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# *GENE EXPRESSION OF NFE2L2 IN MYELODYSPLASTIC SYNDROME PATIENTS – CLINICAL IMPLICATIONS*

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# GENE EXPRESSION OF *NFE2L2* IN MYELODYSPLASTIC SYNDROME PATIENTS – CLINICAL IMPLICATIONS

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## ABSTRACT

**Introduction**: Myelodysplastic syndromes (MDS) are clonal stem-cell disorders that are characterized by ineffective haematopoiesis, peripheral blood cytopenias, and a higher progression to acute myeloid leukaemia (AML). The pathogenesis of MDS is complex and involves multiple genetic and epigenetic events, and although the significant progress in understanding the molecular genetics aberrations in MDS over the last decade its pathogenesis is not yet clear. Oxidative stress (OS), resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant defences, contributes to cell proliferation and damage, as well as to apoptosis and dysfunctional haematopoiesis. Nuclear factor-erythroid 2-related factor 2 (NRF2), encoded by the *NFE2L2* gene, is a key transcriptional activator of the antioxidant response pathway that has been identified as a protector of tumorigenesis. However, enhanced NRF2 activity has been found in a great number of solid and hematologic tumours and has been related with higher survival of neoplastic cells.

**Objectives**: Evaluate the expression levels of *NFE2L2* gene in MDS patients and correlate it with clinical and analytical parameters, exploring its potential role as diagnostic and prognostic biomarker, namely as a predictor of AML transformation.

**Materials and Methods**: Peripheral blood samples were collected from 55 MDS patients and 44 healthy controls. Total RNA was isolated from peripheral blood leukocytes and transcribed to cDNA. *NFE2L2* expression was quantified by real-time PCR. Comparison between groups of patients and controls was performed using nonparametric Mann-Whithney and Kruskal-Wallis tests. The ROC curves analysis were performed. Survival analysis was completed using Kaplan-Meier method.

**Results and Discussion:** Our results show no differences in the expression levels of *NFE2L2* between the MDS patients and the control group (MDS: median 2,29; interquartile range 6,037;

CTL: median 3,40; interquartile range 3,40; $p$=0,816). However, when patients were stratified according to MDS subtypes and compared among them, we found that refractory cytopenia with multilineage dysplasia (RCMD) patients had lower expression levels of *NFE2L2* when compared with the others subtypes (RCMD: median 1,48; interquartile range 1,41; $p$<0,05), which might suggest a higher participation of *NFE2L2*/NRF2 in the pathogenesis of this MDS subgroup. We also observed that *NFE2L2* is overexpressed in MDS patients who progress to AML (AML: median 8,57; interquartile range 13,57; Non-AML: median 2,12; interquartile range 4,03; $p$=0,018), with a sensitivity of 100% and a specificity of 76,5% at a cut-off value of 5,44 ($p$=0,021), therefore it could be used as a potential biomarker to identify MDS patients at high risk of progression to AML. No relations were observed between *NFE2L2* expression pattern and any laboratorial parameter, neither IPSS nor survival.

**Conclusion**: In summary, our results suggest that *NFE2L2* could be used as a new potential biomarker for prediction of AML progression in MDS patients. Over the last decade, significant progress in understanding the molecular genetics aberrations in MDS has been made, however, further studies are needed in order to understand the importance of *NFE2L2*/NFR2 in MDS pathogenesis, particularly in RCDM patients and in high-risk patients.

**KEY-WORDS**

*NFE2L2*/NRF2; Oxidative Stress; Myelodysplastic syndrome; Biomarkers; Prognosis.

**RESUMO**

**Introdução:** A Síndrome Mielodisplásica (SMD) é uma doença clonal que é caracterizada por hematopoiese ineficaz, citopenias periféricas e está associada a elevado risco de progressão para Leucemia Mieloide Aguda (AML). A patogénese da SMD é complexa, estando envolvidos múltiplos eventos genéticos e epigenéticos e, apesar do enorme progresso realizado na ultima década em torno da melhor compreensão dessas anomalias genéticas, continua sem ser esclarecida. O stress oxidativo (SO), que resulta de um desequilíbrio entre a produção de espécies reativas de oxigénio (ROS) e de defesas antioxidantes, contribui para o dano e proliferação celulares, assim como para a apoptose e a hematopoiese ineficaz características das SMD. O nuclear factor-erythroid 2-related factor 2 (NRF2), codificado pelo gene *NFE2L2*, é um dos mais importantes fatores de transcrição envolvidos na resposta antioxidante que tem sido identificado como anticarcinogénico. No entanto, a sobre expressão de NRF2 tem sido observada num grande número de tumores sólidos e hematológicos, que tem sido relacionada com um papel procarcinogénico.

**Objetivos**: Avaliar os níveis de expressão do gene *NFE2L2* nas SMD e compará-los com vários parâmetros clínicos e laboratoriais na SMD, explorando a sua importância como biomarcador no diagnóstico e prognóstico, nomeadamente como preditor de progressão para LMA.

**Materiais e Métodos**: Amostras de sangue periférico foram colhidas de 55 doentes diagnosticados com SMD e 44 controlos saudáveis. RNA total foi isolado de leucócitos derivados de sangue periférico e transcrito em cDNA. A expressão de *NFE2L2* foi quantificada por real-time PCR. A comparação entre grupos de doentes e controlos foi realizada através de testes não paramétricos de Mann-Whithney e Kruskal-Wallis. A análise de sobrevivência foi efetuada recorrendo ao método de Kaplan-Meier e as curvas ROC foram elaboradas.

**Resultados e Discussão:** Os níveis de expressão do *NFE2L2* não apresentaram diferenças

quando comparados entre os indivíduos com SMD e os indivíduos controlos (SMD: mediana 2,29; amplitude interquartil 6,037; CTL: mediana 3,40; amplitude interquartil 3,40; $p$=0,816). No entanto, quando os pacientes com SMD foram estratificados segundo os diferentes subtipos de SMD da classificação da WHO, foi possível observar níveis inferiores de expressão de *NFE2L2* na citopenia refratária com displasia multilinhagem (CRDM) quando comparados com os restantes subgrupos de SMD (CRDM: mediana 1,48; amplitude interquartil 1,41; $p$<0,05), o que parece sugerir uma maior participação do NRF2 neste subtipo de SMD. Foi também possível observar a sobre expressão do *NFE2L2* nos pacientes com SMD que progrediram para LMA (LMA: mediana 8,57; amplitude interquartil 13,57; Não-LMA: mediana 2,12; amplitude interquartil 4,03; $p$=0,018), com uma sensibilidade de 100% e uma especificidade de 76,5%, quando utilizado um valor de cut-off de 5,44 ($p$=0,021). Assim, a expressão de *NFE2L2* poderia ser usada como possível biomarcador na identificação dos pacientes com maior risco de progressão para LMA. Não foram observadas quaisquer relações entre o padrão de expressão do *NFE2L2* e qualquer parâmetro laboratorial, IPSS ou sobrevivência.

**Conclusão**: O *NFE2L2* poderá a vir a ser usado como potencial biomarcador de evolução para LMA nos doentes com SMD. Ao longo da última década, progressos significativos na compreensão das anomalias genéticas têm sido desenvolvidos, no entanto são ainda necessários mais estudos para compreender a verdadeira importância do *NFE2L2*/NFR2 na patogénese das SMD, particularmente nos doentes do subtipo CRDM e nos doentes de alto risco.

**PALAVRAS-CHAVE**

*NFE2L2*/NFR2; Stress Oxidativo; Síndrome Mielodisplásica; Biomarcadores; Prognóstico.

## ABBREVIATIONS

5q-syndrome – MDS with isolated deleted 5q

ALL – Acute lymphoid leukemia

AML – Acute myeloid leukemia

CLL – Chronic lymphoid leukemia

CML – Chronic myeloid leukemia

CTL – Controls

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

HSC – Haematopoietic stem cells

IPSS – International Prognostic Scoring System

KEAP1 – Kelch ECH associating protein 1

LFS – Leukaemia Free Survival

MDS – Myelodysplastic syndrome

MDS-MPN – Myelodysplastic-myeloproliferative neoplasms

MPN – Myeloproliferative neoplasms

NRF2 – Nuclear factor erythroid 2-related factor 2

PB – Peripheral blood

RA – Refractory anaemia

RAEB – Refractory anaemia with excess blasts

RARS – Refractory anaemia with ringed sideroblasts

RBC – Red blood cells

RCMD – Refractory cytopenias with multilineage dysplasia

RN – Refractory neutropenia

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Refractory thrombocytopenia

sAML – Secondary Acute Myeloid Leukaemia

SE – Serum erythropoietin

SF – Serum ferritin

WHO – World Health Organization

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## INTRODUCTION

Myelodysplastic syndromes (MDSs) are a heterogeneous group of clonal stem-cell disorders characterised by an ineffective haematopoiesis leading to peripheral blood cytopenias and most commonly a hypercellular, dysplastic-appearing bone marrow, with an increased propensity for leukemic transformation in a third of patients. (1–7) Besides the cytopenias, the minimal morphologic criterion for the diagnosis is dysplasia in at least 10% of cells of any myeloid lineages. However, such changes can also be seen in other myeloid neoplasms. (2) Generally, is a disease of older people, with a median age at diagnosis of 70–75 years, and only less than 10% occur in individuals younger than 50 years. (1,2,6)

The pathogenesis of MDS has probably age-induced genetic, epigenetic, and immune-mediated changes in haematopoietic stem cells (HSC), which lead to oligoclonal expansion of myelodysplastic stem cells, with defective differentiation, characterised by increased apoptosis of erythroid and myeloid progenitors. (1–4) Microenvironmental changes (probably the high secretion of TNF-α by macrophages) and immune deregulation also contribute to this disease. (3,4,7) However, the cause of MDS is known only in 15% of cases. Environmental factors, including previous use of chemotherapy (specially alkylating agents and purine analogues), radiotherapy and tobacco smoking, and some recognised occupational factors as exposure to benzene and its derivatives had been described as risk factors for MDS development. (1,2)

An abnormal karyotype is shown by conventional cytogenetic analysis in 40-50% of cases of MDS patients at diagnosis. It is characterized by a partial or complete loss or gain of chromosomes, which the most frequent findings are deleted 5q, monosomy 7 or deleted 7q, trisomy 8, deleted 20q and deleted 17p. Cytogenetic analysis has a major prognostic value for myelodysplastic syndromes. (1,6,7)

MDSs are classified in six categories, according to the WHO 2008 criteria: refractory cytopenia with unilineage dysplasia, including refractory anaemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT); refractory cytopenias with multilineage dysplasia (RCMD); refractory anaemia with ringed sideroblasts (RARS); refractory anaemia with excess blasts (RAEB), including RAEB-1 and RAEB-2 based on the marrow blast count being below or above 10%, respectively, but lower than 20%; MDS unclassified and MDS with isolated deleted 5q (5q-syndrome). Recently, a WHO update of this classification has been published (2016), in which the higher-risk patients have been simplified to MDS-Excess Blasts (MDS-EB) 1 or 2; RN or RT has been deemphasized and RCMD and Ring Sideroblasts (RCMD-RS) has been separated from RCMD. (8) In a distinct group, there is the therapy-related MDS and myelodysplastic-myeloproliferative neoplasms (MDS-MPN). (1,2,6,7) Several score models are currently available for MDS risk stratification. The most commonly used and more ancient is the International Prognostic Scoring System (IPSS) that allows the classification of patients in four risk groups (low, intermediate 1, intermediate 2 and high risk) with meaningful differences in overall survival and possibility of progression to acute myeloid leukaemia (AML). This risk stratification score is simple, using only three variables: the percentage of marrow blasts, number of cytopenias and karyotype abnormalities. However, today, the most important prognostic system is the revised IPSS (IPSS-R), which uses less common cytogenetic abnormalities, cytopenias, and blast count for scoring but with new thresholds, which allow a more precise prediction of risk in five categories. (1,2,4–7)

Oxidative stress plays a major role in carcinogenesis. It is caused by an imbalance between reactive oxygen species (ROS) and antioxidant defences, which neutralize the former molecules. (9–14) When the pro-oxidant/anti-oxidant equilibrium is lost, oxidative stress is generated, altering and damaging many intracellular molecules, including DNA, RNA, lipids and proteins. (10) This state of excessive production of ROS and/or deficient production of

antioxidant defences has been observed in several hematopoietic malignancies such as acute and chronic lymphoid leukemias (ALL and CLL, respectively) (15,16), acute myeloid leukemia (AML) (13), chronic myeloid leukemia (CML) (13,16) and MDS (17,18). Cancer cells, which exhibit an accelerated metabolism, demand high ROS concentrations to maintain their high proliferation rate. That is one of the major adaptive advantages that permit cancer cells to increase their metabolic rate and proliferation and to escape free radical damage. (10)

Oxidative stress affects several biochemical pathways that involve key signalling proteins. The most significant effects of oxidants on signalling pathways have been observed in the nuclear factor-erythroid 2-related factor 2 (NRF2) pathway, which regulates oxidative stress. (10,12,19) NRF2, encoded by *NFE2L2* gene, modulates the expression of hundreds of genes, including antioxidant enzymes but also a large number of genes that control processes like immune and inflammatory responses, tissue remodelling and fibrosis, carcinogenesis, and metastasis. (10,20) The NRF2 is a basic region leucine zipper (b-Zip) type transcription factor. Under basal unstressed conditions, Kelch ECH associating protein (KEAP1) binds to NRF2 and promotes its proteasomal degradation through Cullin 3 (Cul-3)–based E3 ligase (Figure 1). (10,12,19–25) Upon exposure to environmental stressors such as ROS, KEAP1 undergoes a conformational change, via modification of critical cysteine thiols, releasing NRF2. (12,19–21,23,24) Free NRF2 translocates to the nucleus and dimerizes with members of the MAF protein family. (19,24,25) This activation results in transcriptional expression of a broad spectrum of protective enzymes including those involved in xenobiotic detoxification, antioxidant response, and proteome maintenance. (12,19–25)

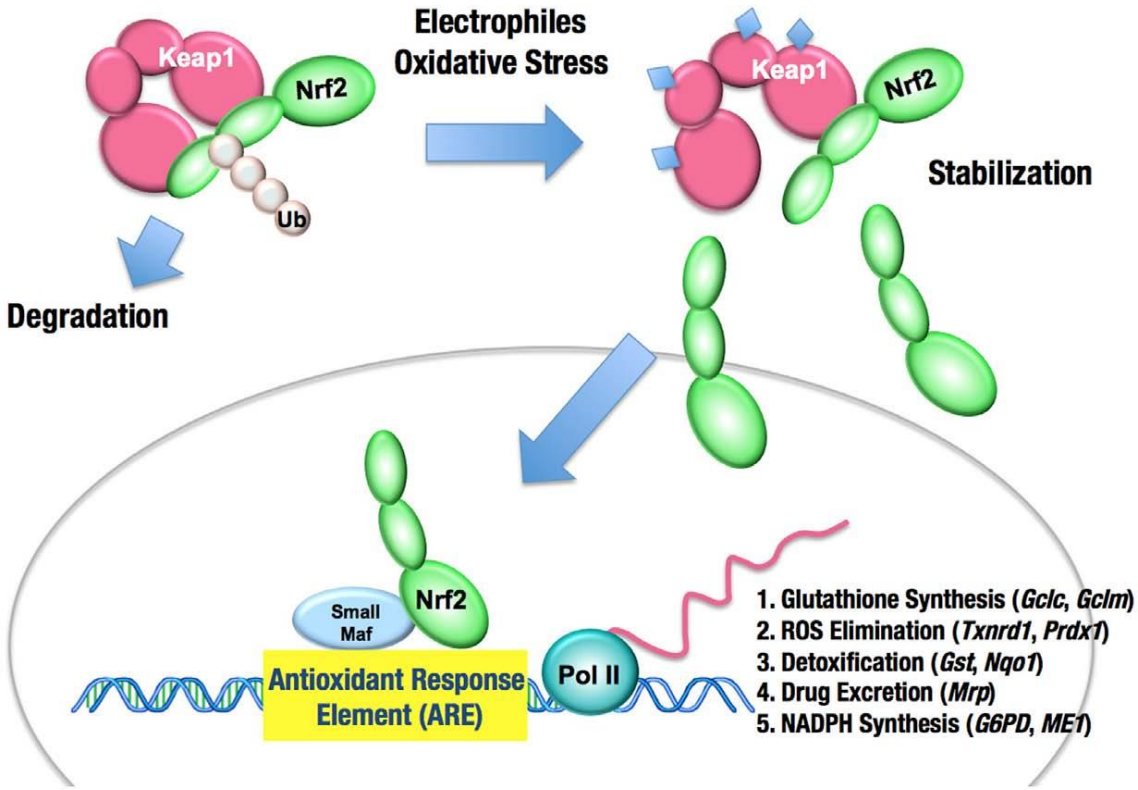



**Figure 1. Schematic model of the NRF2–KEAP1 signaling pathway.** Under basal conditions, NRF2 is constantly ubiquitinated through KEAP1 and degraded in the proteasome. Oxidative stress or electrophiles can cause a disrupt NRF2–KEAP1 binding. Stabilized Nrf2 accumulates in the nucleus and activates many cytoprotective genes. Ub, ubiquitin. From Mitsuishi *et al*. (24)

The impact of NRF2 on cancer is complex. Low levels of NRF2 or loss of NRF2 activity appears to increase ROS production and DNA damage and predisposes cells to tumorigenesis. (12,19,25) However, NRF2 may become protumorigenic if persistently activated, like it was showed in lung, breast, ovarian, endometrial, pancreatic, colorectal, osteosarcoma and prostate cancer cells. (19,21,23) Tumour cells hijack the NRF2 pathway through somatic mutations and epigenetic mechanisms to cause persistent activation of NRF2, resulting in a prosurvival phenotype that modulates anabolic pathways towards promotion of tumour growth and resistance to oxidants and anticancer drugs. (12,19,20,24,25) Enhanced NRF2 activity has been found in a great number of solid and hematologic tumours. (21) In order to broaden the knowledge about the role of *NFE2L2*/NRF2 in MDS patients, the present study focuses on

*NFE2L2* expression levels in MDS patients. Accordingly, our aim with this study was to evaluate the expression levels of *NFE2L2* gene in MDS patients and correlate it with clinical and analytical parameters, exploring its potential role as diagnostic and prognostic biomarker, namely as a predictor of AML transformation.

# MATERIALS AND METHODS

## Ethical Statement

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

## Study Population

To fulfil our objectives, a total of 99 individuals were enrolled in the present study: 55 patients with MDS followed in the Haematology Service of "Centro Hospitalar e Universitário de Coimbra, EPE (CHUC, EPE)" and 44 healthy control individuals. MDS patients were grouped according to the 2008 WHO classification of tumours of haematopoietic and lymphoid tissues, and to the IPSS. (26) We collected demographic characteristics for patients and controls, recorded patient's clinical characteristics, such as laboratorial data, and maintained patient's follow-up in order to collect survival data and transformation to AML.

## Sample Preparation

Peripheral blood samples were collected from patients and controls with EDTA tubes and immediately storage at 4°C until processed as described below. The white blood cells were isolated after mixing EDTA blood with erythrocyte lysis buffer.

**RNA isolation**

Total RNA was isolated from white blood cells with NZYol reagent (NZYTech) according to the manufacturer's instructions. Following RNA extraction, total RNA concentration and purity ($OD_{260}/OD_{280}$) was quantified using Nanodrop 1000 (Thermo Scientific). Extracted RNA was stored at -80°C.

**cDNA Synthesis**

Samples of Total RNA were reverse transcribed with NZY First Strand cDNA Synthesis Kit from NZYTech, according to manufacturer's protocol. For cDNA synthesis a mixture of oligo$(dT)_{18}$ and random hexamers were used as primers. The cDNA was stored at -20°C until Real Time PCR analysis.

**Real-Time PCR**

To analyse the *NFE2L2* expression, 5 µl cDNA was added to Taq SuperMix containing 300 nM forward as well as reversed primers. We used primers for *NFE2L2* (forward: 5'-GCTGTCCTCAATCGTCTCCTT-3'; reverse: 5'-CAACCCTTGTCACCATCTCAG-3') and the housekeeping gene *GUSB* (forward: 5'-CAGGTGATGGAAGAAGTG-3'; reverse: 5'-AAGTAGTAGCCAGCAGAT-3'). All samples were used in duplicate and no template controls were included. The Real-Time PCR was carried out in a CFX96 Touch™ Real Time PCR Detection System (BioRad, USA) in 96-well plates. The thermocycling parameters were one cycle of 30 seconds at 95°C and 40 cycles of 5 seconds at 95°C and 20 seconds at 60°C. The relative experience was calculated with the $2^{-\Delta CT}$ (Livak) method.

**Statistical Analysis**

Statistical analysis of the data was performed with IBM® SPSS® Statistics version 23. We performed descriptive analysis of the characteristics of patients and controls. Normality was assessed by Kolmogorov-Smirnov analysis. For non-normally distributed variables, Mann-Whitney test and Kruskal-Wallis test were performed to assess clinical significance of the difference between two groups (patients *vs* controls; between each two of subgroups of MDS; expression levels of *NFE2L2 vs* IPSS; expression levels of *NFE2L2 vs* ferritin; expression levels of *NFE2L2 vs* erythropoietin) and more than two groups (expression levels of *NFE2L2* vs MDS subgroups; expression levels of *NFE2L2 vs* IPSS; expression levels of *NFE2L2 vs* mortality), respectively. The receiver operating characteristics (ROC) curves analysis was performed to assess the variables' accuracy as diagnostic and evolution biomarker, as well as death predictor. Survival analysis was performed using Kaplan-Meier test. A value of $p < 0.05$ was considered significant.

# RESULTS

## Characteristics of the Study Groups

The present study enrolled a myelodysplastic syndromes patient group ($n$ = 55) and a healthy control group ($n$ = 44) with the characteristics described in Table 1. The MDS group, with a median age of 71,98 years (range 22–89 years), was composed of 30 females (54,5%) and 25 males (45,5%). The four MDS patients that progressed to AML were all males, with an age of 61 years in average (range 22–77). The healthy control group consisted of 22 females (50%) and 22 males (50%) and had a median age of 63,58 years (range 32–92 years). In order to avoid confounding bias and to confirm adequate matching between groups, we assessed differences in the demographic features. However, there were statistically differences, between MDS and controls, in terms of their age ($p$ = 0,006), indicating inadequate age matching. In terms of gender, there were no significant differences ($p$ = 0.653).

According to 2008 WHO classification used at the diagnosis, the MDS subgroup included patients with the following subtypes: 28 patients with RCMD (50,9%), 2 with RA (3,6%), 3 with RN (5,5%), 2 with RT (3,6%), 4 with RARS (7,3%), 5 with RAEB-1 (9,1%), 2 with RAEB-2 (3,6%), 1 with MDS with isolated del(5q) (1,8%) and 8 with MDS-MNP (14,5%). The distribution of MDS patients according to IPSS risk system showed a predominance of low-risk patients, with the following distribution: low-risk, 21 patients (48,837%); 17 patients with intermediate-1 risk (39,535%); 4 with intermediate-2 risk (9,302%); and 1 with high-risk (2,326%), in a total of 43 MDS patients in which was possible to calculate the IPSS.

In 52 MDS patients, we evaluated the existence of cytogenetic abnormalities, 9 of them by FISH and only 43 by conventional karyotype, which have been grouped by their cytogenetic value according to IPSS. The distribution showed a predominance of abnormalities with good

prognostic value (46XX/ 46XY/ 5q abnormalities) (n = 33; 63,5%), but also some patients with intermediate prognostic value abnormalities (t8/ t8; 5q/ t8; -Y/ 46Y, der(X)) (n = 6; 11,5%), with poor prognostic value (complex/ 7q/ 7q; -Y/ t8; 7q; -5) (n = 4; 7,7%) and with normal FISH (n = 9; 17,3%). Four patients from the total of 55 MDS patients (7,3%) evolved to AML (one of them was classified as RAEB-1, other as RAEB-2 and the other two as RCMD). Most MDS patients were still alive (n = 42, 76,4%) and 13 (23,6%) died, considering 44 months of follow-up in average.

**Table 1. Demographic and Clinical Characteristics of MDS patients and controls individuals**

| Characteristics | MDS (*n*=55) | | Controls (*n*=44) | |
|---|---|---|---|---|
| | *n* | % | *n* | % |
| **Demographic data** | | | | |
| Gender | | | | |
| Male | 25 | 45,5 | 22 | 50 |
| Female | 30 | 54,5 | 22 | 50 |
| Age (years) | | | | |
| Median age | 71,98 | | 63,58 | |
| Range | 22–89 | | 32–92 | |
| **Clinical data** | | | | |
| MDS classification | | | | |
| RA | 2 | 3,6 | | |
| RT | 2 | 3,6 | | |
| RN | 3 | 5,5 | | |
| RCMD | 28 | 50,9 | | |
| RARS | 4 | 7,3 | | |
| RAEB-1 | 5 | 9,1 | | |
| RAEB-2 | 2 | 3,6 | | |
| 5q-syndrome | 1 | 1,8 | | |
| MDS–MPN | 8 | 14,5 | | |
| IPSS (*n* = 43) | | | | |
| Low | 21 | 48,837 | | |
| Intermediate-1 | 17 | 39,535 | | |
| Intermediate-2 | 4 | 9,302 | | |
| High | 1 | 2,362 | | |

| | | |
|---|---|---|
| Cytogenic abnormalities (*n* = 52) | | |
| Good prognostic | 33 | 63,5 |
| Intermediate prognostic | 6 | 11,5 |
| Poor prognostic | 4 | 7,7 |
| normal FISH | 9 | 17,3 |
| Evolution to AML | 4 | 7,3 |
| Death | 13 | 23,6 |

*n* – number of cases; % - percentage; MDS – Myelodysplastic Syndrome; RA – refractory anaemia; RT – refractory thrombocytopenia; RN – refractory neutropenia; RCMD – refractory cytopenia with multilineage dysplasia; RARS – RA with ringed sideroblasts; RAEB-1 – RA with excess blasts type 1; RAEB-2 – RA with excess blasts type 2; 5q-syndrome - MDS associated with an isolated del(5q) chromosome abnormality; MDS-MPN – myelodysplastic-myeloproliferative neoplasms.

About the laboratorial parameters, 21 (39%) MDS patients had the serum ferritin elevated (above 300 ng/mL) and none of the patients had ferritin levels below 10 ng/mL, 41 (76%) had serum erythropoietin elevated (above 15 mUI/mL) and 13 (24% had low serum erythropoietin (below 15 mUI/mL), with only 1 patient with erythropoietin level below 3,5 mUI/mL. Patients from the RARS subgroup presented an average of 34,5% of ringed sideroblasts. Other analytical parameters evaluated in MDS patients are presented in Table 2.

**Table 2. Levels of analytical parameters in MDS patients**

| | Median | Interquartile range |
|---|---|---|
| Blasts (%) | 1,0 | 1,0 |
| Folate (ng/mL) | 9,4 | 13,2 |
| Vitamin B12 (pg/mL) | 633,0 | 811,0 |
| Ferritin (ng/mL) | 191,0 | 412,0 |
| Erythropoietin (mIU/mL) | 21,9 | 34,2 |
| LDH (IU/L) | 199,0 | 58,8 |
| β-2 microglobulin (µg/mL) | 2,360 | 1,2 |

**Evaluation of *NFE2L2* gene expression levels amongst MDS patients and controls**

The expression levels of *NFE2L2* gene were compared between patients and control individuals. No statistically differences have been observed between the expression values of *NFE2L2* in MDS patients (median 2,29; interquartile range 6,037) and controls (median 3,40; interquartile range 3,40; U = 1177,0; $p$ = 0,816) (Fig.2).

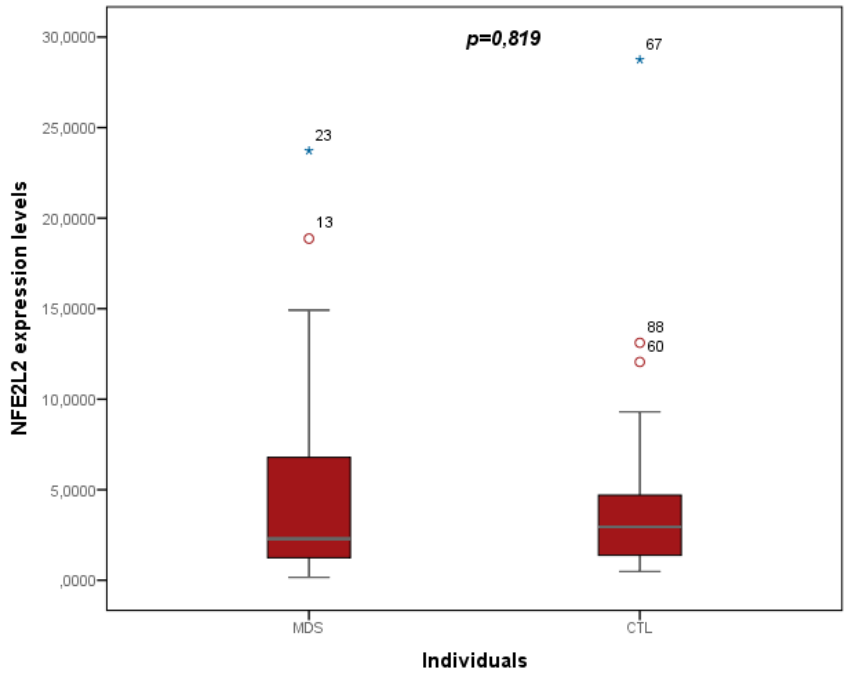



**Figure 2. Analysis of *NFE2L2* gene expression levels in MDS patients and controls.** MDS – myelodysplastic syndrome, CTL – control.

To evaluate if *NFE2L2* gene expression levels could be used as a MDS diagnostic marker, we determined the capacity of *NFE2L2* gene expression levels to discriminate MDS from controls in peripheral blood (PB) by ROC analysis (Fig. 3). With an AUC-value of 0,514 (95% CI: 0,398–0,629; $p$=0,816), the ROC curve shows no statistically significant differences between patients and controls.

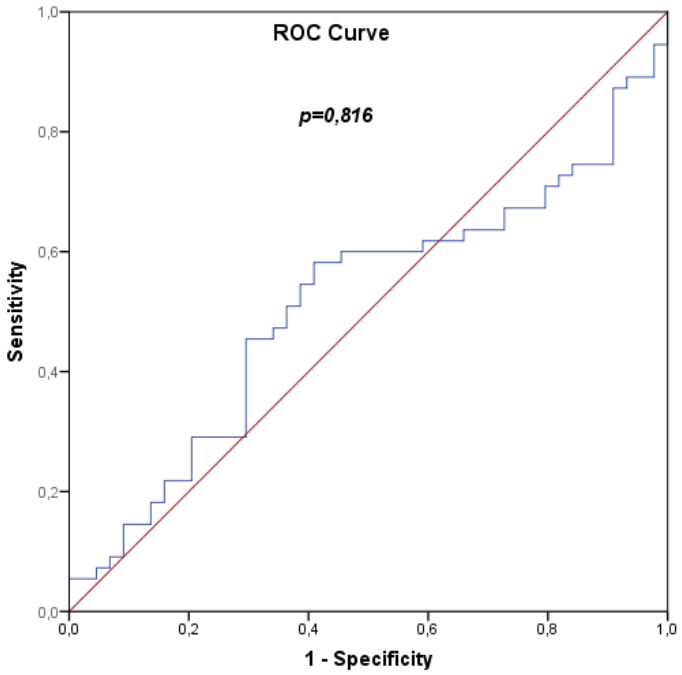



**Figure 3**. **Performance of *NFE2L2* gene expression levels to discriminate MDS patients from controls.**

## Correlation between *NFE2L2* gene expression levels and analytical features of MDS patients

In order to determine if there is any association between *NFE2L2* gene expression levels and some MDS patients' analytical parameters, we analysed the *NFE2L2* expression levels in correlation to serum ferritin (SF), serum erythropoietin (SE) levels and serum LDH.

We organized the ferritin levels in two categories: normal SF (≥10 and <300 ng/mL) and high SF (>300 ng/mL). This study did not found statistically significant differences between the *NFE2L2* expression levels and the ferritin serum levels in MDS patients (normal SF: median 2,47 and interquartile range 6,99; high SF: median 2,12 and interquartile range 3,18; $U$ = 332,0; $p$=0,797).

The erythropoietin levels were organized in two categories: normal SE (≥3,5 and <15 mIU/mL) and high SE (>15 mIU/mL). We found no statistically significant differences between the

*NFE2L2* expression levels and the erythropoietin serum levels in MDS patients (normal SE: median 3,38 and interquartile range 11,49; high SE: median 1,96 and interquartile range 4,75; $U$ = 191,0; $p$=0,127).

The LDH serum levels were organized in two subgroups: normal LDH (<240 IU/L) and high LDH (>240 IU/L). This study also found no statistically significant differences between the *NFE2L2* expression levels and LDH levels in MDS patients (normal LDH: median 2,47 and interquartile range 5,84; high LDH: median 0,80 and interquartile range 4,57; $U$ = 119,0; $p$=0,053).

**Analysis of *NFE2L2* expression according to MDS subgroups**

We analysed the *NFE2L2* gene expression levels in relation in MDS patients' subgroups, according with 2008 WHO classification (Fig. 4). As we can notice in Fig. 4, we observed statistically significant differences between the *NFE2L2* expression levels and the patients of the subgroups ($\chi^2$=17,588; $p$=0,025), revealing that RCMD patients had a lower expression level of NFE2L2 (median 1,48; interquartile range 1,41) when compared with MDS-MPN (median 8,07; interquartile range 15,35; $p$=0,019), RAEB-1 (median 2,45; interquartile range 9,43; $p$=0,006), RN (median 8,28; $p$=0,005) and RA (median 5,35; $p$=0,041). All the other relations between each two MDS subgroups do not show significant differences and are not presented here.

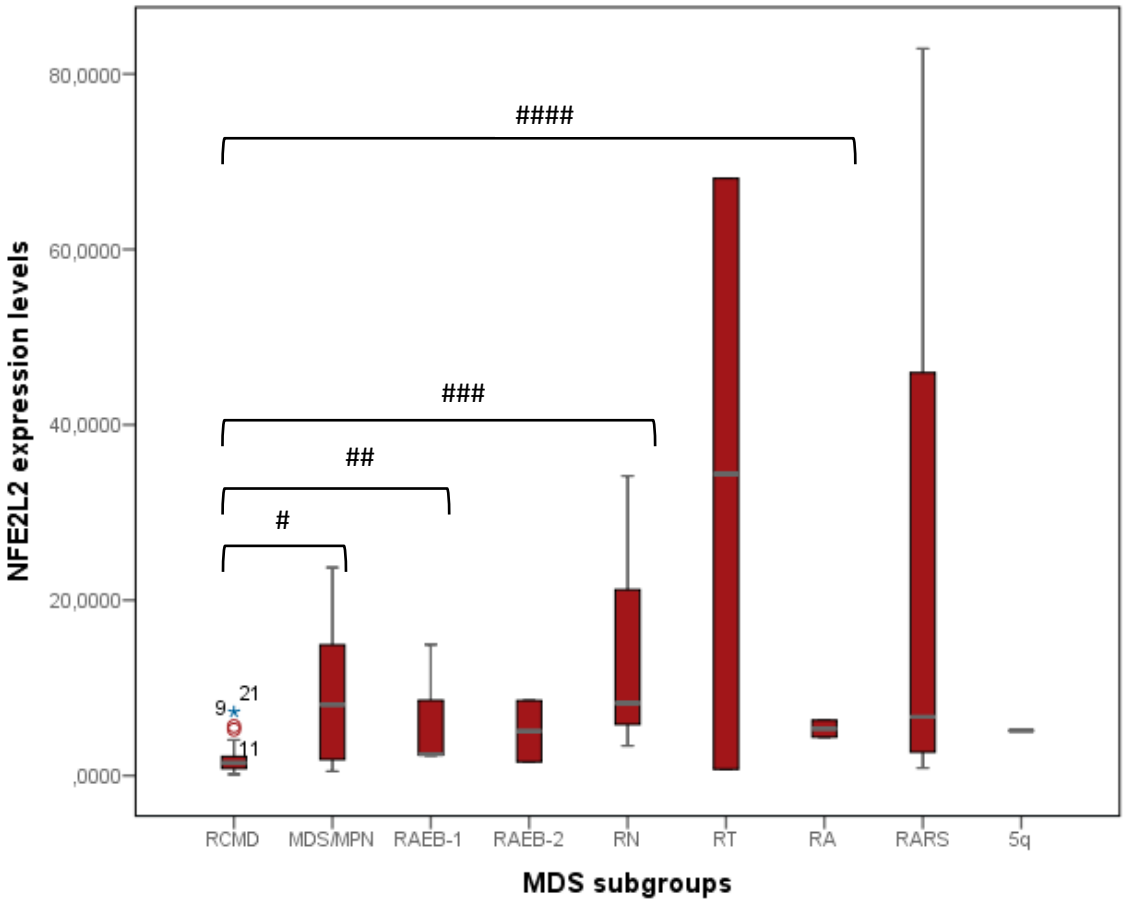
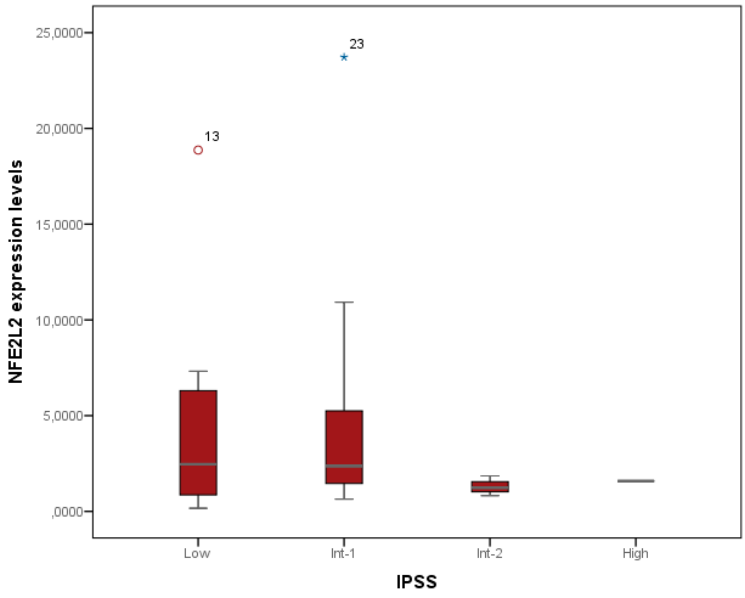


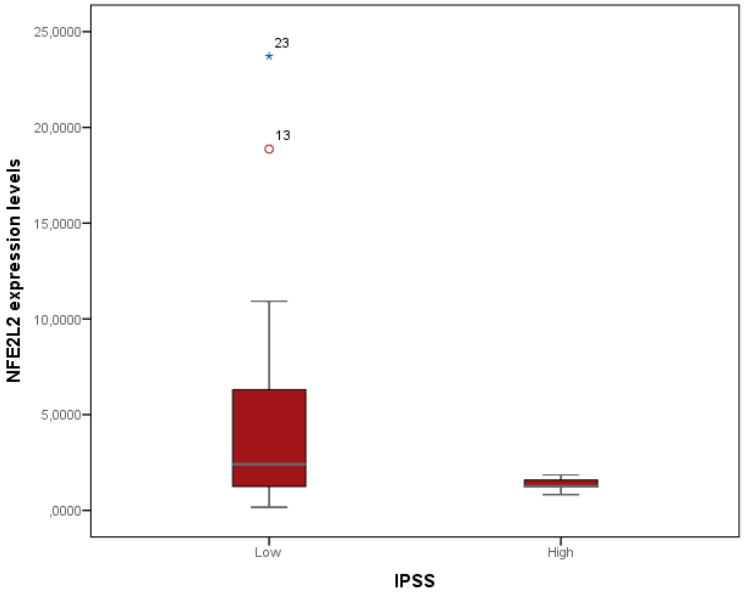

**Figure 4. Analysis of *NFE2L2* expression levels in MDS patients according with WHO 2008 subgroups.** $^{\#}p$=0,019; $^{\#\#}p$=0,006; $^{\#\#\#}p$=0,005; $^{\#\#\#\#}p$=0,041.

## Analysis of *NFE2L2* expression according to IPSS

To determine the contribution of *NFE2L2* in MDS prognosis, patients were grouped according to their IPSS risk (Fig. 5), but no statistically significant differences were observed between the *NFE2L2* expression levels and the patients of the four IPSS subgroups ($\chi^2$=2,770; $p$=0,428). To continue this evaluation, we formed two subgroups: one of low risk (low and intermediate-1) and another of high risk (intermediate-2 and high). However, no significant differences were observed between low and high risk patients (U=53,000; $p$=0,118).




**(A)**




**(B)**

**Figure 5. Analysis of *NFE2L2* expression levels in MDS patients distributed by IPSS risk groups.** In **(A)** we consider the four IPSS patients-groups and in **(B)** we divided patients in two risk groups**.** MDS – myelodysplastic syndrome, Low – low risk, Int-1 – intermediate-1 risk, Int-2 – intermediate-2 risk, High – high risk.

## High *NFE2L2* gene expression levels were associated with MDS progression to AML

Expression levels of *NFE2L2* were compared between patients who progressed to AML and those who did not progressed. We observed that patients who progress to AML have higher

NFE2L2 expression levels (median 8,57; interquartile range 1,41) than those that didn´t progress to AML (median 2,12; interquartile range 4,03); U = 31,0; *p* = 0,018; (Fig.6).

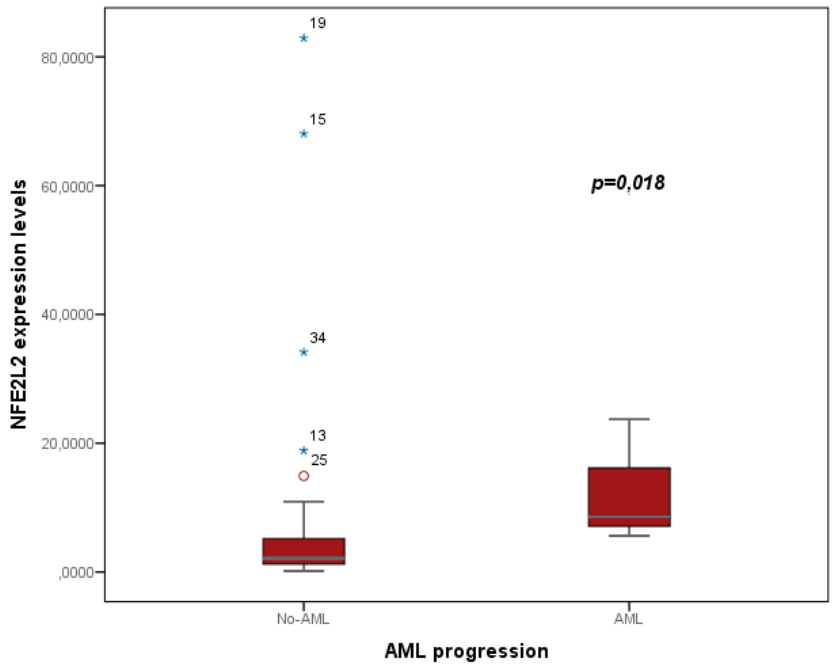



**Figure 6. Analysis of *NFE2L2* gene expression levels in MDS patients according to evolution to AML.** AML – acute myeloid leukaemia.

The ROC curves in Fig. 7 show the statistically significant ability of *NFE2L2* expression to be used as a predictor marker of AML evolution in MDS patients, with an area under the curve (AUC) value of 0,848 (95% confidence interval [CI]: 0,741 – 0,955; *p*=0,021). *NFE2L2* levels greater or equal than 5,4433 were the optimal cut-off value to identify the patients who progress to AML (sensitivity: 100%; specificity: 76,5%; PLR: 65,22; NLR: 47,37), and were associated with a lower time to AML transformation (*p*=0,002, log rank test) (Fig. 8).

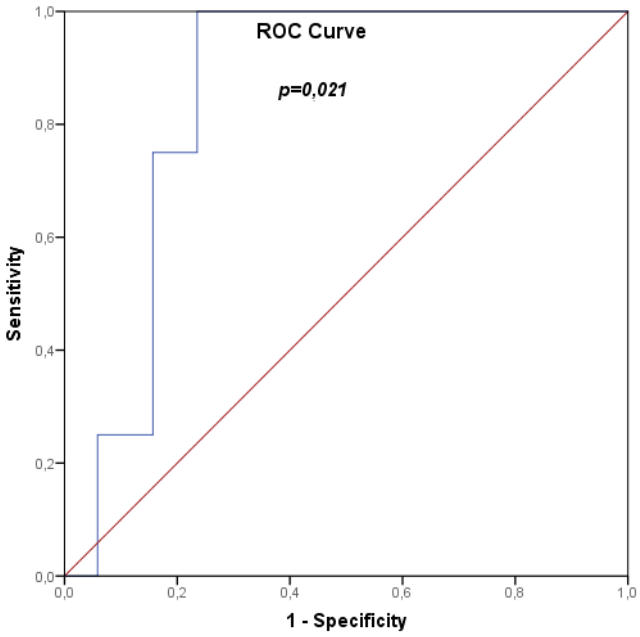



**Figure 7. Performance of *NFE2L2* gene expression levels to discriminate MDS patients who progress to AML.**

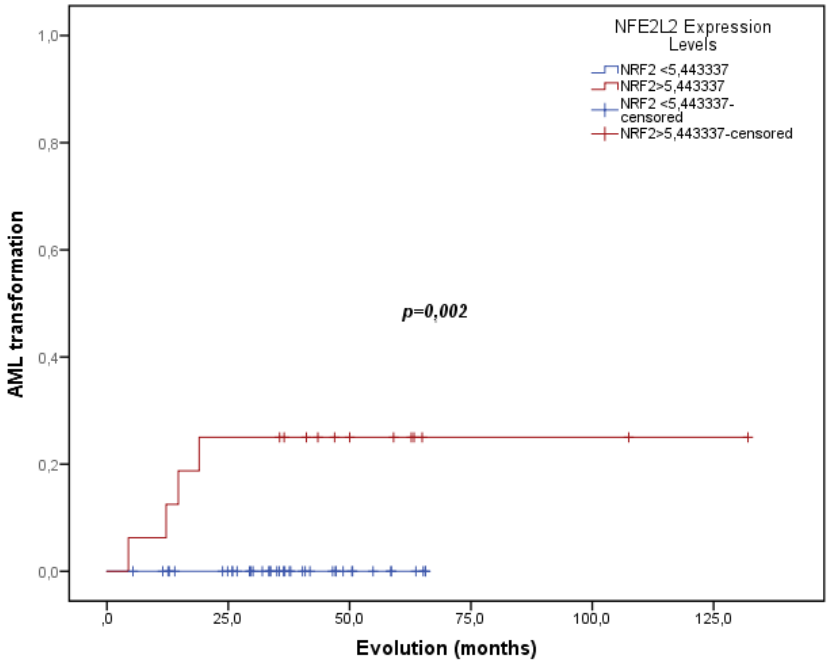



**Figure 8. Time to AML transformation curve of MDS patients, according to *NFE2L2* expression levels.** Survival analysis was performed by Kaplan-Meier method. MDS patients were stratified through the cut-off points obtained from the ROC curves.

### Influence of *NFE2L2* gene expression levels in survival

*NFE2L2* expression levels were analysed as possible survival biomarkers. However, non-valid cut-off value were found, as observed in Fig. 9.

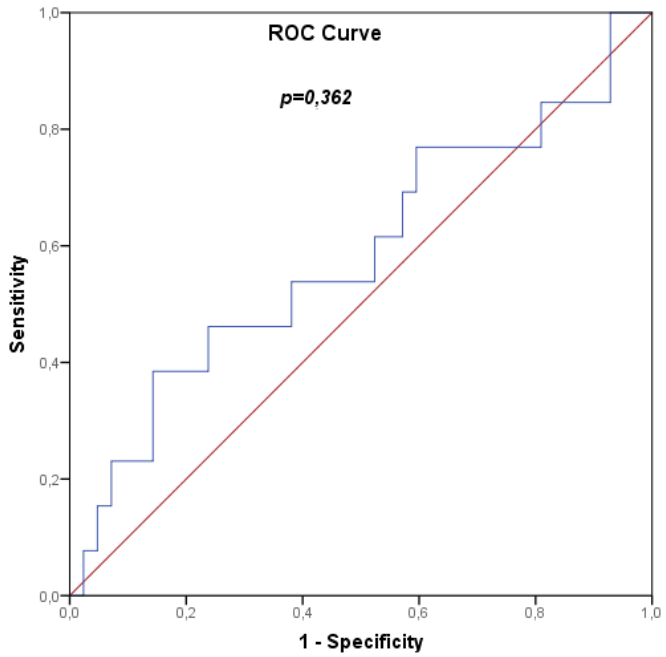



**Figure 9. Performance of *NFE2L2* gene expression levels to discriminate survival in MDS patients.**

## DISCUSSION AND CONCLUSION

It has been well established that oxidative stress (OS) plays a major role in carcinogenesis, with evidence of such importance both in solid tumours, like prostate carcinoma and melanoma, and in several hematopoietic malignancies. (13,15–18) Indeed, several studies revealed markers of OS and of DNA damage in MDS patients (15,17,27–29), such as elevated levels of ROS in red blood cells and platelets (30) and 7,8-dihydro-8-oxoguanine (8-OG) in urine (31), although some of ROS levels are mediated by iron overload (32,33). Elevated ROS levels activate cellular signalling pathways that can affect proliferation or apoptosis depending on the stress levels (9,34), conferring survival advantages to malignant cell population (13). In fact, ROS management is critical for primitive hematopoietic cells, and elevated ROS levels appear to drive HSC out of quiescence and reduce self-renewal capacity, resulting in rapid bone marrow failure. (13,35) This cellular state is modulated by several antioxidant defences, such as superoxide dismutase (SOD), and base excision repair enzymes, like 8-oxoguanine DNA glycosylase (OGG1), as well as by transcriptional factors, such as NRF2. (28)

NRF2, codified by the *NFE2L2* gene, is one of the most critical cytoprotective mechanism to contend the oxidative and xenobiotic stresses. Recent findings suggest that enhanced detoxification of ROS with additional NRF2 functions may in fact be also protumorigenic (35–37), due to somatic mutations of *NFE2L2* and *KEAP1* genes (19,35,38) as well as to epigenetic silencing of *KEAP1* gene. But, also aberrant accumulation of proteins that compete with NRF2 for KEAP1 binding, or oncogene-mediated overexpression of NRF2 may be involved. (19,24,35) Hartikainen *et al*. found a strongly expression of NRF2 in the cytoplasm of breast carcinoma cells, affecting both the cancer predisposition and progression. (35–37) NRF2 was also up-regulated in lung (35,37,38), head and neck (38), skin (37), oesophageal (35,37,38),

larynx (37), stomach (37), hepatocellular carcinoma (37,39), gallbladder (37), ovary (37), endometrial (35) and prostate cancers (35).

In the field of hematopoietic malignancies, higher constitutive *NFE2L2* levels was showed in AML cells (40). Kaufmann *et al.* observed that the *NFE2L2* is often over expressed in myeloproliferative neoplasms (MPN) patients (41). To evaluate such importance in MDS, we compared the expression levels of *NFE2L2* in MDS patients with healthy controls cells and found no significant differences between them, though *NFE2L2* had a lower median expression levels in MDS population. The ROC curves analysis was performed to assess the *NFE2L2* accuracy as possible diagnostic biomarker but we failed to prove it because no significant differences between patients and controls were found. These results may be due to study limitations, namely sample size, incomplete group matching and study design (hospital-based cross sectional study).

As previously explained, oxidative DNA damage has been demonstrated in MDS. (17,18) Novotna *et al.* demonstrated a higher level of DNA breakage in bone marrow cells of patients with RA and RARS subtypes (32), but they only study this two MDS subtypes. Another studies showed that highest oxidative stress levels correlate with an increase of apoptosis susceptibility in RA and RCMD subtypes, as well as in low-risk patients (low- and intermediate-1-risk), which has been translated into cytopenias observed in such patients (17,42). We have investigated the pattern of *NFE2L2* expression in our MDS patients divided into IPSS categories and WHO classification. Based on the previous findings, we expected to observe a different expression of *NFE2L2* in RA, RCMD and low-risk patients, because of its close relationship with oxidative metabolism. However, no differences have been observed within IPSS categories, neither dividing the MDS patients into four subgroups, nor into two (incorporating intermediate-1 into low-risk and intermediate-2 into high-risk groups). Relatively to WHO classification, in fact we observed significant differences between *NFE2L2*

expression levels and MDS subgroups, and found that RCMD patients had lower *NFE2L2* expression levels, when compared with other subgroups as well as to control group. These results seem to correlate with the previously studies that observed highest oxidative stress levels in RCDM patients. (17) This may suggest a different role of *NFE2L2* in RCDM patients, compared with others cancers where *NFE2L2* is overexpressed. Consequently, *NFE2L2* in RCDM patient may have a more important protective role than a protumorigenic one.

Genetic evolution of secondary AML (sAML) is a dynamic process shaped by multiple cycles of mutation acquisition and clonal selection, where the clones present in MDS persist in sAML. (43) So the MDS and AML development may be affected by the same functional pathways, namely OS and KEAP1-NRF2 system. Indeed, a recent study demonstrated that relapse in AML correlates with an escalation of oxidative stress (13), and several genetic mutations may occur in various genes, such as *U2AF1*, *TET2* (43) and *SRSF2* (44), that contribute to a higher rate of progression to sAML. In addition, epigenetic changes have been strongly associated with MDS and AML, such as abnormal methylations of the *TET2*, *IDH*, *ASXL1*, *FANCF*, and *FZD9* genes (44), but also *DNMT3A* mutations, which could induce epigenetic alterations, that often indicate worse overall survival and a more rapid progression to sAML (44). *ASXL1* and *RUNX1* mutations seem to be two major associations in secondary dysplastic AMLs with intermediate cytogenetic risk. (43) Recently it was showed that *NFE2L2* was constitutively active in human AML cells (40), although the precise molecular mechanisms underlying the progression of MDS to sAML are poorly understood. In our study, we observed significant differences in *NFE2L2* expression levels between MDS patients that progressed to AML and those that not progressed, with an overexpression in cells which patients had sAML. This finding agrees with Rushworth *et al.* findings (40). However, we still do not know the underlying mechanism of that overexpression in the AML cells, since there was no relationship between high ROS levels and high nuclear NRF2. On the other hand, it is not known which *KEAP1* or *NFE2L2* somatic

mutations were responsible for those elevated *NFE2L2* gene expression levels (40). We also proved that *NFE2L2* expression levels could be used as a biomarker predictor of AML transformation, using a cut-off value of 5,44, having this test a sensitivity of 100% and a specificity of 76,5%. It is well established in the clinical practise that the prognosis of patients with tumours expressing high levels of NRF2 is poor and is also associated with chemotherapeutic resistance. (19,24,25) Taken together, it is likely that AML cells acquire a growth advantage and chemoresistance via activation of NRF2-dependent defence responses and suggests that the *NFE2L2* expression levels may be appropriate as a prognostic biomarker in one of the prognostic scores actually used, though we need more studies to understand the mechanism under this relation.

Studies have identified the influence of several mutations in overall survival, particularly the mutations in *TP53*, *EZH2*, *ETV6*, *RUNX1*, *ASXL1*, *DNMT3A*, *IDH1/2*, *SRSF2*, *CBL*, *ASXL1* and *STAG2* genes as predictors of shortened survival. (5,6,43,44) Moreover, it was expected a relationship between *NFE2L2* expression levels and survival because of the poor prognosis correlated with AML progression, but no significant different expression levels of *NFE2L2* were found in survival analysis. In our study the use of *NFE2L2* expression levels as a survival biomarker predictor does not seem helpful, probable due to the short follow-up time.

Another aim of our study was also to correlate laboratorial parameters with *NFE2L2* expression levels. In fact, iron regulation in MDS is controversial. Low-risk MDS patients are transfusion-dependent, and, although there is an ineffective erythropoiesis, the transfusion therapy seems to be the main cause of iron overload. (45,46) The increased intestinal iron absorption, caused by ineffective erythropoiesis, hypoxia and, to some extent, to hepcidin suppression, could also contribute to iron overload. (45,47) Recent data suggest a correlation between iron overload and both leukaemia-free survival (LFS) and overall survival. (47) Li *et al.* showed that iron overload is correlated with increased serum ferritin levels (47) and Ghoti *et al.* found a

correlation between the serum ferritin (SF) levels and ROS, in low risk-patients (48). Kikuchi *et al.* showed that baseline SF levels may be a prognostic factor for overall survival and LFS in MDS patients, with LFS and overall survival being significantly longer in a group with low SF level (<500 ng/mL) and SF values significantly higher in the higher-risk MDS patients. (49) Despite that, transfusion dependency was found to significantly worsen the probability of surviving and also increase the risk of progressing to leukaemia (4,45,50). Because of the complexities of iron regulation, the prognostic value of serum ferritin in patients with low- risk MDS not receiving red blood cells (RBC) transfusions is controversial. (47) Although in non-RBC transfusion-dependent lower-risk MDS patients, the progression to AML and overall survival did not significantly differ according to SF, and an increased baseline SF level was correlated with RARS subgroup patients, suggesting that SF levels are a hallmark of dyserythropoiesis in these cases. (51) Both excess of iron has been correlated with OS (46) and SF levels with ROS levels (17,33,46), which might be involved in the MDS disease progression (46). However we have not found a correlation between the SF levels and *NFE2L2* expression levels, using the 300 ng/mL as cut-off value. Therefore, the *NFE2L2* does not seems to correlate with the serum ferritin levels. This also supports the negative impact of iron overload itself on the function of vital organs and on the number of cardiac deaths, which make ferritin an independent prognostic factor for OS (50).

The identification of a few number of features with independent prognostic value, routinely available in all centres, have been assembled in the IPSS (1). Beyond IPSS and the arising of new prognostic systems, namely WHO Prognostic Scoring System and Revised IPSS, other prognostic factors have been identified, such as bone marrow fibrosis, serum LDH levels, β2-microglobulin (52), but also *TP53*, *RUNX1*, or *ASXL1* mutations and age (17). Concerning the serum LDH levels, we did not found a significant difference between them and the *NFE2L2* levels, even though high values of LDH at diagnosis or during follow-up were associated with

an increased probability of AML evolution and decreased probability of survival. (50) Although it is not used in any prognostic score, serum erythropoietin (SE) levels below 500 IU/L are widely accepted as a major predictive factor for response to erythropoiesis-stimulating agents (ESAs) (53) but its importance as a prognostic marker is not fully understood. A 2015 study showed that increased erythropoietin levels at diagnosis can by itself be a poor prognosis factor in MDS patients. According to this study, patients with higher erythropoietin levels (>100 mIU/mL) presented a decreased overall survival. (52) In our study we didn't found any significant differences between the SE levels and *NFE2L2* expression levels. In resume, besides the prognostic value of several laboratorial parameters in MDS, namely SR, LDH and SE, the underlying mechanism remains unknown and is not yet certain that *NFE2L2* as a role on such mechanism.

The present study shows some limitations that prevent us from analysing completely this data and the inadequate age matching does not exclude the possible confounding bias that may exist. One of these limitations is associated with sampling, which predominantly comprises RCMD and low-risk patients, impossibilitating correlations of *NFE2L2* with other MDS subgroups or high-risk patients. Nevertheless, previous reports already indicated that OS was a more common event in low-risk patients. In this context, multicentre studies enrolling a significant number of patients and with a major percentage of high-risk patients will be needed to confirm our results and better understand the role of *NFE2L2* in MDS and sAML.

In conclusion, the present findings indicate that the evaluation of *NFE2L2* expression levels in MDS patients could increase the discriminative power of prognostic scoring systems to detect high-risk features with high probability of AML progression and a poor prognosis, and could be a tool to refine the current prognostics scores.

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**CONFLICTS OF INTEREST**

All authors have no conflicts of interest to declare.

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