective element. In fact, individuals carrying both AA alleles (overdominant and recessivel models) show modest differences between MDS patients and controls. In literature, we found that other polymorphic variant from *SLC19A1* gene was associated with LINE-1 methylation (Tajuddin *et al.*, 2013), so we consider it important to enlarge the number of polymorphic variants per gene.

When analyzing the hypothetical role of *MTRR* gene in disease, we did not find any studies involving rs162036 variant and myeloid neoplasia. However, we found that the homozygous genotype for the mutant allele variant (GG) has previously been associated with spina bifida and congenital heart defects (Shaw *et al.*, 2009), as well as an increased risk for Parkinson's disease among Asian population (Fong *et al.*, 2011). However, it is not described if rs162036 leads to deficient levels of *MTRR* or otherwise differential function.

In the analysis of this polymorphic variant in myelodysplastic syndromes, we did not find any association between rs162036 variants and MDS development. However, we found an association between AG genotype and MDS patients' progression to AML, and this variant was also observed in patients with worse overall survival. Results concerning evolution to AML showed that individuals carrying AG genotype were associated with a significant increase in evolution to AML (P=0.049). According to this results, a worse overall survival related to AG genotype would be expected. Our results showed that, even with no statistical significance, heterozygous individuals for *MTRR* rs162036 had lower overall survival rates. In fact, four of the seven patients who progressed to AML presented AG genotype, and none of the patients who experienced AML transformation did survive.

When evaluating the effect of genetic variants from *MTRR* rs162036 on vitamin B_{12} and folate levels, statistical data revealed that these levels were not influenced by polymorphic variants. However, AG individuals presented substantially higher concentrations of vitamin B_{12} than homozygous MDS patients, so we can hypothesize that higher values of this nutrient may

influence the predisposition of MDS patients to progress to AML and to be associated with worse overall survival.

In the analysis of *CBS* gene, which is the central enzyme in the transsulfuration pathway that irreversibly metabolizes homocysteine to cystathionine, we observed that heterozygous genotype WT-Ins was predominantly associated with MDS patients included in CMML subtype. However, this result lacked in terms of confidence interval, which was not trustworthy and so, larger studies with an increased number of participants are necessary in order to accomplish reliable results.

Despite that, conflicting studies have been reported regarding the biochemical effects of the 844ins68 polymorphism on *CBS* functions. Tsai *et al* (Tsai *et al.*, 1996) demonstrated that carriers of the 844ins68 polymorphism had significantly lower total plasma homocysteine levels and so this polymorphism was associated with higher *CBS* enzyme activities. However, the investigation carried by Silaste *et al* (Silaste *et al.*, 2001) did not show differences in plasma homocysteine levels between patients with or without the polymorphism, and so we hypothesized that it does not affect *CBS* gene activity. More recently, 844ins68 polymorphism has been reported to disrupt the protein, decreasing the functional activity of *CBS* (Gallegos-Arreola *et al.*, 2014). These controversial studies highlight the importance of the quantification of homocysteine levels as an important marker of the influence of polymorphisms in *CBS*, such as 844ins68.

Regarding cytogenetic alterations, our results showed a correlation between the absence of these alterations with the dominant model for *CBS* gene, which establishes the relation between the reference genotype (WT for both alleles) and the heterozygous genotype (WT-Ins). This analysis proved to influence individuals who presented a normal fluorescence in situ hybridization test (FISH) test with the most frequent clonal abnormalities found in MDS (trisomy 8, loss or deletion of chromosome 5, and loss or deletion of chromosome 7) (West *et al.*, 2000). The outcome is not predictive for rare cytogenetic alterations that may be present in patients' karyotype, although not detected by FISH test. However, we can speculate that this polymorphic variant is predictive of a better outcome for the most common cytogenetic alteration in MDS.

Evaluation of the interplay between genetic polymorphisms in enzymes from one-carbon metabolism and IPSS classification among MDS patients did not proved to be statistically significant in any of the studied polymorphic variants, and the same was observed

when we evaluated the influence of polymorphisms according to MDS subtypes. This result may be justified by the small number of patients that each group includes.

After evaluating the individual effect of genetic polymorphisms in MDS for each one of the studied genes, we considered it relevant to study whether genes also interact with each other to modify the risk of MDS outcomes. To this end, genetic profiles were assessed. We performed a combined analysis for *MTR*, *SLC19A1*, *MTRR* and *CBS* genes, to understand if their effect is associated with a higher or lower predisposition to develop MDS. Results were not predictive of the effect of the genetic profiles in modifying risk for MDS development, as none of the 59 resultant profiles showed statistical significance. This can be sustained by the reduced number of patients, that was not enough to establish proper analysis, as many genetic profiles accounted a very small number of individuals.

Overall, in order to perform a more accurate analysis of all parameters discussed in this work, results need to be confirmed in larger, systematic and prospective studies. Additionally, other enzymes from one-carbon metabolism should be introduced in this study, as well as the evaluation of homocysteine levels, which were found to be influenced by genetic variants in several studies concerning one-carbon metabolism. Furthermore, evaluation of the biological and functional consequences of these genetic variants is fundamental, so we can accurately infer about the consequences that they may be associated with. Additionally, in order to properly evaluate progression to AML and overall survival, a continued follow-up of all patients is of utmost relevance.

CONCLUSION

The aim of this study was to evaluate the effect of polymorphic variants of *MTR* (rs1805087 and rs2229276), *SLC19A1* (rs1051266), *CBS* (844ins68) and *MTRR* (rs162936) genes in the pathogenesis of myelodysplastic syndromes (MDS). To this end, we investigated the influence of these polymorphisms on the risk of developing MDS, as well as their role on disease progression to acute myeloid leukemia and on patients' overall survival. As *MTR*, *MTRR*, *SLC19A1* and *MTRR* genes are involved in one-carbon metabolism and this cycle modulates DNA methylation through metabolites such as folate and vitamin B_{12}, we also analysed the relation between polymorphisms and the levels of this nutrients, as well as the methylation degree of *P15*, *P16* and LINE-1, in MDS patients.

Our results reveal that the studied genes may be involved in myelodyasplastic syndromes in different ways. As so:

- Analysis of *MTR* rs1805087 gene variants did not reveal any statistically significant association with MDS;

- The study of polymorphic variants of *MTR* rs2229276 demonstrated that the AG genotype confers a 0.29-fold decreased risk for the development of MDS (CI95% 0.13-0.63; p=0.002). We also concluded that the presence of the G allele is associated with a protective effect (OR=0.33; CI95% 0.15 – 0.72; p=0.005) for the disease development. In addition, statistically significant levels of vitamin B_{12} are found in patients who are homozygous for G allele (P=0.02);

- When evaluating *SLC19A1* rs1051266 polymorphic variants, we verified that individuals carrying GG genotype present significant higher levels of methylation of tumor suppressor gene *P16* compared to those who have AA or AG genotype (OR=3.80; CI95% 1.19-12.09; 0.032);

- The presence of the AG genotype related with the polymorphism rs162036 in *MTRR* gene is associated with a worse prognosis in MDS, as it negatively influences patients' evolution to AML (p=0.049). Additionally, lower overall survival rates are observed in individuals who carry this genotype;

- Individuals carrying the heterozygous genotype (WT-Ins) for the *CBS* 844ins68 variant are significantly associated with a normal karyotype for the most frequent cytogenetic alterations in MDS (OR=0.22; CI95% 0.05-0.96; p=0.044);

- Polymorphisms in enzymes for one-carbon metabolism did not prove to influence folate levels, *P15* and LINE-1 methylation degree in MDS patients.

- Overall, these results allow us to affirm that polymorphisms in genes involved in one-carbon metabolism are predictive of MDS development. Thus, the use of these genetic variants as susceptibility biomarkers can possibly be on the line of future researches.

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