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The role of cereblon in lenalidomide therapy of del(5q) myelodysplastic syndrome

Úloha cereblonu při terapii lenalidomidem u del(5q) myelodysplastického syndromu

Doctoral thesis

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FOREWORD

Myelodysplastic syndrome (MDS) is a hemato-oncologic disease. MDS can be characterized by failure of bone marrow and proliferation of myeloblastic cells. It is associated with older age (around 50 years). Rarely can occur in children, but it is linked to other diseases, as Down syndrome. Myelodysplastic syndrome is not leukemia, it is the stage of so-called pre-leukemia. In 40% transform to acute myeloid leukemia. Clinical symptoms are fatigue, inefficiency (symptoms of anemia), infections, bleeding symptoms, gum hyperplasia, acquired functional and morphological abnormalities of blood cells, less common thrombotic complications, and blood clotting disorders. Course and prognosis are variable, patient survival is around a couple of months to years.

5q- syndrome is the most occurred abnormality in low-risk MDS that is successfully treated with lenalidomide. Lenalidomide improve erythropoiesis and did not affect normal cells in 45%. Unfortunately, not every patient responds to lenalidomide therapy. There is no prognostic signature, which can predict lenalidomide response and determine who should receive the drug and who not. Recent studies show that cereblon can be a biomarker that should predict the efficacy of lenalidomide treatment.

In this thesis, we mainly focused on the analysis of cereblon at nucleotide and protein level in patients with 5q- syndrome, or del(5q) with trisomy 8. In the Introduction, we summarized the recent knowledge about myelodysplastic syndrome (classification, 5q-syndrome, trisomy 8), and agents as erythropoietin and lenalidomide. In addition, we examined the role of nuclear factor erythroid-derived 2-like 2 and arsenic trioxide in myelodysplastic syndrome. The following Results section obtain the major piece of my thesis work was published in the European Journal of Haematology with my co-first authorship. In this project, I prepared samples from peripheral blood and bone marrow for other methods. In this work, I sequenced the splicing variants of cereblon and A/G polymorphism in patients with 5q- syndrome, normal karyotype, and health samples. In the second part of the work, we focused on the continuation of measurements of cereblon expression at nucleotide and protein levels. Furthermore, we compare our outcomes with the level of hemoglobin. Also, we measured the level of nuclear factor erythroid-derived 2-like 2 in patients that were treated with erythropoietin and lenalidomide. The outcome of this study could be useful material for further research for the pathogenesis of MDS. Moreover, we measured arsenic trioxide alone or its combination with lenalidomide,

erythropoietin, and prednisone in MDS-L and SKM-1 cell lines. Finally, in the Discussion part, we compared our data with other published studies and discussed them.

ABSTRACT

Myelodysplastic syndrome (MDS) with deletion of the long arm of the chromosome 5 (5q- syndrome, del(5q)) can be characterized by anemia, macrocytosis, a normal or high platelet count, and hypolobulated megakaryocytes in the bone marrow. 5q- syndrome belongs to low- risk MDS, which means low risk to transform to acute myeloid leukemia. 5q- syndrome is associated with female predominance and older age. Another sign is transfusion burden that is treated by erythropoiesis-stimulating agents (ESA) as erythropoietin (EPO). Moreover, the response of MDS patients is around 30-60% with the median of the response being ~24 months. The second line of treatment is lenalidomide (LEN) which is a derivate of teratogenic analog thalidomide. LEN increases erythropoiesis and inhibits the growth of del(5q) erythroid progenitors in vivo and it does not have a significant effect on the growth of normal CD34+ progenitors or cytogenetically normal progenitors in MDS with del(5q) clones.

LEN is used as therapy in multiple myeloma, myelodysplastic syndrome, and lymphoma. LEN is an expensive agent and not every MDS patient responds to this therapy. This is a reason why is a need to find a biomarker for the determination of successful treatment. Some multiple myeloma studies showed that cereblon can be the biomarker that is needed. Cereblon (CRBN) is part of the E3 ubiquitin ligase CRL4 complex (cullin 4-RING E3 ubiquitin ligase complex), containing cullin-4A (CUL4A), damaged DNA binding protein (DDB1), and regulator of cullins 1 (ROC1). The function of CRBN in the CRL4 complex as a substrate receptor is to attach proteins, determined for polyubiquitination and subsequent degradation in 26S proteasome. This E3 ubiquitin ligase $CRL4^{CRBN}$ ubiquitinates CRBN, DDB1, and some endogenous proteins in the absence of lenalidomide. LEN binds to a specific hydrophobic pocket in the exons 10 and 11 of CRBN and changes the specificity of the $CRL4^{CRBN}$. In multiple myeloma, CRBN targets Ikaros and Aiolos proteins for ubiquitination, and in myelodysplastic syndrome is the target casein kinase 1A1.

In studies in multiple myeloma was proven that high CRBN expression leads to a good response to LEN therapy and improved patients´ life. The lower CRBN expression leads to the failure of therapy and progresses disease to high-risk MDS (MDS-EB-1 or MDS-EB-2) or acute myeloid leukemia. Furthermore, some patients stop responding to LEN therapy, and in these cases is added EPO or EPO and prednisone (PRED) that show temporally the effect of therapy.

In my thesis, we mainly focused on confirmation CRBN as the prognostic factor in human MDS samples at mRNA and protein levels. In the first part of the study, we verified that the high level of CRBN mRNA and protein CRBN in mononuclear cells isolated from the peripheral blood and bone marrow is associated with good response, and the lower level of CRBN mRNA and protein CRBN means the failure of LEN therapy or its combination (LEN+EPO or LEN+EPO+PRED). We validated our results with doctors and with the level of hemoglobin. We found out that CRBN protein in 5q- syndrome patients predict a quicker response on LEN therapy than CRBN mRNA. We confirmed that LEN binds to CRBN at exon 10 and 11. It was important to confirm the binding site of LEN because if exons 10 and 11 are missing, LEN cannot be bound and it also means the failure of treatment. Sardnal and his colleagues reported that A/G polymorphism has a prognostic signature in LEN treatment in MDS patients with normal karyotype. Unfortunately, we did not confirm this statement.

In the second part of this thesis, we focused on the role of nuclear factor erythroid-derived 2-like 2 (Nrf2). Nrf2 binds to a putative binding site in CRBN promoter and Nrf2 stimulates CRBN gene transcription under hypoxia/ reoxygenation conditions in neuronal cells. We confirm our assumptions that Nrf2 expression follows or overtakes the level of CRBN mRNA in the majority of MDS samples on EPO or LEN+EPO therapy. The level of Nrf2 mRNA did not follow CRBN mRNA in causes who failure LEN+EPO therapy.

The third part was experimental on MDS-L and SKM-1 cell lines when we monitored the effectiveness of arsenic trioxide (ATO). According to publications, ATO is effective with other agents. We made 19 groups, where we followed LEN, EPO, PRED, and ATO alone or its combination. Moreover, our result did not correspond at nucleotide and protein levels.

Key words: myelodysplastic syndrome, 5q- syndrome, cereblon, lenalidomide, nuclear factor erythroid-derived 2-like 2, erythropoietin, arsenic trioxide

ABSTRAKT

Myelodysplastický syndrom (MDS) s delecí dlouhého raménka chromozomu 5 (5q- syndrom, del(5q)) může být charakterizován anémií, makrocytózou, normálnímim nebo zvýšeným počtem krevních destiček a hypolobulovanými megakaryocyty v kostní dřeni. 5q- syndrom se řadí mezi nízko rizikové MDS, které ojediněle transformují do akutní myeloidní leukémie (AML). 5q- syndrom je spojen se starším věkem a převládá u žen. Dalším znakem je transfúzní závislost, která je léčená léky stimulujícími erytropoézu (ESA), například erytropoetin (EPO). Navíc, odpověď pacientů je jenom 30-60% s mediánem odpovědi ~24 měsíců.

Druhou linií léčby je lenalidomid (LEN), což je derivát teratogenního analogu thalidomid. LEN zvyšuje erytropoézu a inhibuje růst erytroidných progenitorových buněk del(5q) in vivo a nemá signifikantní efekt na růst normálních progenitorových buněk CD34+ nebo cytogeneticky normálních progenitorových buněk u MDS se klony del(5q).

LEN je využíván jako léčba u mnohočetného myelomu, myelodysplastického syndromu a lymfomů. LEN je drahý lék a ne u každý MDS pacient odpovídá na terapii. Toto je důvod proč je důležité najít biomarker pro určení úspěšné terapie. Některé studie u mnohočetného myelomu ukazují, že cereblon může být tímto biomarkrem, který je zapotřebí. Cereblon (CRBN) je součástí komplexu E3 ubiguitin ligázy CRL4 (cullin 4-RING E3 ubiquitin ligase complex) obsahující CUL4A (cullin-4A), DDB1 (damaged DNA binding protein 1) a ROC1 (regulator of cullins 1). Funkce CRBN v komplexu CRL4 jako substrátového receptoru slouží k připojení proteinů pro polyubikvitinaci a následnou degradaci v 26S proteazomu. V nepřítomnosti lenalidomidu, E3 ubiquitin ligáza $CRL4^{CRBN}$ ubikvitinuje CRBN, DDB1 a některé endogenní proteiny. LEN se váže na specifickou hydrofobní kapsu v CRBN exonech 10 a 11 a mění specificitu $CRL4^{CRBN}$. U mnohočetného myelomu, cílem CRBN pro ubikvitinaci jsou proteiny Ikaros a Aiolos a u myelodysplastického syndromu je cílem kasein kináza 1A1.

V studiích týkajících se mnohočetného myelomu bylo popsáno, že vysoké hladiny CRBN exprese vedou k dobré odpovědi na léčbu LEN a zkvalitňují život pacientů. Nízké hladiny CRBN exprese vedou k selhání terapie a progresi do vysoko rizikových MDS (MDS-EB-1 nebo MDS-EB-2) nebo AML. Někteří pacienti přestanou odpovídat na léčbu LEN a v těchto případech je přidán EPO anebo EPO a prednison (PRED), které vykazují dočasný účinek terapie.

V mé práci jsme se hlavně zaměřili na potvrzení CRBN jako prognostického faktoru u MDS vzorků jak na mRNA, tak na proteinové úrovni. V první části studií jsme potvrdili, že vysoká hladina CRBN mRNA a proteinu CRBN v mononukleárních buňkách izolovaných z periferní krve a kostní dřeně je spojená s velmi dobrou odpovědí a snížená hladina CRBN mRNA a proteinu CRBN vede k selhání LEN terapie nebo kombinace LEN s dalšími látkami. Naše výsledky jsme potvrdili s doktory a výsledky měření hladiny hemoglobinu. Objevili jsme, že hlasdina CRBN jako proteinu u pacientů s 5q- syndromem předpovídá rychlejší odpověď na LEN terapii než hladina CRBN mRNA. Potvrdili jsme, že LEN se váže na CRBN v exonech 10 a 11. Bylo důležité potvrdit vazebné místo LEN, protože když chybí exon 10 a 11, LEN se nemůže vázat a také to značí selhání léčby. Sardnal a jeho kolegové popsali, že A/G polymorfismus by mohl být prognostický faktor u MDS pacientů s normálním karyotypem. Bohužel, nepotvrdili jsme toto tvrzení.

V druhé části této práce jsme se soustředili na úlohu Nrf2 (nuclear factor erythroid-derived 2-like 2). Nrf2 se váže na domnělé vazebné místo v CRBN promotoru a Nrf2 stimuluje transkripci CRBN génu za podmínek hypoxie/ reoxygenace v neuronových buňkách. Potvrdili jsme náš předpoklad, že exprese Nrf2 sleduje nebo předbíhá hladiny CRBN mRNA u většiny MDS vzorků na EPO nebo LEN+EPO terapii. Hladina Nrf2 mRNA nenásledovala CRBN mRNA v případe selháni LEN+EPO terapií.

Třetí část byla experimentální na liniích buněk MDS-L a SKM-1, kde jsme sledovali účinnost oxidu arsenitého (ATO). Podle publikací, ATO je účinný v kombinaci s jinými léky. Vytvořili jsme 19 skupin, kde jsme sledovali LEN, EPO, PRED a ATO samotné nebo jejích kombinace. Naše výsledky nesouhlasily s předpokládanými hladinami exprese analyzovaných genů na mRNA nebo proteinové úrovni.

Klíčové slova: myelodysplastický syndrom, 5q- syndrom, cereblon, lenalidomid, nuclear factor erythroid- derived 2-like 2, erytropoetin, oxid arsenitý

List of abbreviations

+8	trisomy 8
11 β-HSD- enzyme	11 β-hydroxysteroid dehydrogenase
AA	amino acid
AER	apical ectodermal ridge
AKT	protein kinase B
AML	acute myeloid leukemia
AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANC	absolute neutrophil count
APL	acute promyelocytic leukemia
ARE	antioxidant response elements
ASXL1	ASXL transcriptional regulator 1
ATO	arsenic trioxide
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCOR	BCL6 corepressor
BM	bone marrow
BK_{Ca}	large- conductance calcium-activated potassium channel
β-TrCP	β- transducing repeat-containing protein
CALR	calreticulin
CAPN1	calpain-1 protein
CASP-8	caspase 8
CBL	Cbl proto- oncogene
CDC25A	cell division cycle 25 homolog A
CDC25C	cell division cycle 25C
CD147	basigin, EMMPRIN
CDR	common deleted region
CEBPA	CCAAT/enhancer- binding protein alpha
CFU-E	colony-forming unit-erythroid
CRBN	cereblon
CRL4	cullin 4-RING E3 ubiquitin ligase complex
CSNK1A1	casein kinase 1α

CUL4A	cullin-4A
DDB1	DNA damage binding protein 1
del(5q)	deletion of the long arm of chromosome 5
DNMT3A	DNA methyltransferase 3 alpha
DNMT inhibitors	DNA methyltransferase inhibitors
EB-1	refractory anemia with excess blasts-1
EB-2	refractory anemia with excess blasts-2
EPO	erythropoietin
EPOR	erythropoietin receptor
ERK	extracellular signal-regulated kinase
ESA	erythropoietin stimulating agent
ETV6	ETS variant transcription factor 6
EZH2	enhancer of zeste homolog 2
FAB clasification	French-British-American classification
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FDA	U.S. Food and Drug Administration
Fgf	fibroblast growth factor
Fgf8	fibroblast growth factor 8
Fli-1	Friend leukemia virus integration 1
FLT3	FMS- like tyrosine kinase 3
FPN1	Ferroportin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA2	GATA- binding factor 2
GC	glucocorticoid
GSK-3	glycogen synthase kinase 3
Gstp1	glutathione S transferase P
GVHD	graft-versus-host disease
G3BP1	Ras GTPase-activating protein-binding protein 1
hCRBN	human cereblon
hTERT	human telomerase reverse transcriptase
HIF	hypoxia- inducible factor
HIF2	hypoxia-inducible factor 2 alpha
HNF-4A	hepatocyte nuclear factor 4 alpha

HIV	human immunodeficiency virus
HO 1	hemo oxigenase
HPSC	human pluripotent stem cells
HR	hazard ratios
HSC	hematopoietic stem cells
IDH1/2	isocitrate dehydrogenase 1 and 2
IKZF1	Ikaros
IKZF3	Ailos
IL-1	interleukin 1
IL-2	interleukin 2
IL-6	interleukin 6
IL-8	interleukin 8
IMiD	imunomodulatory drug
Int-1	intermediate 1
int-2	intermediate 2
IPSS	International Prognostic Scoring System
IPSS-R	revised International Prognostic Scoring System
IRF4	interferon regulatory factor 4
JAK2	Janus kinase 2
Keap1	Kelch-like ECH associated protein
KRAS	Kirsten rat sarcoma virus
LEN	lenalidomide
MCT1	monocarboxylate transporter 1
MDM2	mouse double minute 2 protein
MDS	myelodysplastic syndrome
MDS/MPD	myelodysplastic/myeloproliferative neoplasm
MEIS2	Myeloid Ecotropic Insertion Site-2
miRNA	small non-coding RNA
MLD	multilineage dysplasia
ML835	inhibitor Nrf2
MM	multiple myeloma
MPL	myeloproliferative leukemia virus oncogene
NF1	neurofibromin 1
NF-κB	nuclear factor-kappa B

NO	nitric oxide
non-5q	patients with normal karyotype
NRAS	neuroblastoma RAS viral oncogene homolog
Nrf2	nuclear factor erythroid 2-related factor
NQO1	(NAD(P)H quinone oxidoreductase 1
OS	overall survival
PB	peripheral blood
PBS	phosphate-buffered saline
PCR	polymerase change reaction
PEL	primary effusion lymphoma
PINK1	PTEN-induced kinase 1
PI3K	phosphatidylinositol 3 kinase
PLZF	promyelocytic leukemia zinc finger
PP2A	protein phosphatases 2A catalytic domain alpha
PRED	Prednisone
PTEN	phosphatase and tensin homolog
RA	refractory anemia
Rabex-5	Rabaptin-5-associated exchange factor for Rab 5
RARS	refractory anemia with ring sideroblasts
RARS-T	refractory anemia with ring sideroblasts with thrombocytosis
RCMD	refractory anemia with multilineage dysplasia
RCUD	refractory cytopenias with unilineage dysplasia
REMS	Thalidomide Risk Evaluation and Mitigation Strategy
RN	refractory neutropenia
RNF41	RING finger protein 41
ROC1	RING finger protein
ROS	reactive oxygen species
RPS14	ribosomal protein S-14
RT	refractory thrombocytopenia
RUNX 1	Runt- related transcription factor 1
SALL-4	Spat-like transcription factor 4
SBTI	soybean trypsin inhibitor
SDS PAGE	sodium dodecylsulphate-polyacrylamide

	gel electrophoresis
SF3B1	splicing factor 3B subunit 1
SRSF2	serine/arginine-rich splicing factor
STAG2	stromal antigen 2
STAT5	Signal transducer and activator of transcription 5
S100A8	S100 calcium-binding protein A8 or myeloid-related protein 8
S100A9	myeloid-related protein 9
TET2	Tet methylcytosine dioxygenase 2
TF	transcriptional factors
TIRAP	Toll-IL-1 receptor domain-containing adaptor protein
TL	Triptolide
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-alpha
TOP2B	DNA topoisomerase II beta
TRAF6	TNF-receptor associated factor 6
TRF	transfusion
Trx/TrxR system	Thioredoxin/ Thioredoxin reductase system
UA2F1	U2 small nnuclear RNA auxiliary factor 1
WHO	World Health Organization
WNT	Wingless/ integrated
ZFP91	zinc finger protein 91

1. LITERATURE REVIEW

1.1. Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is a heterogeneous clonal pre-malignant hematopoietic disorder, which is characterized by peripheral blood cytopenias, ineffective hematopoiesis, hypercellular bone marrow with morphologically defined dysplasia of one or more lineages [Ganguly and Kadam, 2016]. Around 40% of MDS patients transform to acute myeloid leukemia (AML) [Pellagati and Boultwood, 2015].

1.2. Epidemiology

Incidence is approximately 4 per 100 000 men and women per year and is connected by older age [Germing et al., 2013]. The median age of MDS patients is approximately 70 years. There are around 10% of patients under the age of 50 [Germing et al., 2013]. MDS can be caused by previous chemotherapy (secondary MDS), radiation, or benzene exposure [Parylo et al., 2017].

1.3. Classification of MDS

French-British-American (FAB) classification by Bennet et al. gave the foundation of classification of MDS in 1976. The World Health Organization (WHO) changed a little FAB classification in 1999 and revised it in 2008. The WHO divided MDS groups from FAB to more subtypes of MDS according to peripheral and bone marrow (BM) findings (Table 1) [Vardiman, 2012].

Table 1: WHO classification of MDS [Vardiman, 2012]

Disease	Blood findings	Bone marrow findings
Refractory cytopenias with unilineage dysplasia (RCUD), refractory anemia (RA), refractory neutropenia (RN), refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia No or rare blasts (<1%)	Unilineage dysplasia: ≥10% of the cell in one myeloid lineage < 5% blasts 15% of erythroid precursor are ring sideroblasts

Refractory anemia with ring sideroblasts (RARS)	Anemia No blasts	≥15% if the erythroid precursors are ring sideroblasts < 5% blasts
Refractory anemia with multilineage dysplasia (RCMD)	Cytopenia No or rare blasts No Auer rods <1x10⁹/L monocytes	Dysplasia in >10% of cells in 2 or more lineages < 5% blasts in BM No Auer rods <1x10⁹/L monocytes
Refractory anemia with excess blasts-1 (EB-1)	Cytopenia < 5% blasts No Auer rods < 1x10⁹/L monocytes	Unilineage or multilineage dysplasia 5-9% blasts in bone marrow No Auer rods
Refractory anemia with excess blasts-2 (EB-2)	Cytopenia 5-19% blasts Auer rods <5 % myeloblasts < 1x10⁹/L monocytes	Unilineage or multilineage dysplasia 0-19% blasts in bone marrow Auer rods <10% myeloblasts
MDS, unclassifiable	Cytopenias < 1% blasts	Unequivocal dysplasia in less than 10% of cells in one or more myeloid lines when accompanied by a cytogenetic abnormality < 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(5) is the sole cytogenetic abnormality

		No Auer rods

In 1997 was developed the International Prognostic Scoring System (IPSS, Table 2) and was revised in 2012 (IPSS-R- revised IPSS, Table 3). This scoring system is an important guide for untreated patients. It is an internationally accepted standard risk assessment system in MDS [Mohammad, 2018; Schanz et al., 2012; Greenberg et al., 2012].

Table 2: Survival and AML evaluation according to IPSS [Mohammad, 2018].

International Prognostic Scoring System (IPSS)					
Prognostic variable	0.0	0.5	1.0	1.5	2.0
Blasts in BM	<5	5-10	-	11-20	21-30
Karyotype	Good	Intermediate	Poor	-	-
Cytopenia	0/1	2/3	-	-	-

Risk category	**Total score**	**Median survival without treatment**	**25% AML progression without treatment**
Low	0	5.7	9.4
Intermediate-1	0.5-1.0	3.5	3.3
Intermediate-2	1.5-2.0	1.1	1.1
High	>2.5	0.4	0.2

Table 3: Survival and AML evaluation according to IPSS-R [Mohammad, 2018].

Revised International Prognostic Scoring System							
Prognostic variable	0	0.5	1.0	1.5	2	3	4
Cytogenetic	Very good		Good		Intermediate	Poor	Very poor
Blasts in BM	≤2	-	>2-<5		5-10	>10	
Hemoglobin	≥10		8-<10	<8			
Platelet	≥100	50-<100	<50				
ANC (Absolute neutrophil count)	≥0.8	<0.8					

Risk category	**Total score**	**Median survival without treatment**	**25%AML progression without treatment**
Very low	≤1.5	8.8	Not reached
Low	>1.5-≤ 3	5.3	10.8
Intermediate-1	>3-≤ 4.5	3.0	3.2
High	>4.5-≤ 6	1.5	1.4
Very high	>6.0	0.8	0.7

The last scoring group is cytogenetic prognostic classification, which uses a more detailed analysis of cytogenetic abnormalities and prognostic meaning than IPSS-R (Table 4) [Schanz et al., 2012].

Table 4: Cytogenetic classification of MDS by Schanz et al. [Montalban-Bravo and Garcia-Manero, 2017]

Prognostic groups	**Chromosomal categories**	**Median survival time**
Very good n=80 (2,9%)	**Single:** del(11q) -Y	**Median overall survival (OS):** 60,8 months **Hazard ratios (HR)** 0,47 (0,3-0,7)
Good n=1844 (65,9%)	**Single:** Normal der(1,7) del(5q) del(12p) del(20q) **Double:** Double incl. del(5q)	**Median OS:** 48.5 months **HR (Ref.)** 1,00 (0,8-1,3)
Intermediate n=578 (20,7%)	**Single:** -7/7q- +8 iso(17q) +19 +21 Any other clones **Double:** Any other double	**Median OS:** 25 months **HR** 1,59 (1,4-1,9)
Poor n=101 (3,6%)	**Single:** der(3)(q21)/der(3)(q26) **Double:** Double include -7/7q-	**Median OS:** 15 months **HR** 2,83 (2,2-3,7)

	Complex: 3 abnormalities	
Very poor n=196 (7%)	**Complex:** >3 abnormalities	**Median OS:** 5,7 months **HR** 4,37 (3,5-5,5)

1.4. Pathogenesis

MDS patients can have single/multiple or no chromosomal aberrations, which arise before or during the treatment. Simple chromosomal aberrations may involve a numerical change (trisomy, monosomy), a structural abnormality, or balanced translocation [Zhang et al., 2018]. The common abnormality among MDS patients is monosomy 5/del(5q), monosomy 7/deletion of the long arm of 7, trisomy 8, deletion of the long arm of chromosome 20, deletion of the short arm of chromosome 17, and deletion of the long arm of chromosome 11. Chromosomal abnormalities are demonstrated in 50% of de novo cases, and 80% of cases are related to therapy [Kawankar and Vundinti, 2011; Pellagati and Boultwood, 2015].

In this chapter, we will also introduce our target group of MDS patients with 5q-syndrome and trisomy 8.

1.4.1. 5q- syndrome

MDS patients with deletion of the long arm of chromosome 5 (del(5q), 5q- syndrome) were described by Van den Berghe et all, in 1974. 5q- syndrome is defined by anemia, macrocytosis, a normal or high platelet count, and hypolobulated megakaryocytes in bone marrow [Boultwood et al, 2010]. Another sign of 5q- syndrome is a female predominance, transfusion dependence, and rare transformation to AML. Naturally, patients with complex karyotypes have a higher possibility to progress to high-risk MDS or AML [List et al, 2018]. The median survival in MDS patients with del(5q) is higher than 5 years. Patnaik et al have reported that dysgranulopoiesis has an impact on the survival of these patients [Patnaik et al, 2010]. The frequency of 5q- syndrome is around 20-30% of patients, but only around 5% of these subjects belong to the classification of MDS with isolated del(5q) [Gokalp-Yasar and Liu, 2013; List et al., 2018].

There are two common deleted regions (CDRs) in 5q- syndrome- proximal CDR at 5q31 with a minimal deletion of 1MB leading to presumed loss of a tumor suppressor gene(s), and the distal CDR has 1,5MB interval at 5q32-33. 5q31 is connected with poor prognosis and progression to high-risk MDS (RAEB1 or RAEB2) or AML. The distal CDR includes 40 genes and 33 of these genes were found to be expressed in CD34+. What is important to mention is the absence of mutations in CDR and the expression of genes in this region is monoallelic [Sallman et al., 2014; Gaballa and Besa, 2013].

5q- syndrome is linked to haploinsufficiency of ribosomal protein S-14 (RPS14), encoding a component of the 40S ribosomal subunit (determinant of hypoplastic anemia) and casein kinase 1α (CSNK1A1, CK1α) [Caceres et al., 2013; Pellagati and Boultwood, 2015].

Casein kinase 1α encodes a serine/threonine kinase and is a tumor suppressor gene [Tan et al., 2016]. Further, CSNK1A1 has a regulatory role in the β-catenin and p53 signaling pathways [Bello et al., 2015]. Constitutive activation of the β-catenin leads to increasing hematopoietic stem cells (HSC) and subsequently to apoptosis, HSC depletion, and bone marrow failure [Schneider et al, 2014]. Mutations of CSNK1A1 are rare in 5q- syndrome (approximately 7%) and Schneider et al. have indicated that these lesions are genetic drivers of clonal dominance. MDS patients have missense mutations which are targeted glutamic acid E98 in exon 3 and aspartic acid D140 [Bello et al., 2015; Schneider et al., 2014; Heuser et al., 2015]. The complete loss of CSNK1A1 is not tolerated by MDS cells with del(5q) but it can be tolerated 50% expression of this gene [List et al., 2018].

RPS14 is associated with heterodimer S100A8/S100A9 in monocytes, erythroblasts, macrophages, granulocytes, and the inflammatory environment. S100A8 (myeloid-related protein 8) and S100A9 (myeloid-related protein 9) are members of S100 calcium-binding proteins and they were characterized as new endogenous TLR4 (Toll-like receptor 4) activators. Proteins p53 and S100A8 must be active because inactive forms cause a block in erythroid differentiation. The same importance has ribosomal insufficiency, which disrupts ribosome integrity and this situation leads to autologous degradation of the human homolog of the mouse double minute 2 protein (MDM2), resulting in stabilization of p53. In mice, MDM2 regulated p53 as an E3 ubiquitin ligase, which participates in the ubiquitination of p53 and its degradation in the proteasome. MDM2 promoter is activated by Fli-1 (Friend leukemia virus integration 1). Fli-1 is a member of the ETS family of transcription factors and functions as a potential oncogene

and its expression is increased in 5q- syndrome [Schneider et al., 2016; Dessing et al., 2014; Caceres et al., 2013; Pellagati and Boultwood, 2015; Ribezzo et al., 2019, and Neuwirtova et al., 2013]. The relationship between p53 and MDM2 is more explained in the chapter- Lenalidomide.

In this region is also located G3BP1 (Ras GTPase-activating protein-binding protein 1) which regulates the activity of p53 deletion involving protein interaction of G3BP1-p53 and deubiquitination by regulating the ubiquitin-specific peptidase USP10. Haploinsufficiency and downregulation of G3BP1 predict a poor prognosis in MDS patients with del(5q) [Hosono, 2019].

Phosphatases PP2A (protein phosphatase 2A catalytic domain alpha) and CDC25C (cell division cycle 25C, dual-specificity phosphatase) and small non-coding RNA (microRNA or miRNA) have another critical role at the 5q locus. For 5q- syndrome is typical the downregulation of miRNA 145 and miRNA 146a [Duong et al., 2012].

Besides, the overexpression was found in the Toll-IL-1 receptor domain-containing adaptor protein (TIRAP) and TNF-receptor associated factor 6 (TRAF6), which is connected to congenital immune signaling. The low levels of miRNA 145, miRNA 146, and upregulation of TRAF6 cause thrombocytosis and hypolobated micromegaryocytes. The low level of expression of TIRAP and TRAF leads to upregulation of interleukin 6 (IL-6). miRNA 145 does not have any mutation in 5q-syndrome. Differentiation of megakaryocytes is associated with the Fli-1 mRNA, which is the target of miRNA 145. The low expression of miRNA 145 increases the level of Fli-1, which leads to the enhancement of megakaryocyte production and erythroid progenitors. Fli-1 mRNA is higher in 5q- syndrome than in cohorts without del(5q). MDS patients are linked also to miRNA 143, miRNA 378, miRNA 150, and miRNA 34a [Duong et al., 2012; Ebert, 2011; Neuwirtova et al., 2013; Gaballa and Besa, 2013 and Kumar et al., 2011].

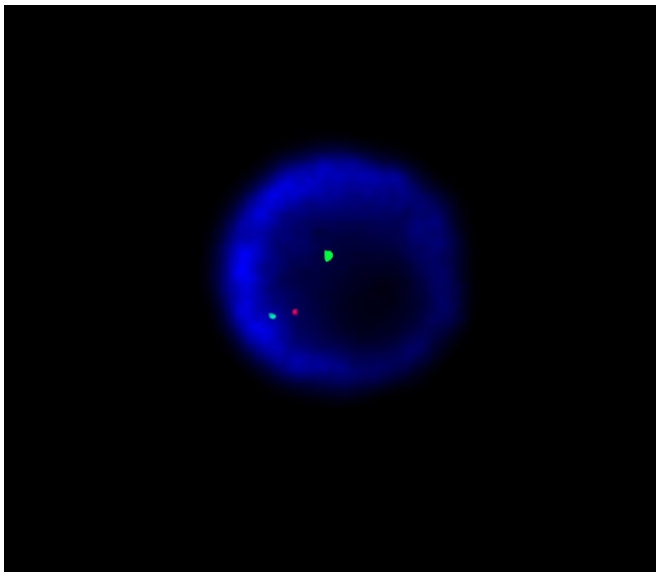


Fig.1: Figure 1 presented pathological cell with del(5q,31). The deletion is visualized by fluorescence in situ hybridization using Vysis LSI 5q EGR1 so/D5S23 sg (Abott Vysis Inc.). The figure was published in the diploma work of Bokorova, Medirex, Bratislava, Slovakia, 2012.

1.4.2. Trisomy 8

Trisomy 8 (+8) is the common chromosome aberration in MDS (approximately 5-10% of MDS patients), which is included in the MDS intermediate cytogenetic risk group according to IPSS (IPSS-R) [Saumell et al., 2015; Konuma 2017]. The median survival with trisomy 8 is 25 months [Wesner et al., 2018]. In these causes was found up-regulation of anti-apoptotic proteins and resistance to apoptotic stimuli [Zahid et al., 2017].

As far as therapy, these patients have an amazing response to immunosuppressive treatment, which is up to 67%. Furthermore, it can be linked to inflammatory or autoimmune features (approximately 15-20% of cases) [Wesner et al., 2018; Zahid et al., 2017].

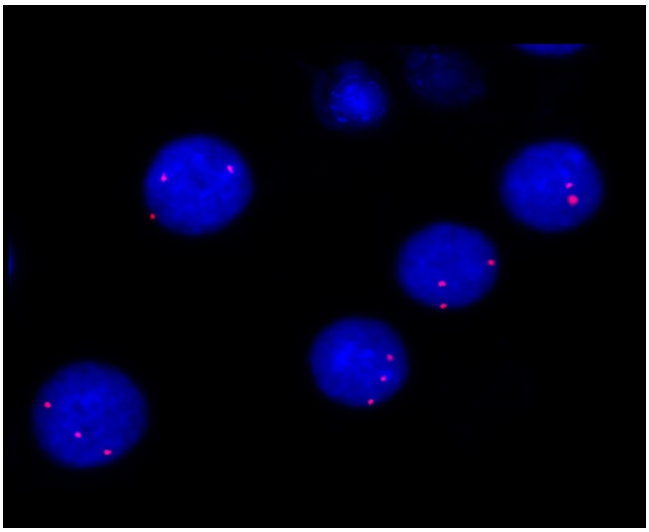


Fig.2: Pathological cell with trisomy 8. Deletion is visualized by fluorescence in situ hybridization using Vysis CEP 8 so (Abott Vysis Inc.). The figure was obtained in Medirex, Bratislava, Slovakia (2011-2012), during Bokorova master´s degree, but it was not included in the thesis.

1.5. Somatic mutations

Except for chromosomal abnormalities, mutated genes are associated with RNA splicing, DNA methylation, chromatin modification, transcription factors, signal transduction, and cohesin complex. Table 5 is a summary of the common mutations in MDS.

Table 5: Molecular classification [Harada and Harada, 2015]

Mutated genes	**Associated phenotypes**	**MDS types**	**Frequency in MDS**	**Effect on outcome**
RNA splicing (mutually exclusive)			60-70%	
SF3B1 (splicing factor 3B subunit 1)	Ring sideroblasts	RARS, RCMD-RS	15-30%	Good
SRSF2 (serine/arginine-rich splicing factor)		RCMD, RAEB	10-20%	Poor

U2AF1 (U2 small RNA auxiliary factor 1)		RCMD, RAEB	5-10%	Poor
ZRSF2		RCMD, RAEB	5-10%	None
DNA methylation (TET2 and IDH1/2 are exclusive)			40-50%	
TET2 (Tet methylcytosine dioxygenase 2)	Myeloid dominancy	All MDS, normal karyotype	20-30%	None
IDH1/2 (isocitrate dehydrogenase 1 and 2)		RCMD, RAEB	5%	Poor
DNMT3A (DNA methyltransferase 3 alpha)		All MDS	10%	None
Chromatin modification			20-30%	
ASXL1 (ASXL transcriptional regulator 1)		RCMD, RAEB	15-20%	Poor
EZH2 (enhancer of zeste homolog 2)	-7/7q	RCMD, RAEB	5%	Poor
BCOR (BCL6 corepressor)		RCMD, RAEB	5%	Poor
Transcriptional factor			20-30%	

RUNX1 (runt-related transcription factor 1)		RCMD, RAEB	10%	Very poor
CEBPA (CCAAT/enhancer-binding protein alpha)	Thrombocytopenia	RCMD, RAEB	<5%	None-poor
ETV6 (ETS variant transcription factor 6)		RCMD, RAEB	<5%	Poor
Signal transduction (mutually exclusive)			20-30%	
NRAS (neuroblastoma RAS viral oncogene homolog)/KRAS (Kirsten rat sarcoma virus)		All MDS	10%	Poor
CBL (Cbl proto-oncogene)		All MDS	5%	Poor
JAK2 (Janus kinase 2)	Megakaryocytosis	All MDS	5%	None
NF1 (neurofibromin 1)		All MDS	<5%	Poor
FLT3 (FMS-like tyrosine kinase 3)		All MDS	<5%	Poor
Cohesin complex (mutually exclusive)			10%	
STAG2 (stromal antigen 2)		RCMD, RAEB	5-10%	None-poor
TP53	Complex karyotype	RAEB, isolated del(5q)	10%	Very poor

1.5.1. *TP53* and the product of its expression p53

The tumor suppressor protein p53 was discovered in 1979. The protein is located on chromosome 17p13.1, contains 11exons, and has ~53kDa. In normal cells, the half-life of p53 is 6-20 min. [Saha et al., 2015; Xiong et al, 2020; Zhang, 2018]. *TP53* encodes cytoplasmic protein p53, which is a homotetramer, consisting of 393 amino acids. P53 has an N-terminal region, the DNA binding domain, nuclear localization signaling domain, and C-terminal domain. P53 gene is very well known for its important role in a cell cycle- G1 checkpoint halts cell division when DNA damages are found and it stimulates repair enzyme activity at the same time. In conclusion, p53 is involved in the cell cycle, apoptosis, senescence, and DNA repair [Tan et al., 2019; Yao and Lima, 2014; Zhang, 2018; Kamaraj and Bogaerts, 2015].

The frequently mutated in p53 are Arg248, Arg273, Arg175, Gly245, Arg249, and Arg282. In MDS, these mutations are found in intermediate or higher risk causes. The role in low-risk MDS is not clarified and mutations of p53 are associated with poor prognosis. [Yao and Lima, 2014; Kamaraj and Bogaerts, 2015]. TP53 plays a significant role in lenalidomide therapy because p53 mutations cause lenalidomide resistance. Moreover, the function of p53 is required for the cytotoxic effects of this agent following casein kinase 1A1 degradation [Martinez-Høyer and Karsan, 2020]. More about lenalidomide is in Chapter about lenalidomide.

1.6. Cereblon

One of the possible prognostic factors can be cereblon (CRBN). The correlation of cereblon upregulation and the better response of patients, who were treated by lenalidomide (LEN), was demonstrated in multiple myeloma (MM) [Heintel et al., 2013].

The human cereblon gene (*CRBN*) is located on chromosome 3 at 3p26 and the product of its expression was found in the cytoplasm, nucleus, and peripheral membrane. The size of the genomic DNA of CRBN is 30 111 bases and contains 11 exons coding 442 amino acids (AAs), its molecular weight is ∼ 51 kDa. CRBN consists of the N-terminal part (237 AA) of ATP (adenosine triphosphate)- dependent Lon protease domain without the conserved Walker A and Walker B motifs, 11 casein kinase II phosphorylation sites, 4 protein kinase C phosphorylation sites, 1 N- linked glycosylation site, and 2 myristoylation sites. Cereblon can regulate the large-conductance calcium-activated

potassium channel (BK_{Ca}) and regulates its surface expression. CRBN binds to α1 subunits of 5' adenosine monophosphate-activated protein kinase (AMPK), which regulates the homeostasis of metabolism by monitoring the ratio level of AMP (adenosine monophosphate)/ATP. Interaction between CRBN and AMPK can control metabolism better when mammals have serine at position 366. Moreover, CRBN has an important role in cell proliferation, apoptosis, and the regulation of ion transport. CRBN in connection with the E3 ubiquitin ligase complex targets ion channels for ubiquitination in proteasome thereby creating the ion balance and decreasing the ion diseases. Human CRBN is expressed in the kidney, placenta, leukocytes, skeletal muscle, ovary, pancreas, brain, colon, retina, spleen, prostate, testis, small intestine and is also involved in endoplasmic reticulum stress and oxidative stress [Chang and Steward, 2011; Sawamura et al., 2018; Lee et al., 2012; Shi and Chen, 2017; Onodera et al., 2019].

Cereblon is an autosubstrate and receptor for substrates of the cullin-RING E3 ubiquitin ligase CRL4 complex. The CRL4 consists of cullin-4A (CUL4A), RING finger protein (ROC1), and DNA damage binding protein 1 (DDB1). Cereblon functions as one of the substrates tagging proteins for polyubiquitination and degradation in 26S proteasome [Díaz-Rodríguez and Pandiella, 2016].

CSNK1A1 is targeted by the same E3 ubiquitin ligase CRL4 complex as cereblon. CSNK1A1 is bound to LEN and CRBN through the beta-hairpin loop in CSNK1A1 consisting of 35-41 residues. It was identified the specific glycine (Gly40 of CSNK1A1) which is required in the surface-turn of $CUL4A^{CRBN}$ substrates. The importance of the specific glycine was demonstrated by the loss of CRBN and CSNK1A1 binding activity [Asatsuma-Okumura et al., 2019].

Lenalidomide is bound to cereblon through glutarimide ring at exons 10 and 11, for binding is needed His380, Trp382, Trp402 and Phe404, these AAs arrange binding through van der Waals contact. However, only Trp388 and Tyr386 are important for the direct binding process of LEN to CRBN [Guirguis and Ebert, 2015; Asatsuma-Okumura et al., 2019]. The IMiDs (immunomodulatory drugs) binding site is C–terminal β-sheet. The other potential binding ligand is uridine. CRBN binds uridine in the same way as thalidomide and with comparable affinity. The connection of CRBN and uridine cause the same teratogenic effects as IMiDs [Hartmann et al., 2015]. Myeloid Ecotropic Insertion Site-2 (MEIS2) is the endogenous substrate of CRBN. LEN or other IMiDs can prevent the interaction of CRBN with MEIS2 and block its degradation. Therefore, CRBN/MEIS2 could be important for therapeutic relevance and enhance the anti-MM

activity of IMiDs [Abruzzese et al., 2019]. Except for MEIS2 and uridine, there are other substrate proteins, which can bind to cereblon, such as the acetylated form of glutamine synthase, Rabex-5 (Rabaptin-5-associated exchange factor for Rab 5) [Gemechu et al., 2018]. Zhou and Xu have shown that CRBN protects against DNA damage-induced apoptosis, but it does not affect the degradation and protein level of p53 and BAX. CRBN KO mice increase the interaction of p53, Bcl-2, and Bcl-X_L and decrease the level of *CRBN* (p53) mRNA indicating that CRBN can regulate the transcription-independent function of p53 [Zhou and Xu, 2019].

Sardnal et al. have analyzed the A/G polymorphism located at the site 29 nucleotides before the transcriptional start site of the *CRBN* gene as a biomarker of lenalidomide responders in low/int-1-risk MDS without del(5q). Their results indicated that MDS patients without del(5q) and with G allele have a better response to LEN treatment than with A allele. The G allele was not linked with a different level of CRBN mRNA in comparison to allele A [Sardnal et al., 2013].

Moreover, nuclear factor erythroid 2-related factor 2 /Nrf2/ binds antioxidant response elements /ARE/ site in the upstream promoter region of mouse *CRBN* enhance expression of CRBN through hypoxia-reoxygenation in neuroblastoma cells. The overexpression of Nrf2 triggers the expression of the endogenous *CRBN* gene [Lee et al., 2010].

1.7. Nuclear factor erythroid 2-related factor 2

Gene for nuclear factor erythroid 2-related factor 2 (*Nrf2*, *NFE2L2*) is located on chromosome 2 at 2q31.2. Its transcript is a part of the cap-n-collar subfamily of the basic region-leucine zipper-type transcription factors. Nrf2 includes seven embedded contact homology (Nrf2-ECH) Neh domains. The Neh1 domain contains a basic region-leucine zipper structure, this domain is needed for dimerization of Nrf2 with small Maf proteins and binding to DNA. Neh2 domain, an N-terminal regulatory domain, interacts with E3 ligase adaptor, Kelch-like ECH associated protein (Keap1) and mediates Nrf2 degradation in 26S proteasome under normal physiological conditions. The alternative mechanism of Nrf2 degradation is through the N6 domain, which is mediated by glycogen synthase kinase 3 (GSK-3) and β- transducing repeat-containing protein (β-TrCP). The C terminal Neh3, Neh4, and Neh5 are linked to Nrf2 dependent transactivation mediating the interaction of Nrf2 with other co-activators. Furthermore, Neh5 is responsible for the cytoplasmic localization of Nrf2. The Neh7 is included in the repression of transcriptional

activity of Nrf2 by the retinoid X receptor α. Except for Keap1, the expression of Nrf2 is also regulated by the extracellular signal-regulated kinase (ERK). Moreover, Nrf2 has a half-life of about 20 - 30 min., due to constant degradation by 26S proteasome. Under the normal, non-stressed conditions, low levels of Nrf2 provide the basal expression of its target genes. In the past, scientists predicted the molecular weight of Nrf2 around 55-65 kDa based on its 2- kb open reading frame. The new data showed that the biological relevant species of Nrf2 migrate between ~ 95 and 110 kDa. The constitutive activation of Nrf2 is caused by somatic mutations in Nrf2 or Keap1, and loss of Keap1 expression in different types of tumors, which allow Nrf2 to escape Keap1- mediating degradation. The high level of Nrf2 is connected to chemoresistance in cancer [Saha et al., 2020; Robledinos- Antón et al., 2019; Wu et al., 2017; Vomund et al., 2017; Lau et al., 2013].

The activation of the Nrf2 signaling pathway can be the mechanism of EPO (erythropoietin) neuroprotection. Genc et al. have not observed a change in expression of Nrf2 or Keap1 in EPO-treated neuroblastoma cells. The activation of Nrf2 is important for nuclear translocation. It seems that EPO does not interact directly with Keap1. On the other hand, the translocation of Nrf2 into the nucleus after EPO treatment through binding to ARE is associated with the upregulation of HO 1 (heme oxygenase) mRNA. This mechanism can be linked by cytoprotective responses against oxidative stress [Jin et al., 2011; Genc et al., 2010].

Besides, Nrf2 is expendable for murine development, growth, and erythropoiesis, where is requested for the protection of hematopoietic stem cells against oxidative stress. Activity and plentiful Nrf2 are regulated at the transcriptional, post-transcriptional, and post-translational levels. Nrf2 has controlled a lot of genes coding for anti-oxidant, cytoprotective, and anti-inflammatory proteins, such as heme oxygenase 1, NAD(P)H: quinone, peroxiredoxin, glutathione peroxidase, thioredoxin antioxidant system, and glutathione S transferase P (Gstp1) [Zakharova et al., 2018; Tonelli et al., 2018; Tsai et al.,2013].

Furthermore, Nrf2 has a binding activity to the Nrf2 binding site in the many regulatory regions of heme biosynthetic genes, such as the porphobilinogen deaminase gene and the enhancer region of the β-globin gene. Nrf2 also regulates the expression of ferritin and ferroportin (FPN1). Nrf2 is a part of nitric oxide (NO) - induced FPN1 expression and iron efflux to avoid intracellular pathogen proliferation. NO is a mediator of the immune response [Kasai et al., 2018]

Nrf2, RUNX (Runt-related transcription factor), and Fli1 participate in controlling the late megakaryocyte transcription program through additional transcriptional factors (TFs) such as TFs which bind the Fox motif increased near Nrf2 binding sites. Nrf2 is participated in late megakaryocyte maturation, while defective Nrf2 in mutated mice is arrested in platelet assembly. RUNX and Fli1 play role in an early stage of megakaryocyte maturation. In addition, loss of Nrf2 activity does not impact only megakaryocytes, but also erythroid and dendritic cells [Zang et al., 2016; Murakami et al., 2014]. Nrf2 regulates differentiation of hematopoietic stem cells through activation of target genes which are connected to cytokine and/or chemokine signaling pathways than modulating intracellular levels of ROS (reactive oxygen species). ROS is important for the differentiation and proliferation of human pluripotent stem cells (HPSC) [Murakami et al., 2014].

Remarkably, arsenic trioxide (ATO) can activate the Nrf2/HO 1 signal pathway. Hu et al. have noticed better survival in graft-versus-host disease (GVHD) mice which were treated by phosphate-buffered saline (PBS) + ATO than GVHD mice treated by ML835 (inhibitor Nrf2) + ATO [Hu et al., 2019].

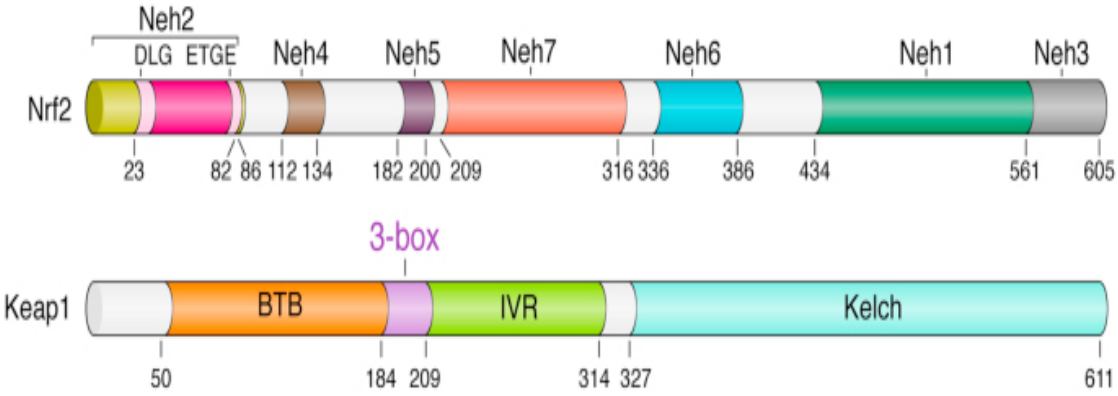


Fig.3: Domain architecture of Nrf2 and Keap1 proteins [Canning et al., 2015].

1.8. Therapy

Erythropoiesis stimulating agents (ESAs) are the first line of therapy for anemia. A lot of MDS patients with del(5q) remain transfusion-dependent, which is dangerous because of iron overloading. The second line of therapy is lenalidomide alone or its combination with erythropoietin with or without prednisone.

1.8.1. Erythropoietin

The relationship between oxygen in blood and erythropoiesis was described by Francois Gilbert Viault in 1980. Carnot and Deflandre noted the mechanism for hypoxic induction of erythropoiesis in 1906. In 1943, Krumdieck and in 1953, Erslev modified Carnot and Deflandre´s experiments [Bunn, 2013].

Erythropoietin (EPO, epoietin)) is located on chromosome 7q22, contains five exons and one splicing variant [Debeljak, 2014]. EPO is an acidic glycoprotein, which is expressed in several tissues (brain, liver, spleen, lung, and testis). Its molecular weight is 30.4 kDa with 165 amino acid residues chain, which creates four antiparallel α helices, two β sheets, and two intrachain disulfide bridges. The carbohydrate part consists of three N-glycans and one O-glycan. The N- glycans protect EPO from proteases and modulate its receptor binding affinity [Jelkman, 2013; Lamon and Russell, 2013].

EPO receptor (EPOR) is a member of the cytokine class I receptor family and creates homodimers [Jelkman, 2011]. EPO has two binding sites, high-affinity G151, and low-affinity R103. It is unknown if cells can express both EPO receptors (EPORs) and their effect on cells. EPO signal pathway leads through JAK2/STAT5 (signal transducer and activator of transcription 5), phosphatidylinositol 3 kinase (PI3K), RAS/MAP kinase pathway, and protein kinase C pathway. EPO effects are dependent on activation of Janus kinase 2 (JAK2) and the nuclear translocation of nuclear factor-kappa B (NF-κB) and EPO resistance is demonstrated by absent or defective phosphorylation of the STAT5 pathway. EPO can reduce the inflammatory signal through upregulation of anti-apoptotic proteins (Bcl2 and Bcl-XL) and inhibition of pro-apoptotic proteins- cytochrome c and caspase 3 from the mitochondrial membrane by upregulation PI3K and protein kinase B pathway [Debeljak et al., 2014; Broxmeyer, 2013; Patel et al., 2011; Santini, 2011].

EPO has an anti-inflammatory effect, which is associated with decreasing pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and increased expression of the protective cytokines [Patel et al., 2011].

The levels of EPOR mRNA and EPO are regulated by levels of oxygen through the activity of hypoxia-inducible transcription factors – HIFs (hypoxia-inducible factors). Involved transcription factors are GATA 2 (GATA- binding factor 2), HIF 2 (hypoxia- inducible factor 2 alpha), HNF-4α (hepatocyte nuclear factor 4 alpha), and NF-κB. EPOR is expressed by the colony-forming unit-erythroid (CFU-Es) and downregulated during the erythroid differentiation [Debeljak et al., 2014; Jelkmann, 2011; Chen et al., 2018].

Anemia is noticed in inherited hematopoietic diseases, chronic diseases, for example, kidney, heart, inflammatory bowel diseases, rheumatoid arthritis, human immunodeficiency virus (HIV), and cancer [Chen et al., 2018].

Anemic patients with bone marrow hypoplasia show higher plasma EPO levels than patients with hemolytic anemia. Also, the level of EPO can be increased by chemotherapeutics [Jelkmann, 2011]. EPO is used as a first-line treatment for anemia in MDS [Castelli et al., 2014].

EPO can be in three forms: epoietin α (Eprex®, Binocrit®), epoietin β (NeoRecormon®) and darbopoietin α (Aranesp®) [Castelli et al., 2014].

1.8.2. Lenalidomide

Lenalidomide (Revlimid®, Celgene, LEN) is immunomodulatory drug (IMiD), which is a derivative of thalidomide. During the Second World War, thalidomide was tested as an antidote to neurotoxins. In 1950, this treatment was used for anxiety, nausea-related conditions, morning sickness in pregnant women. Unfortunately, thalidomide affected babies, who were born with malformations (limb deformations- dysmelia, stunted limb growth, including congenital heart disease, ear, and eye damage, internal organ damage), or increased the incidence of miscarriage [Short et al., 2013; Lu et al., 2014; Ruchelman et al., 2013, Gao et al., 2020].

After this incident, scientists have reported that mice and rats are less sensitive to thalidomide than other unhuman non-human species (for example zebrafish, rabbits, bacterias, etc.) [Vargesson, 2015]. To be able to use mice as animal models, scientists have developed humanized mice carrying a humanizing mutation $CRBN^{I391V}$ at the IMiD binding region. The single AA substitution in humanized cereblon mice, $CRBN^{I391V}$ can cause sensitiveness CD4+ T cells to IMiDs therapy, which are originally resistant. In humanized cereblon mice is higher degradation of its targets than in WT mice (degradation of IKZF1 (Ikaros), IKZF3 (Aiolos), CSNK1A, ZFP91- zinc finger protein 91, Gstp1). Human cereblon (hCRBN) mice have a higher production of IL-2 and strongly inhibit TNF-α. There is a possibility that other receptor apart from CRBN is involved in the inhibitory effects of LEN or other IMiDs against the production of TNF-α. In vitro data of Fink research group shows that the haploinsufficiency of CSNK1A1 in cells is required for sensitivity to LEN therapy. [Gemechu et al., 2018; Fink et al., 2018].

Numerous studies have hypothesized about the molecular mechanism of the teratogenic effects of thalidomide. There is a lot of possibilities, which can be combined. The candidates are MEIS2, CD147, SALL4, p63.

MEIS2 negatively regulates limb outgrowth, which can downregulate the target of thalidomide. This hypothesis has not been investigated by any suitable animal models [Asatsuma-Okumura et al., 2020].

Another candidate is CD147 (EMMPRIN, basigin), which is a transmembrane glycoprotein and it is in complex with monocarboxylate transporter 1 (MCT1). IMiDs compete with CD147-MCT1 for cereblon binding to exert anti-tumor effects. IMiDs destabilize CD147-MCT1 and mediate teratogenic activity through this mechanism in lymphoid and myeloid cells [Eichner et al., 2016].

Scientists discovered phenotypic similarities between newborns affected by thalidomide and patients with mutations in transcription factor p63. In the apical ectodermal ridge (AER), fibroblast growth factor 8 (Fgf8) is a part of the pathway involving p63. FgF8 is included in limb development. Interesting, isoforms of ΔNp63α (limb development and epidermal proliferation) and Tap63α (heart and cochlea development and quality control in oocytes) are thalidomide-dependent neosubstrate of the $CRL4^{CRBN}$ responsible for teratogenicity in zebrafish. In fact, p63 is a non- C2H2 zinc finger-type neosubstrate, where glycine was important for degradation. The mutant ΔNp63α and Tap63α were not degraded by thalidomide [Asatsuma-Okumura, 2020; Asatsuma-Okumura, 2019; Gao et al., 2020].

Spat-like transcription factor 4 (SALL4) is a C2H2 zinc finger transcriptional factor and it is used in embryonic development in mice and humans. The mice studies have shown nonsense or frameshift mutations in a single allele of *SALL4* gene case development defects. The second C2H2 zinc finger mediates thalidomide-induced CRBN binding and mutations of G416A blocks thalidomide-induced SALL4 ubiquitination and degradation. The level of SALL4 protein is reduced in tissue from thalidomide-treated rabbits but not in mice. [Belair et al., 2020 and Gao et al., 2020, Matyskiela et al., 2018]. Using zebrafish as a model organism for limb teratogenicity is controversial. SALL4 proteins are different in humans and animals suggesting another protein/ other proteins cause(s) teratogenicity. Another teratogenicity potential has transcription factor-promyelocytic leukemia zinc finger (PLZF, ZBTB16, or ZFP145), which is involved in the developmental process, such as hematopoiesis, limb skeletal formation, spermatogenesis, and immune regulation. Yamanaka et al. suggest that degradation of

PLZF, SALL4, and 5-hydroxythalidomide produce CRBN- dependent teratogenic phenotypes in species sensitive to thalidomide [Yamanaka et al., 2021].

Thalidomide induces oxidative stress, which is also associated with the teratogenic activity of the drug. Teratogenic activity leads to the production of ROS, DNA damage, and interference of intracellular signalings such as Fgf (fibroblast growth factor), WNT (Wingless/Integrated), and AKT (protein kinase B). The production of ROS causes oxidation or alteration of glutathione content [Meganathan et al., 2012].

Therefore, lenalidomide as a member of the second generation of IMiDs was approved by the Food and Drug Administration. Thalidomide prescription is strictly controlled by the Thalidomide Risk Evaluation and Mitigation Strategy (REMS). Lenalidomide therapy is highly effective for transfusion-dependent MDS patients with del(5q), MDS patients with trisomy 8 or with combination +8 and del(5q), multiple myeloma, other B-cell neoplasms, chronic lymphocytic leukemia [Drevon et al., 2018; 2013; Fink and Ebert, 2015; Asatsuma-Okumura et al., 2020].

The difference between LEN, thalidomide, and pomalidomide (Fig.4) is the presence or absence of an amino group or a carbonyl group. Thalidomide contains a phthaloyl ring and a glutarimide ring with a chiral carbon, which racemizes in vivo, and only the (S)-enantiomer is thought to be teratogenic. LEN is the same as thalidomide, except for LEN has modified phthaloyl moieties [Hartman et al., 2014 and Chamberlain et al., 2014].

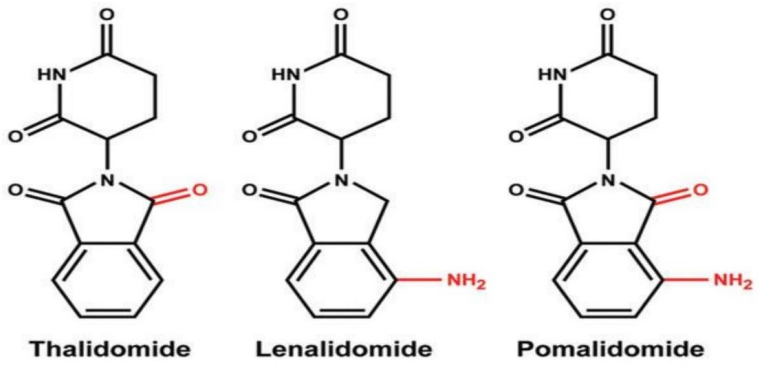


Fig.4: Chemical structures of thalidomide, LEN and promalidomide [Fuchs et al., 2014].

Lenalidomide has anti-angiogenic and anti-neoplastic activity, which inhibits proliferation and induces apoptosis of hematopoietic tumor cells. Also, LEN stimulates interleukin 2 (IL-2), interleukin 6 (IL-6), and other cytokines and inhibits tumor necrosis

factor α (TNF-α) production in monocytes [Cruz et al., 2016; Krönke et al., 2014; Chaulet et al., 2014].

Side effects of LEN are neutropenia and thrombocytopenia during the first 8 weeks of treatment, diarrhea, muscle cramps, rash, infection, and fatigue. Neutropenia and thrombocytopenia caused by LEN are less frequent in MDS patients without del(5q) than in 5q-syndrome. Thalidomide can be associated also with an increase in venous thromboembolism and arterial events [Toma et al., 2016; Holstein et al., 2018; Jan et al., 2021]. Around 60-70% of MDS patients with del(5q) and 25-27% MDS patients with normal karyotype respond to LEN therapy. Complete cytogenetic response achieves approximately 20% of treated patients [Jonasova et al., 2018; Lodé et al., 2018]. In clinical MDS trials were recommended 10mg dose of LEN, which can be connected to a better outcome, reduction of del(5q) clone [List et al., 2018]. Long-term use of LEN has been linked with an increased presence of second solid tumors and hematological malignancies, especially MDS and AML [Jan et al., 2021].

However, the molecular mechanism of LEN is not completely understood. LEN reduced expression of p53 in erythroid precursors in successful treatment, but in LEN resistance is regenerated of accumulation of p53. Patients with lenalidomide treatment gain drug resistance during 2-3 years and 40% of treated cohorts still progress to AML by five years [Caceres et al., 2013, Lodé et al., 2018]. TP53 mutations have an influence on LEN treatment when patients with the mutations are linked with an absence of cytogenetic response. Depletion of IKZF1 by LEN in del(5q) MDS cells leads to RUNX upregulation and megakaryocyte differentiation and sequencing clinical samples shows that RUNX mutations are associated with LEN resistance.

In newly diagnosed MM patients are CRBN mutations rarely present, but IMiD-treated patients have a higher frequency of CRBN mutations. CRBN can be linked by other genomic (structural variations, copy loss) or transcriptomic aberrations (epigenetic, RNA splicing/stability). The low level of CRBN and transcript splice variations have been associated with acquired IMiD resistance [Jan et al., 2021; Gooding et al., 2021]. Thus, there is the possibility that the cancer cells can develop resistance by upregulating the expression of unrelated substrates. CRBN competes with other substrate receptors for access to the limiting concentration of the CRL4. The expression profile of these substrates and the CRL4 might determine the efficiency of thalidomide analogs- induced degradation. This hypothesis is an object of research [Jan et al., 2021]. Of note, another study suggests U2AF1 (U2 small nuclear RNA auxiliary factor 1) mutations can be

responsible for poor response of LEN in del(5q) and non-del5q- and the mutations in DEAD-box RNA helicase genes (DDX41, DDX54, DHX29) in non-del5q- MDS. In fact, p53 probably is not related to thrombocytopenia [Mallo et al., 2013; Garcia-Manero et al., 2020]. Recently, Tochigi and his colleagues have reported that aromatase is a novel IMiD- dependent cereblon substrate in humans. Aromatase is degraded by IMiD through interaction with CRBN and inhibited autocrine estradiol signaling-dependent proplatelet formation in megakaryocytes. Degradations of aromatase by IMiDs cause thrombocytopenia in patients with multiple myeloma [Tochigi et al., 2020].

Lenalidomide inhibits directly phosphates CDC25C (cell division cycle 25C) and indirectly phosphatases PP2A (protein phosphatases 2A catalytic domain alpha) and CDC25A (cell division cycle 25 homolog A). CDC25C is dephosphorylated by PP2A. Inhibition of phosphatases by LEN causes G2/M arrest and induction of apoptosis in hematopoietic cells. PP2A haplodeficient cells are associated with autoubiquitination of MDM2 and stabilization of p53. PP2A dephosphorylated MDM2, inactive MDM2 cannot bind p53 and TP53 keep inactivate [Abou Zahr et al., 2014; Wei et al., 2013; Giagounidis et al., 2014]. The high level of PP2Acα causes repair p53 expression in erythroid cells and leads to LEN resistance in 5q-syndrome. The low levels of PP2Acα phosphatase mRNA enhance LEN sensitivity in MDS cells without del(5q) [Sallman et al., 2014].

The important role in LEN treatment has CSNK1A1. Krönke et al. have reported decreased CSNK1A1 protein levels (both, in mutated and wild type form) in MDS patients with del(5q). Interestingly, in patients with multiple myeloma are decreased Ikaros and Aiolos that contain both an N-terminal zinc finger (ZF) DNA-binding domain and a C-terminal ZF protein-protein interaction domain. The decreased amount of CSNK1A1 proteins is without modifying of CSNK1A1 mRNA level [Krönke et al., 2015; Powell et al., 2019]. Haploinsufficiency of CSNK1A1 probably leads to sensitivity to LEN treatment, contrariwise, the high level of CSNK1A1 is linked to LEN resistance [Guirguis and Ebert, 2015; Lu et al., 2014].

Fang et al have reported, mechanism of LEN is connected with the calcium-dependent pathway in MDS and AML, which causes initiating apoptosis. After LEN treatment, they have shown an increasing rate of calpain-1 protein (CAPN1) by high-throughput proteomic mass spectrophotometry. This team has supposed that the expression of CAPN1 and cytosolic calcium flux can predict the responsiveness of LEN [Fang et al., 2016].

The mechanism of LEN in MDS patients with normal karyotype (non-5q-) is different than in MDS patients with del(5q). The function of LEN leads through EPOR/STAT 5 pathway [Komrokji and List, 2010]. LEN increases JAK2/STAT5 phosphorylation in response to stimulation of EPO. LEN stabilizes and upregulates also the expression of JAK2/EPOR complex through inhibition of the E3 ubiquitin ligase activity of RING finger protein 41 (RNF41) which polyubiquitinates EPOR and marks it for degradation in proteasome [Basiorka et al., 2016].

LEN affects CFU-E colonies and erythropoiesis, but it does not influence the expression of ribosomal genes. Further, LEN does not kill hematopoietic stem cells in MDS patients with del(5q) and non-5q- patients, LEN reduces TRF (transfusion) necessary without leading to cytogenetic remission, implying that stimulates erythropoiesis in MDS cells. Narla et al. have reported that corticosteroids and LEN have effects on erythropoiesis and improve the life of MDS patients. Apart from this, LEN combined with EPO increases erythroid response in non-5q- patients more than LEN alone. LEN affects raft formation, which is connected to EPOR by incorporation of JAK2, STAT5, and Lyn kinase. The result is the enhancement of EPOR signaling. Jonasova et al. have reported also good effects of LEN+EPO or LEN+ EPO+ Prednisone (corticosteroid) in those MDS subjects without LEN response [Narla et al., 2011; Jonasova et al., 2018; Toma et al., 2016; McGrraw et al., 2014].

Zhou et al. illustrated that inhibition of the caspase 8 (CASP-8) activity increases CRBN protein and can cause benefits to the effect of LEN treatment and proliferation in multiple myeloma (MM) [Zhou et al., 2020]. Another study has shown that loss of DNA topoisomerase II beta (TOP2B) mediates the sensitization of MM cells for IMiD therapy. The phenotype seemed to be independent of an increase in CRBN activity or changes with IKZF1/3-IRF4 (interferon regulatory factor 4)-MYC expression [Costacurta et al., 2020]. A further study suggests that cellular antioxidative capacity has shown LEN sensitivity in MM cells expressing CRBN with the highest capacity to decompose H_2O_2 by hydrogen peroxidase. It was discovered that Trx/TrxR (thioredoxin/thioredoxin reductase) system-mediated intracellular H_2O_2 decomposition is inhibited by LEN, but the mechanism is unclear yet. Mountjoy et al. tested bone marrow plasma samples of 33 patients with MM. Cells with high antioxidative capacity generate more oxidized flavin adenine dinucleotide (FAD) and MM cells with increasing FAD after H_2O_2 were counted as IMiD resistant. MM cells without a change in FAD were counted as IMiD sensitive.

This reaction was generated visually by oxygen-containing bubbles and compared with flow cytometry [Sebastian et al., 2017; Mountjoy et al., 2020].

Thalidomide analogs mark the number of proteins for ubiquitination and subsequent degradation in proteasomes. All these proteins marked for degradation are still not known. The proteins which have been found had a structural degron with beta-hairpin, which included a pinnacle glycine motif (primarily the form Cys2His2 /C2H2/ zinc finger domain). C2H2 zinc finger domain binds to composite a thalidomide analog-CRBN surface [Jan et al., 2021; Fuchs and Bokorova, 2020].

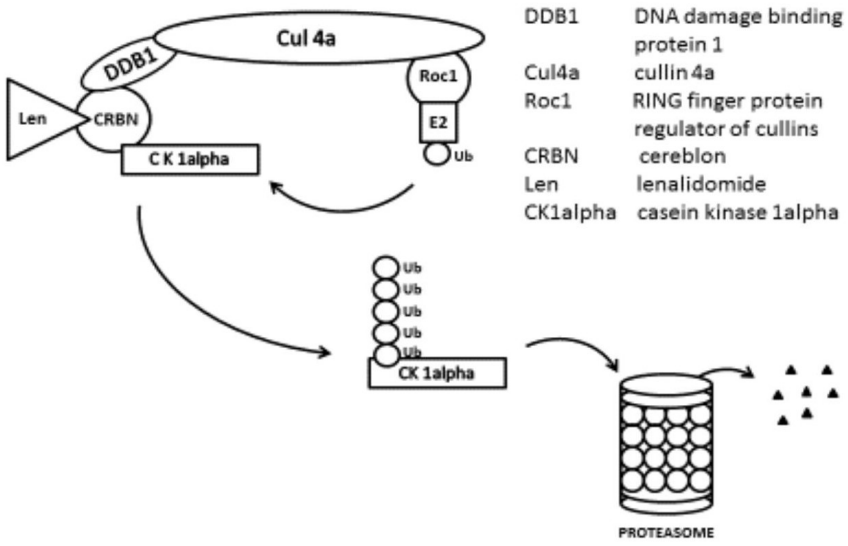


Fig.5: Molecular mechanism of Lenalidomide through cereblon [Fuchs and Bokorova, 2020].

1.8.3. Prednisone

Prednisone (PRED) belongs to glucocorticoids. Glucocorticoids (GCs) were discovered in 1940 and are used in the treatment of plenty of disorders, such as asthma, inflammatory, and immune/rheumatologic diseases. Glucocorticoids are a family of steroid hormones produced by the adrenal cortex and plasma concentration is followed by the hypothalamic-pituitary-adrenal axis. The adrenal cortex consists of 3 cellular zones, responsible for the synthesis of a specific class of steroidal hormones. Synthesis of GCs is induced with cholesterol and culminate in mineralocorticoid, glucocorticoid, and androgen production [Ciriaco et al., 2013; Becker, 2013]. GCs have anti-

inflammatory, immunosuppressive, anti-proliferative, and vasoconstrictive effects [Liu et al., 2013]. Hench et al., have discovered a high level of GCs in the blood of Gushingoid patients in 1949, thereby showing anti-inflammatory effects in these patients [Becker, 2013]. The immune-modulating mechanism of GCs is to trigger apoptosis in T cells and monocytes/macrophages. In addition, apoptosis caused by GC was found in plenty of cells, such as osteoblasts, muscle, and gastric epithelial cells. On the contrary, GCs prevented apoptosis in hepatocytes and adipocytes [Ryu et al., 2017].

The side effects of GCs are obesity, insulin resistance, glucose intolerance, and dyslipidemia [Peng et al., 2016].

Prednisone has high glucocorticoid activity and/ or mineralocorticoid features and is used as an anti-inflammatory and immunosuppressive agent [Liu et al., 2013]. PRED is activated by the enzyme 11 β-hydroxysteroid dehydrogenase (11 β-HSD). There are two types of 11 β-HSD: 1.) 11 β-HSD-1 is reductase and converts prednisone (inert) into active prednisolone 2.) 11 β-HSD-2 is oxidase and converts prednisolone to prednisone. It protects the mineralocorticoid receptor from occupation by cortisol and prednisolone [Schijvens et al., 2019]. The mechanism of effectiveness of PRED in the regulation of immunologic homeostasis is not completely clear [Qian-Fu et al., 2019].

Low-dose Prednisone can prevent the synthesis of pro-inflammatory factors such as IL-6, IL-1 (interleukin 1), and TNF-α. PRED inhibits also the binding of immunoglobulin with the cell surface, inhibits the immune complex traveling through the membrane, decreases immunoglobulin concentration and alexin [Qian-Fu et al., 2019]. The genomic mechanism associated with changes in gene expression is needed in low-dose treatment. The non-genomic mechanism is mediated through high-dose therapy. The low-dose therapy leads glucocorticoid molecules through the cell membrane, binding to the inactive cytosolic glucocorticoid receptor. This formation is changed from inactivated to activated glucocorticoid- cytosolic glucocorticoid receptor complex, which is translocated into the nucleus. The high dose therapy leads through the cytosolic receptor, through the membrane glucocorticoid receptor through non-specific interactions with cell membranes and organelles, including mitochondria [Van der Goes et al., 2014].

Erythropoiesis stimulating agents (such as EPO), androgenic steroids (such as Prednisone), and immunosuppressive therapy (such as Lenalidomide, Pomalidomide, and Thalidomide) are used against anemia. Androgenic steroids suppress anemia by stimulating erythropoietin, enhancing iron use, and preventing telomere loss [Luo et al., 2018].

Lenalidomide in combination with Prednisone leads to better erythroid response than Lenalidomide alone in MDS patients with normal karyotype. There is no additional toxicity and less grade of thrombocytopenia and neutropenia [Komrokji et al., 2018].

1.8.4. Arsenic trioxide

Arsenic trioxide (Trisenox®, ATO, As_2O_3) is used in traditional Chinese medicine. It is used worldwide as a treatment for acute promyelocytic leukemia (APL), multiple myeloma (MM), MDS, and non-Hodgkin's lymphoma [Dawood et al., 2018; Xu et al., 2014].

ATO has anti-angiogenesis, apoptotic effects, and some effects on the microvascular density of bone marrow during remission induction [Alimohhaddam, 2014]. Also, ATO can be involved in the accumulation of intracellular hydrogen peroxide, release cytochrome c, inhibition of glutathione peroxidase, generation of ROS, induction of autophagy, inhibition of Notch pathway, repression of β-catenin, inhibition of the Hedgehog pathway through reduction of Gli transcriptional effector [Roboz et al., 2011; Cholujova et al., 2017].

ATO with a combination of other agents showed effectiveness in therapy for a variety of human cancers. Hua et al. used a combination of ATO and Triptolide (TL) and found out upregulation of Bax and caspase-3, downregulation of Bcl2 compared to ATO or TL alone in MDS SKM-1 cells. Therefore, the level of ROS was increased after the combination of ATO + TL in SKM-1 cells [Hua et al., 2016]. Patients with high-risk MDS or AML can be treated by ATO and cytarabine, which can produce complete remissions [Roboz et al., 2011]. In MDS, a simple tri-lineage response to ATO alone is produced in approximately 20%, thereby is prevented transformation to leukemia and MDS relapse thereby is prevented transformation to leukemia and MDS relapse [Sekeres et al., 2011; Roboz et al., 2011; Zeng et al., 2016]. The side effect of using ATO alone/ daily causes lethal hepatic damage in the clinic [Wu et al., 2017].

The mechanism of ATO in MDS is still not clarified [Galimberti et al., 2012]. In vitro data show ATO supports apoptosis by inhibiting telomerase activity and transcription of the *hTERT* (human telomerase reverse transcriptase) gene. ATO inhibits other transcription factors, such as Sp-1, NF-κB, AP-1 in MUTZ-1, and SKM-1 cell lines. Furthermore, ATO inhibits activation of NF-κB and downregulates Bcl-XL, Bcl-2, XIAP, and FLIP in MDS [Xu et al., 2014].

Another in vitro data show ATO downregulates Cdc25C increasing the cytostatic effect of LEN in MM cells (U266 and RPMI 8226), and did not downregulate or inhibit PP2A. However, the combination of LEN + ATO keeps downregulation of IL-6 [Wang et al., 2013]. Moreover, ATO increases the level of CRBN in U266, and RPMI 8226 cell lines, the high level of CRBN leads to increased sensitivity of LEN. This therapy, in G0/G1 phase, increases the percentage of cells with a decrease in proliferative phase-S and G2/M phase, which causes increased inhibition of MM cell viability [Jian et al., 2017].

2. Aims of the works

1. The first aim is to compare the levels of cereblon mRNA in MDS patients with isolated del(5q) with other low-risk MDS patients without del(5q) and with healthy controls.

2. The second aim is to confirm that the successful lenalidomide therapy of del(5q) MDS is dependent on the level of cereblon mRNA in both bone marrow and peripheral blood mononuclear cells and cereblon as protein similarly as in patients with multiple myeloma.

3. The third aim is to measure the levels of full-length cereblon mRNA and protein cereblon through the lenalidomide treatment and to find whether a sudden decrease of the CRBN expression is associated with resistance to lenalidomide therapy.

4. The fourth goal is to study the mechanism by which erythropoietin, prednisone, and arsenic trioxide added to lenalidomide can reverse resistance to lenalidomide in cultures of human cells lines derived from MDS patients (MDS-L cells and SKM1 cells).

5. Effect of combinations of lenalidomide with erythropoietin or prednisone or their combination in the case of appearance of lenalidomide resistance in the course of lenalidomide therapy of low-risk MDS patients with del(5q) will be studied.

6. The role of NRF2 in the expression of *CRBN* gene in the course of EPO and the combination of EPO plus lenalidomide therapy in examined samples from MDS patients and experiments with cell lines (MDS-L cells and SKM1 cells).

3. MATERIALS AND METHODS

3.1. Patients

Lenalidomide treated cohort consisted of 83 patients with 5q- syndrome or combination of deletion of chromosome 5 (del(5q)) with trisomy 8. LEN treatment has not been approved for non-5q- patients in the Czech Republic. We have obtained 7 non-5q- patients on LEN treatment, thanks to the international clinical trial (NCT01029262). All MDS samples were obtained from the Institute of Hematology and Blood Transfusion and the General University Hospital of Prague during 2006 and 2021. The selection criteria were that the EPO group had a hemoglobin level below 100g/L at beginning of treatment and the LEN group was EPO resistant and transfusion-dependent. For the classification of transfusion dependency, there was the requirement of at least two transfusions per month for at least eight weeks before the initiation of therapy. After patients stopped responding to LEN treatment, they were treated by a combination of LEN + EPO or LEN+ EPO + PRED. LEN dose was 5mg, 10mg, or 25mg (only in the case of MM+MDS together)- it depended on the response of patients, but starting level was 10mg. The dose of EPO before LEN was 300 mU/mL. After EPO was added to lenalidomide, the dose was 40,000 - 80,000 units per week. Starting dose of Prednisone was administered at a dose of 20mg/day, the level was decreased to 5mg/day in all responding patients.

3.2. IN VITRO STUDIES

3.2.1. Preparations of patient samples:

Chemicals and materials:

1x Phosphate-buffered saline: 2.7mM potassium chloride (KCl), 1.8mM potassium dihydrogen phosphate (KH_2PO_4), 137mM sodium chloride (NaCl), 10mM disodium phosphate (Na_2HPO_4). The chemical's pH was adjusted to 7.4 and the solution was sterilized by autoclaving.

Lysis solution for the isolation of total RNA: 114mmol/L ammonium chloride (NH_4Cl), 1mmol/L sodium bicarbonate ($NaHCO_3$), 1mmol/L EDTA (pH 8.0).

Lysis solution for the isolation of genomic DNA: 10mmol/L Tris (pH 9.0), 50mmol/L KCl, 0.1% Nonident P-40.

Peripheral blood (PB) and bone marrow (BM) were stored in EDTA test tubes. Mononuclear cells were processed by using Ficoll-Paque PLUS (GE Healthcare Bioscience AB, Upsala, Sweden) and washed by 1x Phosphate-Buffered saline (PBS). The remaining red blood cells were lysed by lysis solution. The mononuclear cell amount of PB or BM was measured by Mindray BC-3000 Plus auto hematology analyzer.

The total RNA from mononuclear cells was prepared by using RNA STAT-60 (GeneTiCA) following the manufacture's protocol. The concentration and purity of RNA were measured by NanoDrop™ One Spectrophotometer (Thermo Fisher).

Mononuclear cells for the isolation of genomic DNA were lysed by another lysis solution (see above). To 200µl of the lysate (5 x 10^6 cells) was added 4µl of proteinase K (20mg/mL). The lysate was incubated at 37°C during the night, and proteinase K was inactivated for 10 min. at 90°C.

3.2.2. Real-time quantitative PCR (polymerase chain reaction)

The equal amount of RNA (2µg per reaction) was reverse transcripted using SuperScript™ II RNase H reverse transcriptase (Thermo Fisher) according to the manufacture's protocol. Specific gene expression was measured using the specific TaqMan assay. cDNA was diluted in RNase-free water. Diluted cDNA was amplified in the presence of primers pair (TaqMan assay) and TaqMan™ Universal PCR Master Mix (Thermo Fisher) in Rotor-Gene Q (Qiagen) following manufacture's protocol.

The volume of RNA for the synthesis of cDNA was calculated:
The corresponding volume of RNA = 2µg RNA / concentration of the measured RNA.

The list of assays that were used:
Human cereblon assay (cat. no. Hs00372271_m1, Thermo Fisher), the amplicon length is 118bp.

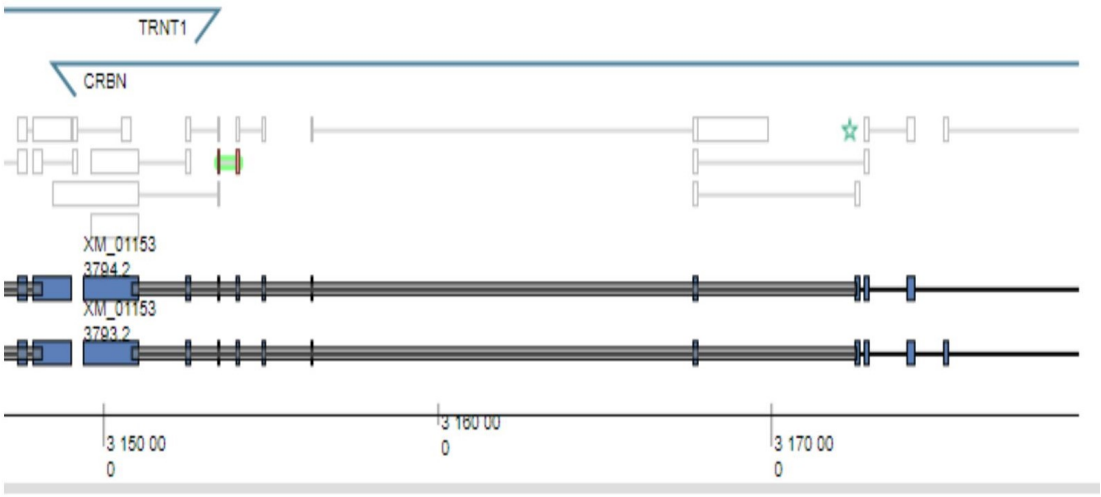


Fig.6: CRBN genomic map obtained from Thermo Fisher, link is below: https://www.thermofisher.com/taqman-gene-expression/product/Hs00372271_m1?CID=&ICID=&subtype=

Human nuclear factor erythroid 2 like 2 assay (cat.no. Hs00975961_g1, Thermo Fisher). The amplicon length is 74bp.

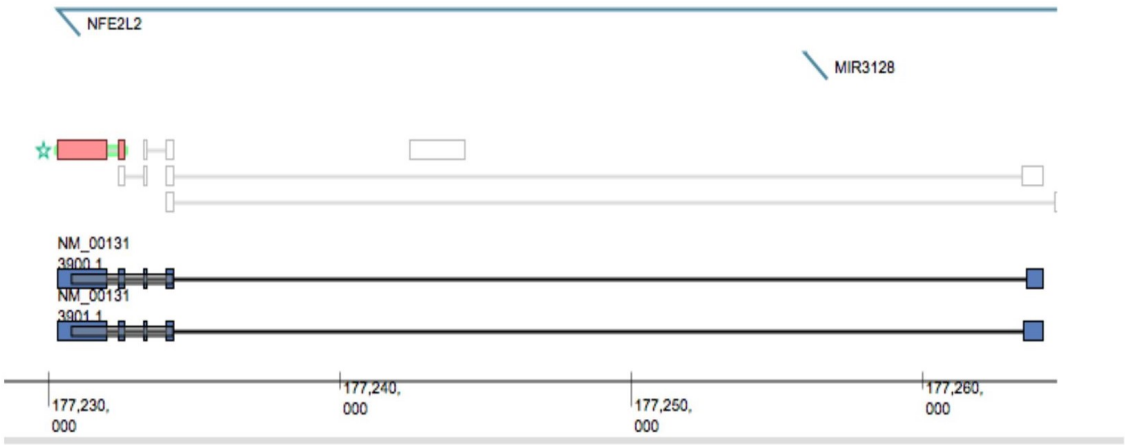


Fig.7.: Nrf2 genomic map obtained from Thermo Fisher, link is below: https://www.thermofisher.com/taqman-gene-expression/product/Hs00975961_g1

Human PTEN induced putative kinase 1- PINK1 (cat.no._Hs00260868_m1, Thermo Fisher- best coverage). The amplicon lenght is 104bp.

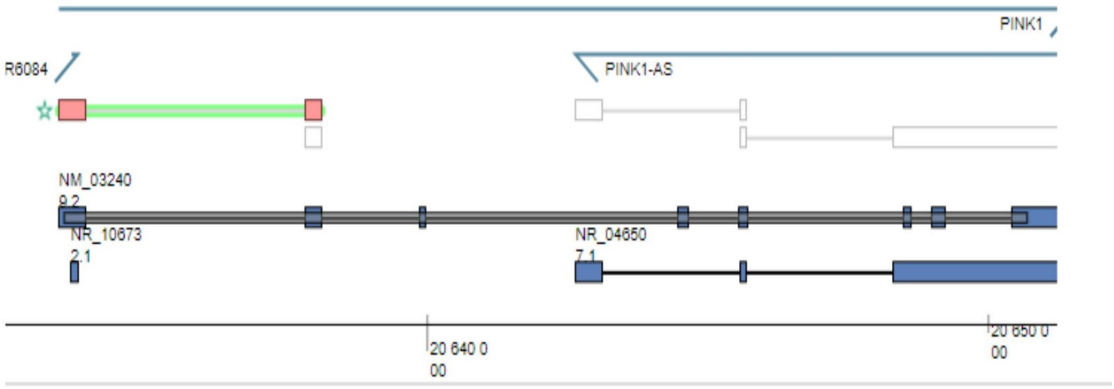


Fig.8: PINK1 genomic map obtained from Thermo Fisher, link is below: https://www.thermofisher.com/taqman-gene-expression/product/Hs00260868_m1?CID=&ICID=&subtype=

The measured data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which gene expression was measured by the specific assay (cat. no. 4352934E, Human GAPD (GAPDH) Endogenous Control (FAM™/MGB probe, non-primer limited, Thermo Fisher)). The experiments were designed in duplicate, and the evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the control.

3.2.3. DNA sequencing

Chemicals and materials:

Distilled water

Isopropanol

70% Ethanol

PrimeSTAR HS DNA polymerase (TAKARA BIO INC., Japan)

5x TBE buffer- 450mM Tris-Borate, 10mM EDTA (pH 8.3) in distilled water.

6x DNA Loading Dye (Thermo Fisher) - dye for electrophoresis

Agarose for electrophoresis- 2% agarose in 0.5 TBE buffer

SYBR Green I (Invitrogen)

0.5M EDTA (pH 8.0) – EDTA is diluted in distilled water

3M sodium acetate (pH 5.2) - sodium acetate is diluted in distilled water

10M ammonium acetate- ammonium acetate is diluted distilled water with 6x loading Dye (electroelution of cDNA or DNA from agarose gel)

Electrophoretic marker- Thermo Scientific GeneRuler 50bp DNA Ladder (Thermo Fisher)
Beckman Coulter quick-start kit

The PCR amplification of MDS cDNA or DNA was produced on a PCR machine (PTC-200, Peltier Thermal Cycler, BioTech and DNA Engine, Peltier Thermal Cycler, BIO-RAD). The PCR mix consists of 2x PrimeStar GC buffer, dNTP (2,5mM ATP, GTP, CTP, UTP), 2μM forward and reverse primer, PrimeSTAR HS DNA polymerase (2,5U/μL), and H_2O_2. To the master-mix is added 3μl DNA or cDNA. The list of PCR cycles is below.

The PCR products were separated on 2% agarose gel and bands were visualized using SYBR Green I staining. The PCR products were electroeluted from the agarose gel. The relative intensity of products was measured using the FLA-5100 fluorescent image analyzer (Fujifilm Life Science, Stamford, CT, USA) and the bands were quantified with AIDA analysis software (version 4.23.035, Raytest isotopenmessgeräte GmbH, Straubenhardt, Germany). The pieces of PCR products were purified from agarose and precipitated with isopropyl alcohol and washed with 70% ethanol. The precipitated PCR products were produced by Genome Lab DTCS Quick Start Kit (Beckman Coulter Ireland, Inc., Mervue, Galway, Ireland) according to the manufacture´s protocol and sequenced by Beckman Coulter CEQ 3000 DNA sequencer (Beckman Coulter, Inc., Brea, CA, USA).

To evaluate the results, GenBank Accessions NC_000003.12 (Homo sapiens, GRCh38 primary assembly) and NM_016302.3 (Homo sapiens, CRBN transcript variant 1 mRNA) were used.

The list of primers for detection of A/G-polymorphism:
CRBN1F: 5'- GCAGGCCTGTAATTGTCCCT -3'
CRBN1R: 5'- GCAACAGAGCAGCGAAGAAA -3'
The list of primers for detection of splicing variants:

FCRB1ex: 5'- TCCTTTGCGGGTAAACAGAC -3'
FCRB5ex: 5'- AGTCAGATGGAATCCAGCAAGC -3'
FCRB7ex: 5'- CAAGAAACAGCTACGTGAATGG -3'
RCRB5ex: 5'- GCAACACACATTCGGGAAGA -3'

RCRB7ex: 5'- CCCATTCACGTAGCTGTTTC -3'

RCRB11ex: 5'- TTCATCTTCAGTGTCTGGGATC -3'

PCR amplification for detection of A/G polymorphism (DNA)

1. 98°C for 4 minutes
2. 98°C for 30 seconds
3. 60°C for 50 seconds
4. 72°C for 50 seconds
5. 98°C for 30 seconds (39 times)
6. 72°C for 10 minutes
7. 4°C for forever

PCR amplifications for detection of splicing variants (cDNA)

1. 98°C for 4 minutes
2. 94°C for 30 seconds
3. 55°C for 50 seconds
4. 72°C for 50 seconds
5. 94°C for 30 seconds (39 times)
6. 72°C for 10 minutes
7. 4°C for forever

Sequencing PCR

1. 96°C for 20 seconds
2. 50°C for 20 seconds
3. 60°C for 4 minutes
4. 96°C for 20 seconds (39 times)
5. 4°C for forever

3.2.4. Immunoblotting

Chemicals and materials:

Lysis solution of proteins: 50mM Tris- hydrochloric acid (Tris-HCl) pH 7.4, 50mM sodium fluoride (NaF), 5mM sodium pyrophosphate, 1mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1% Triton X-100, 1mM dithriotreitol (DTT), 1mM

benzamidine, 1mM phenylmethane sulfonyl fluoride (PMSF), 5µg/mL soybean trypsin inhibitor (SBTI)

2x Laemli sample buffer: 50mM Tris-HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate (SDS), 200mM DTT, 0.05% bromophenol blue

Separating gel: final concentration of acrylamide was different according to the molecular weight of the protein of interest, 10%, and 12% polyacrylamide gels were prepared from 30% acrylamide/bis acrylamide solution, 1.5M Tris (pH 8.8.), 10% ammonium persulfate (APS), 10% SDS, TEMED, H_2O_2

5% Stacking gel: 30% acrylamide/bis acrylamide solution, 1M Tris (pH 6.8.), 10% APS, 10% SDS, TEMED, H_2O_2

Spectra™ Multicolor Broad Range Protein Ladder (cat.no. 26623, Thermo Fisher, 10 to 260kDa)

1xRunning buffer: 25mM Tris base, 200mM glycine, 0.1% SDS

1x Blotting buffer: 25mM Tris base, 200mM glycine, 20% methanol

Amersham™ Protran™ Premium 0.45µm NC, nitrocellulose Blotting Membrane (GE Healthcare Life Sciences)

Washing buffer: PBS, 0.05% Tween-20

Ponceau S solution: 0.1% Ponceau S in 5% acetic acid

Blocking buffer: 5% milk in washing buffer (PBS/Tween-20)

SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher)

Syngene G:box- gel imaging system for fluorescence and visible aplications

After centrifugation at 14 000rpm for 10 min., cells were lysed by another lysis solution (see Chemicals and materials). The concentrations of protein were measured by the BCA (bicinchoninic acid) method (Pierce Biotechnology Inc., Rockford, USA). To the cell lysates, 2x Laemmli sample buffer was added and then proteins were separated by SDS PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). For SDS PAGE were used 10% and 12% polyacrylamide gels, the same amount of protein was loaded into each well. After electrophoresis, proteins were transferred from the gel onto the nitrocellulose membrane by the wet transfer method. Further, the membrane was stained using Ponceau S for showing the effectivity of the protein transfer and blocked 1h with blocking buffer. Proteins were detected using specific primary antibodies (whole night, 4ºC) combined with horseradish peroxidase-conjugated secondary antibodies (2h,

4ºC). Peroxidase activity was detected by SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher)-according to the manufacture's protocol and visualized by Alliance Q9 Advanced, Uvitec (Baria). GAPDH was used as a marker of equal loading.

The list of antibodies used for immune detection:

Primary antibodies:

- CRBN rabbit polyclonal antibody (cat. no. PA5-38037, Thermo Fisher)
- Nrf2 (A-10) mouse monoclonal antibody (cat. no. sc-365949, SANTA CRUZ BIOTECHNOLOGY, INC.)
- HMOX1 rabbit polyclonal antibody (cat. no. PA5-27338, Thermo Fisher)
- GAPDH mouse monoclonal antibody (cat. no. 39-8600, Thermo Fisher)

Secondary antibodies:

- Goat anti-mouse IgG (H+L) (cat. no. G-21040, Thermo Fisher)
- Goat anti-rabbit IgG (H+L) (cat. no. UB280570, Thermo Fisher)

3.3. Applicated drugs

Lenalidomide (CC-5013, Selleckchem, Germany) and Prednisone (S1622, Selleckchem, Germany) were solved in dimethyl sulfoxide (DMSO). Recombinant human erythropoietin (329871-50UG, MERCK) was solved in water. In preparation of arsenic trioxide stock solution (1mM; 19.784 mg for 100ml), we had to first dissolve in 10 ml 10% NaOH and then neutralize with a strong acid (HCl).

The concentration of LEN in our experiments was used 10μM according to multiple myeloma and AML cell lines studies. The concentration of ATO was prepared in various groups- 0.05μM, 0.1μM, 0.5μM, 1μM following previous studies. In the study groups using combinations of ATO with other drugs, 0.1μM concentration of ATOwas used. The concentration level of PRED was 100μM, and EPO was 20U. The applicated concentration of drugs was calculated for 10ml RPMI 1640 media.

3.4. Cells and culture

SKM-1 cell line was established from the peripheral blood of a 76-year-old man with acute monoblastic leukemia (AML M5) following MDS. Cytogenetics is human hypodiploid karyotype with 8% polyploidy- 43(38-43)<2n>XY, +1, -12, -14, -20, -21, t(1,19)(q21,q13), del(2)(p11), del(9)(q12), add(17)(p1?)- sideline with idem, der(10)t(10,?21)(p11,q11), -21. SKM-1 cell lines were cultured in RPMI 1640 with glutamax and 25mM HEPES (Thermo Fisher) containing 10% fetal bovine serum (FBS) (Thermo Fisher), 100 000 units/l of penicillin (Sigma Aldrich), and 50 mg/l of streptomycin (Sigma Aldrich) at 37°C with 5% CO_2. The doubling time of cells is 48 hours and they were harvested at a concentration of 2.5 x 10^6 cells/ ml. The cell line was kindly provided by RNDr. Denisa Imrichova, Ph.D. (Slovak Academy of Sciences, Bratislava, Slovakia) and prof. MUDr. Tomas Stopka, Ph.D. (Biocev, Prague, Czech Republic).

The MDS-L cell line was established from the bone marrow of MDS patients with del(5q) by Dr. Tohyama's laboratory (Kawasaki Medical School, Kurashiki, Okayama, Japan). Cytogenetics is 49, XY, +1, der(5)t(5,19), -7, +8, -12, der(13)t(7,13), der(14)t(12,14), der(15)t(15,15), +19, +20, +21, der(22)t(11,22). In addition, this cell line shows growth inhibition by LEN treatment, susceptibility to DNA methyltransferase (DNMT) inhibitors. Maybe MDS has genetic instability and their daughter cells show reduced maturation.

The MDS-L cells were cultured in RPMI 1640 with glutamax and 25mM HEPES (Thermo Fisher). The medium was supplemented with 10% FBS, 50μM 2-mercaptoethanol, 100 U/ml IL-3, 100 000 units/l of penicillin, and 50 mg/l of streptomycin. The cell line was kindly provided by Dr. Tohyama (Kawasaki Medical School, Kurashiki, Okayama, Japan).

The cells of SKM-1 and MDS-L were stained by Trypan Blue and counted by hemocytometer.

3.5. Statistical analysis

The GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Mann-Whitney U- a non-parametric test was used for the

comparison of groups in our experiments, which were performed in at least three biological replicates. $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. Statistical analysis obtained MDS patients

The MDS cohort treated by EPO can be divided into two groups: patients with normal karyotype (non-5q-) and patients with isolated del(5q) or in combination with trisomy 8. Non-5q- samples were obtained only from the Institute of Hematology and Blood Transfusion (ÚHKT). The acquired MDS group had 14 people, of which were four women and ten men. Two of them died (no hematological issue), ten (71.43%) were non-responders. Four (28.57%) are constantly continuing in treatment, three of this group with the response and they are TRF independent, one of them continue with EPO, but there is no response, anymore.

The second group with isolated del(5q) or in combination with +8 was obtained from the ÚHKT and the General University Hospital of Prague on LEN therapy. The department of cytogenetic evaluated 71 (85,54%) 5q- patients and 12 (14,46%) patients with combination del(5q) and +8 according to the WHO classification. In conclusion, every MDS group was unsuccessfully treated by EPO, which leads to TRF dependence and LEN treatment.

As I mentioned in Chapter 3 of this thesis, seven non-5q- samples on LEN treatment were obtained thanks to the clinical trial (NCT01029262) only from the General University Hospital of Prague. Unfortunately, the acquired MDS cohort did not respond to LEN treatment or its combination (+EPO, or +EPO+PRED).

The LEN response rate was 66,27% (55 of 83 evaluated patients), with 32,53% complete and 14,46% cytogenetic response. MDS patients belong to the IPSS low/Int-1 (intermediate- 1) risk categories had a better response on LEN treatment than IPSS Int-2 (intermediate- 2)/high. Three patients in remission stopped with treatment, and they returned to LEN treatment after anemia appeared again (patients were in remission a couple of months or years). One of them had refused LEN treatment at the time of this analysis, she was dead.

During unsuccessful treatment, erythropoietin was added to LEN. PRED was added to LEN+EPO, if the level of hemoglobin was constantly low.

If this combination of drugs has not increased the level of hemoglobin, treatment was ended. If an MDS patient is a suitable candidate for another clinical trial, she/he will continue in this treatment. In fact, 21 LEN patients have died during treatment, but it was

not connected to this drug. It was caused by aging-associated diseases, for example, heart disease, or transformation to high-risk MDS, or AML.

Tab.6: Characteristics of MDS patients with 5q- syndrome and del(5q) + trisomy 8 in isolated clones

Number of patients	83
Female/Male	69/14
TRF dependence	55
EPO before LEN	37
IPSS-low	51
IPSS-int.1	23
LEN	83
LEN+EPO	33
LEN+EPO+PRED	15
Discontinuation of treatment-no response	26
Complete response	27
Cytogenetic response	13
Hematologic improvement	28
Re-LEN	3
Death	21
Transformation	10

4.2. Comparison of level CRBN mRNA in chosen groups

We chose five groups, where we compared the levels of CRBN mRNA without LEN treatment. Our groups included MDS patients with 5q- syndrome, MDS with (del5q) and +8, the cohort of MDS patients with trisomy 8, low-risk MDS patients with normal karyotype, and healthy controls. Human cereblon assay (cat. no. Hs00372271_m1, Thermo Fisher) was used in this comparison of the mentioned groups.

It is visible in the picture, the highest level of CRBN mRNA in PB mononuclear cells have groups with isolated deletion of the long arm of the chromosome 5 (84 patients), almost the same level has been detected in the group with del(5q) and trisomy 8 (20 patients) and lower level in following other groups - trisomy 8 (12 patients), non-5q- with

NK (NK-normal karyotype- 62 patients) and healthy control (CTRL- 49 patients) (Figure 9).

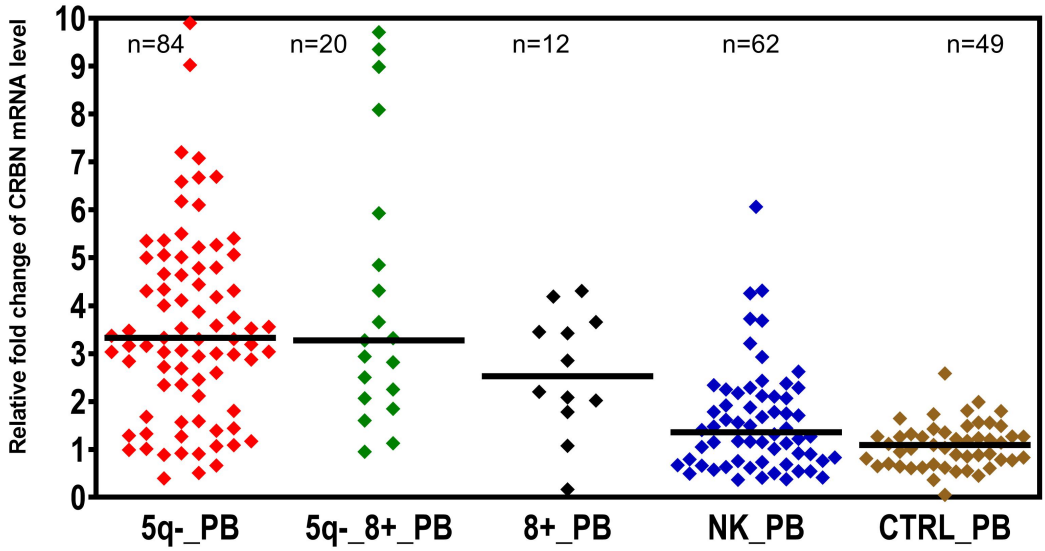


Fig.9: The level of CRBN mRNA in peripheral blood (PB) mononuclear cells of chosen groups.

We detected CRBN mRNA levels also in MDS bone marrow (BM) samples, where we found slightly different results. There is a possibility that a higher level of CRBN mRNA in cohort with del(5q) and +8, and trisomy 8 show that there is some inflammatory process, which can increase the level of CRBN mRNA. We did not focus on this idea. Nevertheless, there is a constantly higher level of CRBN mRNA than in non-5q- and healthy control.

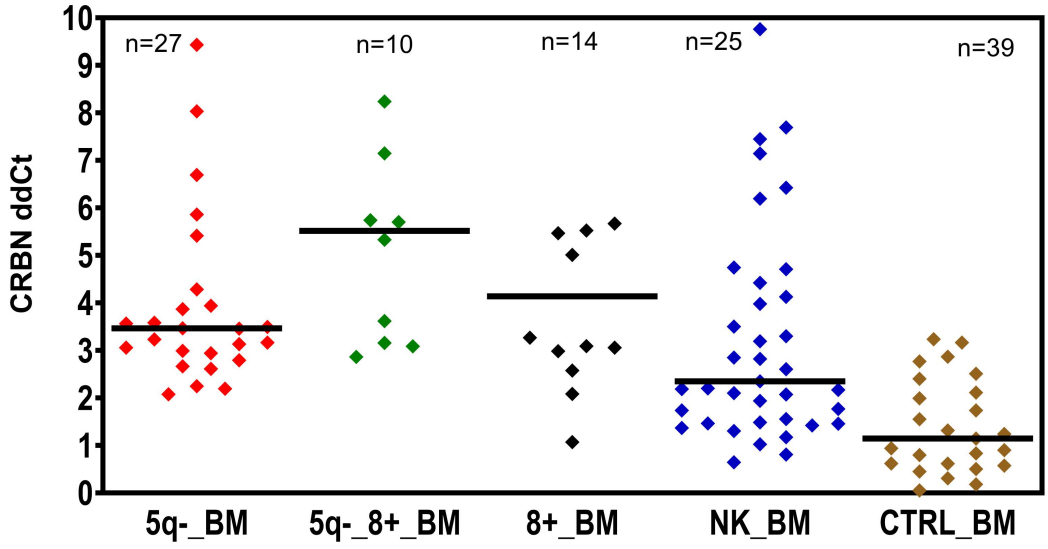


Fig.10: The level of CRBN mRNA in BM mononuclear cells of chosen groups.

We also measured the level of CRBN protein in twenty-three 5q- patients, six patients with del(5q) with trisomy 8, one trisomy 8, eight non-5q- patients with NK, and seven healthy controls (Fig.11). Interestingly, proteins outcomes were different in comparison with mRNA. The lower level had constant healthy control, whereas the cohort of 5q-syndrome was slightly higher. Approximately the same CRBN level was cohort del(5q) with trisomy 8, trisomy 8, and non-5q-. CRBN protein was measured without LEN therapy. Is a known poor correlation between protein and nucleotide levels. Our results can be caused by post-translation modification or different sensitivity of methods (qRT-PCR is a more sensitive method than western blott).

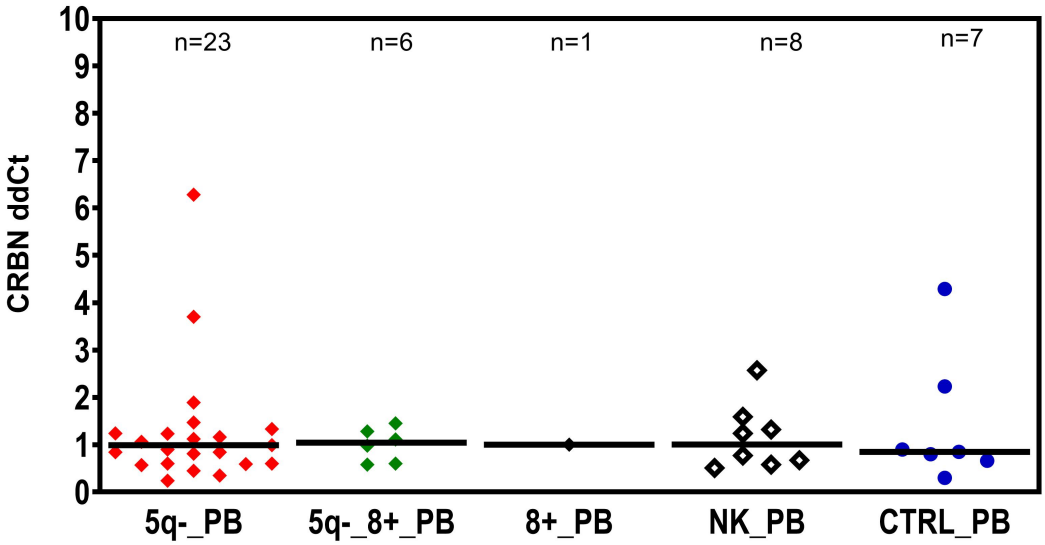


Fig.11: The level of CRBN protein in PB lysate of chosen groups.

We compare three commercial assays for the level of CRBN mRNA detection in the bone marrow and peripheral blood. The used assays were Thermo Fisher TaqMan assays Hs00372271_m1 (primers in exons 8 and 10); Hs01020590_m1 (primers in exons 5 and 6), and Hs02568574_s1 (primers in exon 11). The best of these assays were Hs00372271_m1 (primers in exons 8 and 10), which was used for detection of expression in patients with del(5q) alone or with trisomy 8.

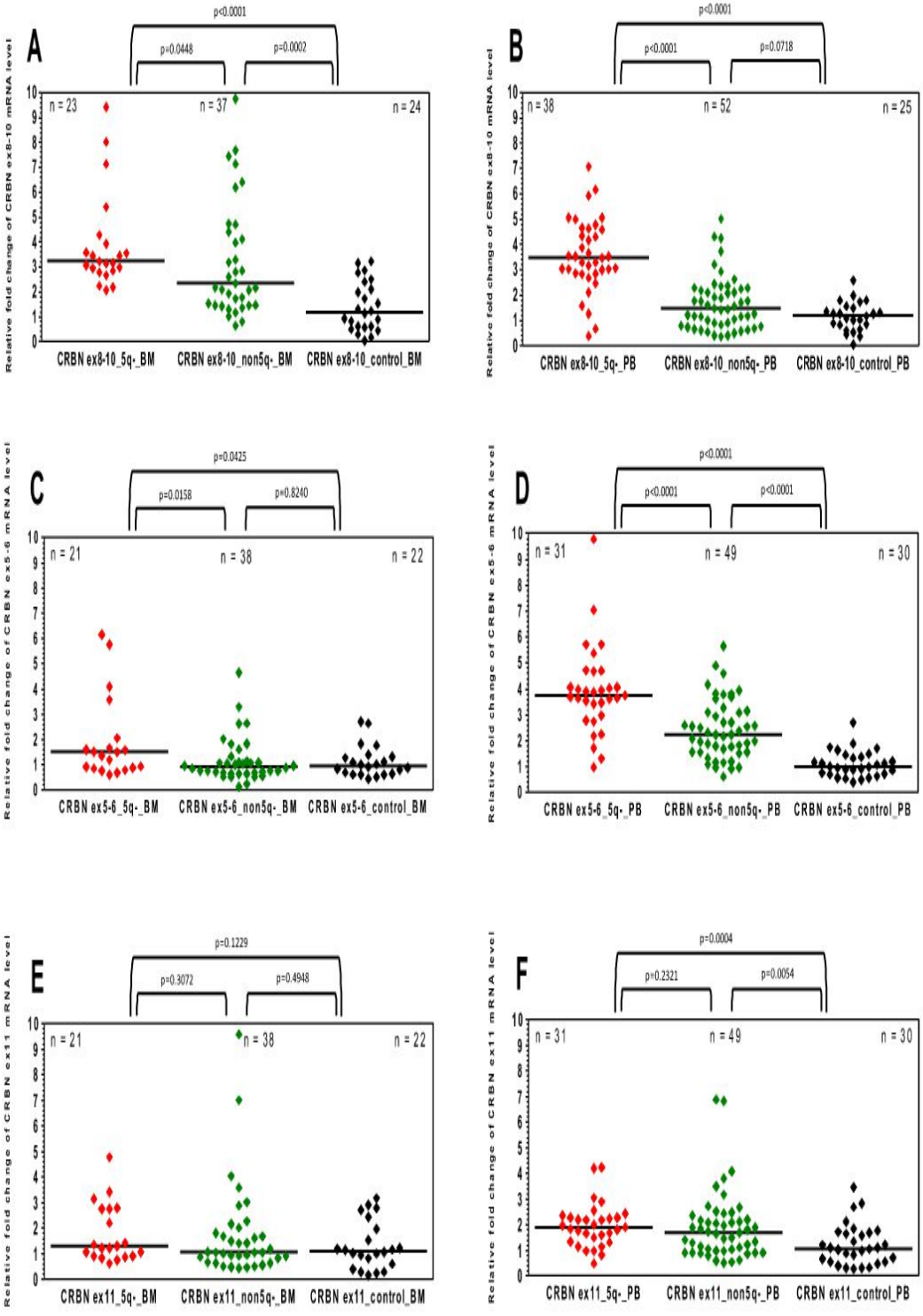

A
p<0.0001
p=0.0448
p=0.0002
Relative fold change of CRBN ex8-10 mRNA level
n = 23
n = 37
n = 24
CRBN ex8-10_5q-_BM
CRBN ex8-10_non5q-_BM
CRBN ex8-10_control_BM
B
p<0.0001
p<0.0001
p=0.0718
Relative fold change of CRBN ex8-10 mRNA level
n = 38
n = 52
n = 25
CRBN ex8-10_5q-_PB
CRBN ex8-10_non5q-_PB
CRBN ex8-10_control_PB
C
p=0.0425
p=0.0158
p=0.8240
Relative fold change of CRBN ex5-6 mRNA level
n = 21
n = 38
n = 22
CRBN ex5-6_5q-_BM
CRBN ex5-6_non5q-_BM
CRBN ex5-6_control_BM
D
p<0.0001
p<0.0001
p<0.0001
Relative fold change of CRBN ex5-6 mRNA level
n = 31
n = 49
n = 30
CRBN ex5-6_5q-_PB
CRBN ex5-6_non5q-_PB
CRBN ex5-6_control_PB
E
p=0.1229
p=0.3072
p=0.4948
Relative fold change of CRBN ex11 mRNA level
n = 21
n = 38
n = 22
CRBN ex11_5q-_BM
CRBN ex11_non5q-_BM
CRBN ex11_control_BM
F
p=0.0004
p=0.2321
p=0.0054
Relative fold change of CRBN ex11 mRNA level
n = 31
n = 49
n = 30
CRBN ex11_5q-_PB
CRBN ex11_non5q-_PB
CRBN ex11_control_PB

Fig.12: The comparison of three commercial assays for the detection of CRBN levels in bone marrow mononuclear cells (A, C, E) and in peripheral blood mononuclear cells (B, D, F). Thermo Fisher TaqMan assays Hs00372271_m1 (primers in exons 8 and 10; A and B); Hs01020590_m1 (primers in exons 5 and 6; C and D), and Hs02568574_s1 (primers in exon 11; E and F) were used [Jonasova A, Bokorova R., et al. 2015].

We detected splicing variants CRBN mRNA without exon 8 or 10 or both exons 8 and 10 by sequencing analysis of CRBN cDNAs obtained by reverse transcription of the CRBN mRNA isolated from individual MDS patients and amplification of these CRBN cDNAs by PCR. Therefore, we used in all analyses of CRBN mRNA levels TaqMan assay Hs00372271 which detected the full-length CRBN mRNA. This assay does not detect splicing variants without exon 8 or 10 or both exons 8 and 10.

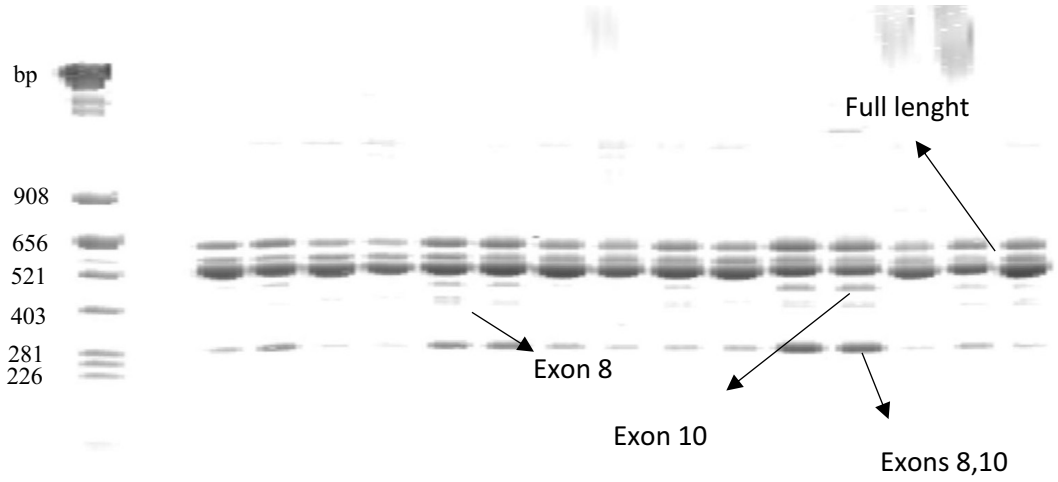


Fig.13: Representation of full- length CRBN mRNA and CRBN mRNA splice variant with deleted exon 8, exon 10, and both exons 8, 10.

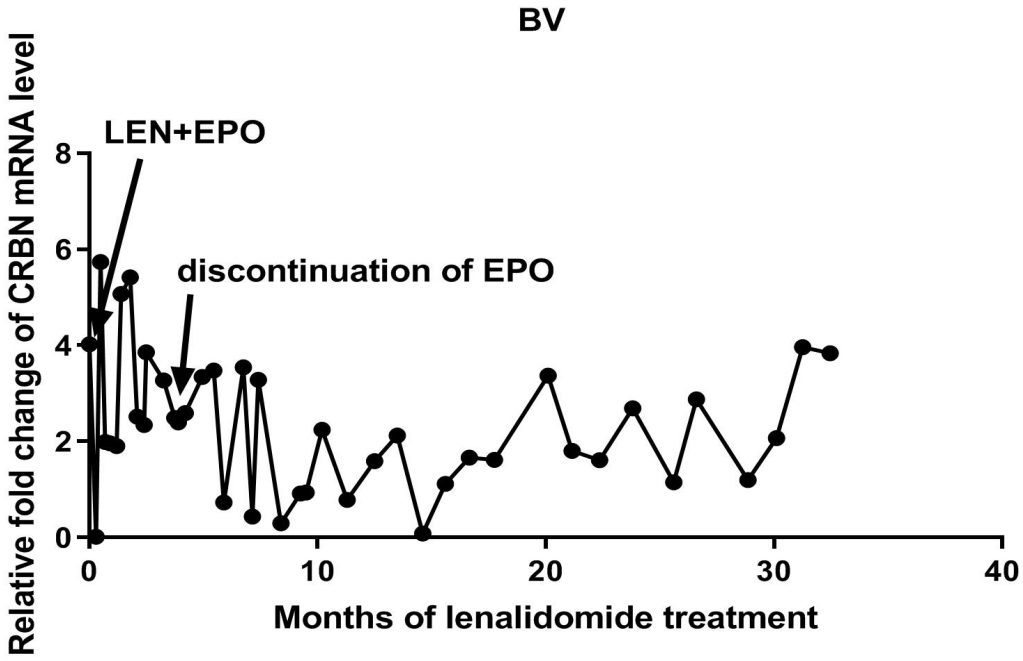
4.3. Cereblon as a prognostic factor for the success of lenalidomide therapy

At first, we analyzed the level of CRBN mRNA before, during, and after LEN treatment in the MDS cohort with isolated 5q- syndrome or 5q- and trisomy 8 in individual clones. The level of CRBN was measured in PB and BM by qRT- PCR (quantitative real-time polymerase reaction).

We measured the level of CRBN mRNA in 83 patients on LEN therapy in 5q- syndrome alone or in combination with trisomy 8 (Chapter 4.1.).

This study has shown, the level of CRBN mRNA is important for LEN treatment. The high level of CRBN mRNA showed successful treatment, while a low level of CRBN mRNA predicted failure in treatment and the possibility to transform from low-risk MDS to high-risk or AML.

A.)



B.)

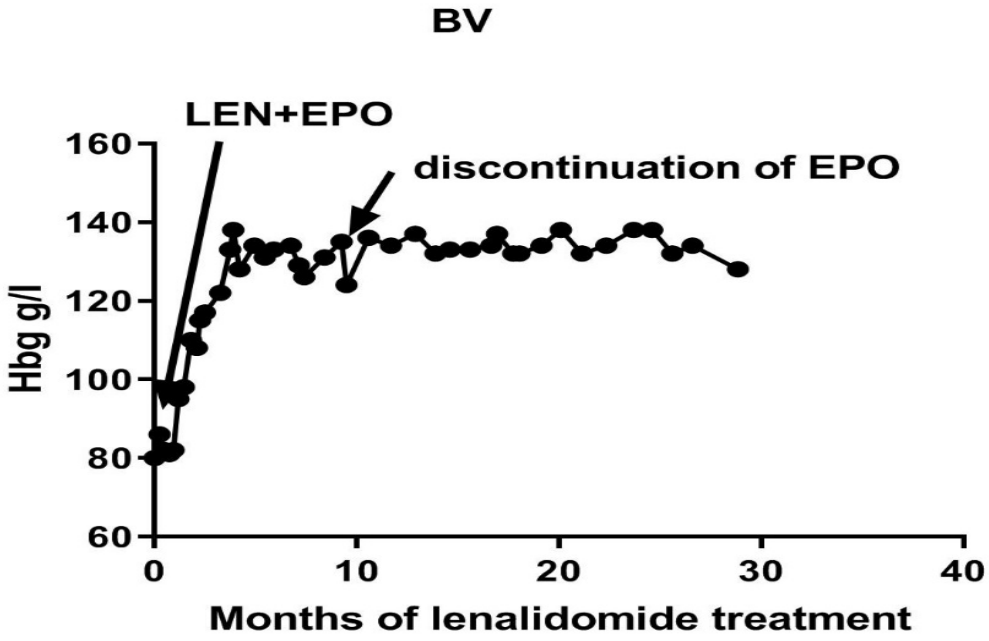
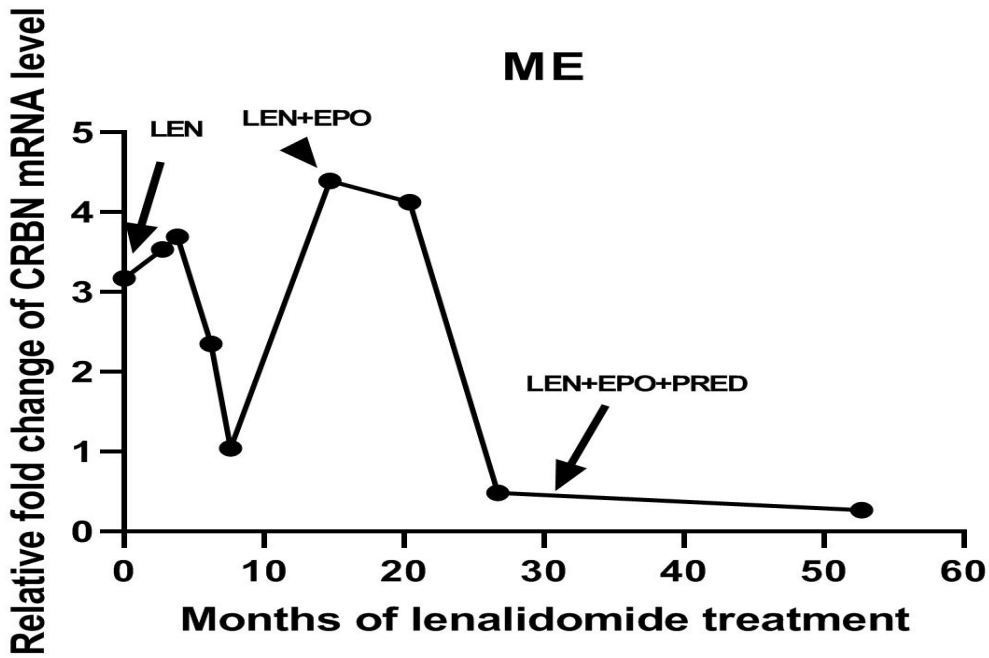


Fig.14: Picture A.) shows CRBN mRNA level of PB before and during LEN treatment. Before LEN, the female patient with 5q- syndrome was treated by EPO, in this therapy had continued with LEN. The second arrow showed the time when MDS patients stopped EPO treatment, she has continued only with LEN. Picture B.) shows hemoglobin in the same patient as verification of the efficacy of therapy and our results in the measurement of CRBN mRNA level.

A.)



B.)

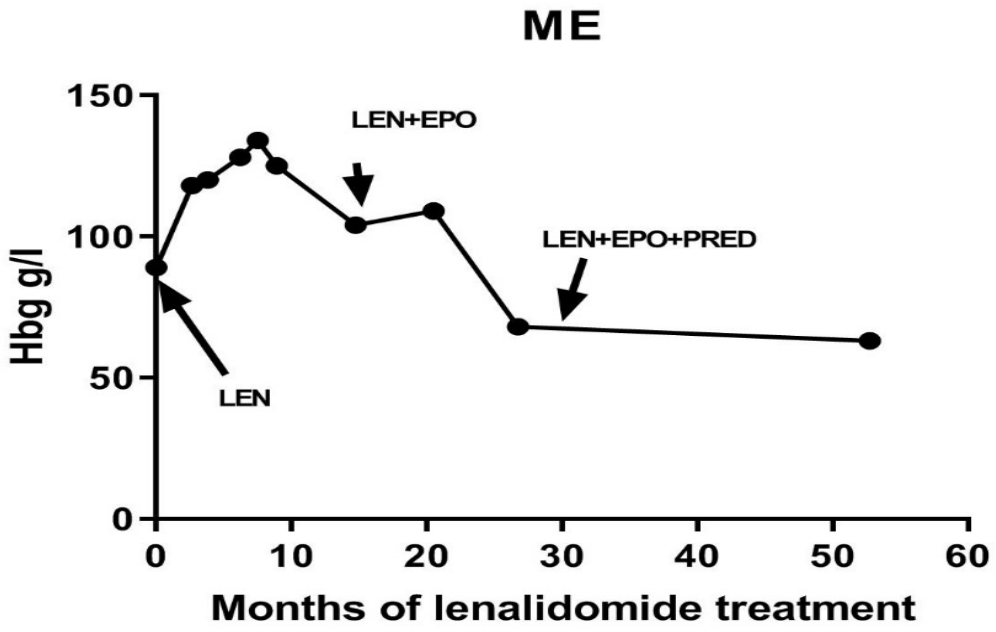
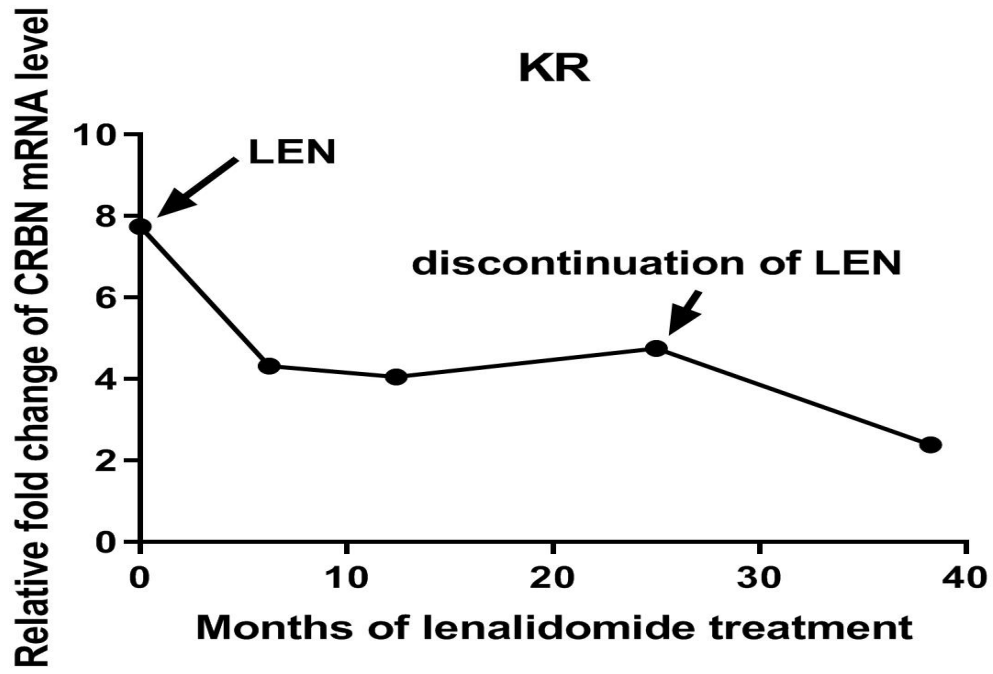


Fig.15: Figure A.) shows PB of 5q- syndrome with three-combination of treatment. The lower level of CRBN mRNA has indicated failure of therapy. Picture B.) shows

hemoglobin in the same patient as verification of the efficacy of therapy and our results in the measurement of CRBN mRNA level.

A.)



B.)

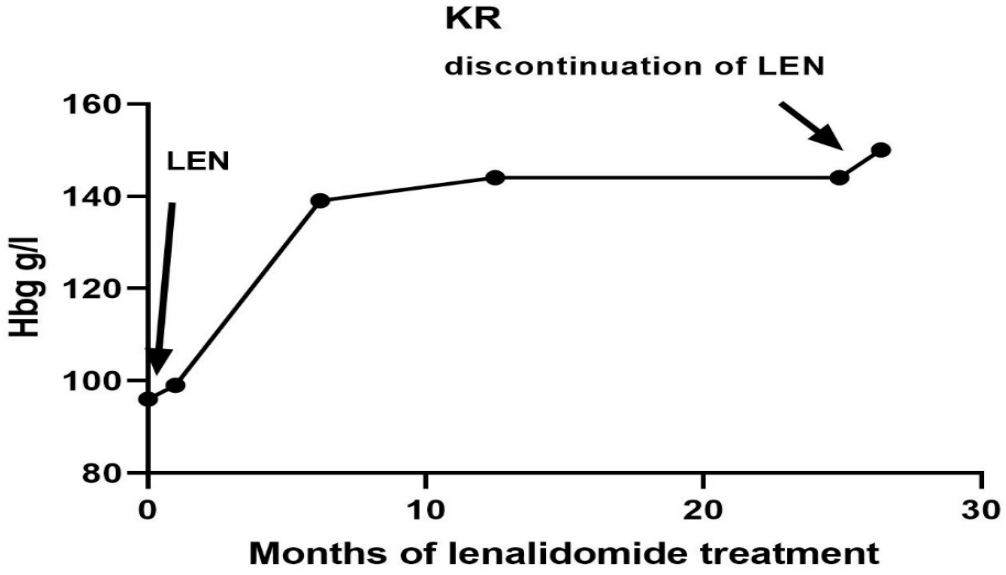
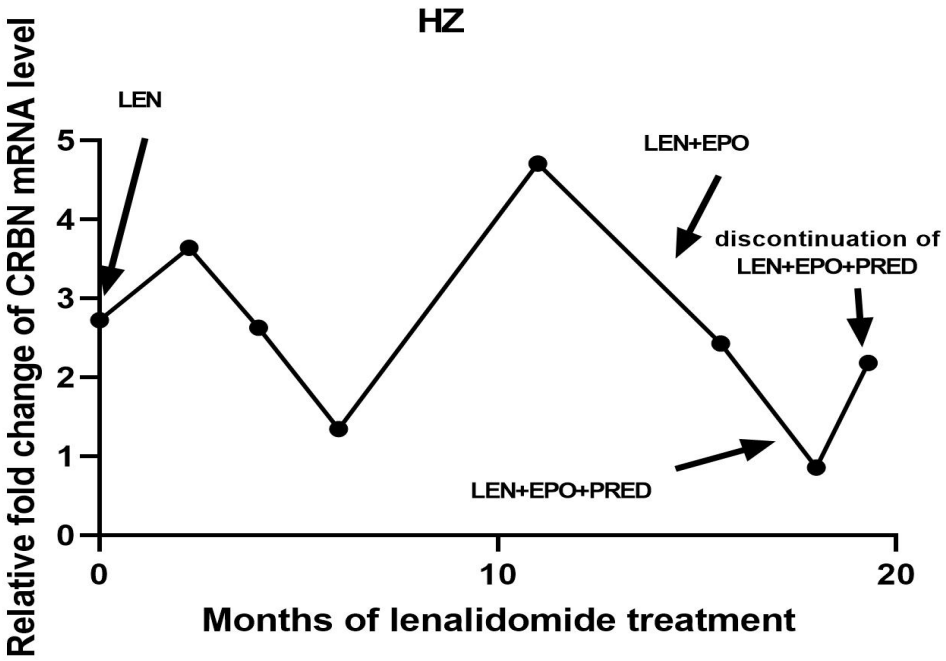


Fig.16: 5q- syndrome with isolated clone 8, the patient completed her treatment because she obtained hematology and cytogenetics responses. After one year of discontinuation of LEN, we have observed decreased level of CRBN mRNA. After three years without treatment, the patient has relapsed and returned to LEN treatment. Picture A.) illustrates the course of CRBN mRNA during LEN therapy. Picture B.) shows hemoglobin in the same patient as verification of the efficacy of therapy and our results in the measurement of CRBN mRNA level.

A.)



B.)

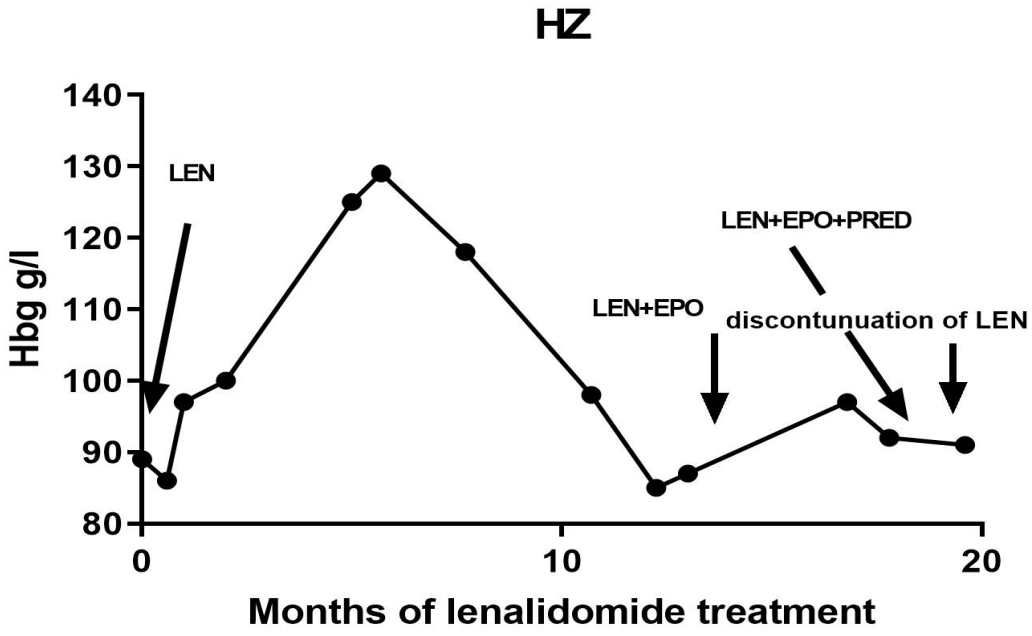


Fig.17: MDS-RCMD, del(5) (13q33,q31), the level of CRBN mRNA before, during, and in time of discontinuation of LEN. At the time of biopsy of BM, the patient transformed to MDS-EB-1. Picture B.) shows hemoglobin in the same patient as verification of the efficacy of therapy and our results in the measurement of CRBN mRNA level.

We have found articles about Nrf2 and its connection with cereblon and EPO. These researches were provided on mice cells. According to these articles, we thought that the level of Nrf2 mRNA and CRBN mRNA is the same. Our next goal is to prove that we were right.

We choose a cohort of non-5q- on EPO treatment, 5q- syndrome, or combination of del(5q) with trisomy 8 on EPO, LEN, LEN+EPO treatment. As I mentioned above, the Czech Republic has not approved LEN treatment of non-5q- patients. We obtained a small group of these samples, where were approved this therapy by the health insurance. The level of Nrf2 mRNA was measured in PB mononuclear cells, not in BM.

Patients with del(5q), del(5q) with trisomy 8, and with normal karyotype on EPO, LEN+EPO, or LEN+EPO+PRED therapy have corresponded course of the levels of Nrf2 mRNA and CRBN mRNA in 70,27% of cases. EPO-treated patients (5q- syndrome, del(5q) with trisomy 8) had the same course or forgone course of Nrf2 mRNA expression levels compared with CRBN mRNA expression levels in 66,7%. The course of Nrf2

mRNA levels corresponded to course of CRBN mRNA expression in 71.43% of cases in LEN treated cohort (MDS with 5q- syndrome, del(5q) with trisomy 8). We measured one non-5q- patient on LEN therapy (Fig.20), and the courses of Nrf2 mRNA levels and CRBN mRNA levels corresponded. The rest of the MDS group was on EPO treatment. This group predominantly had the same or the overtaken course of the expression of Nrf2 mRNA compared with the course of CRBN mRNA expression in 85,71% cases. In some cases that did not correspond to it, we found out that patients did not respond to treatment.

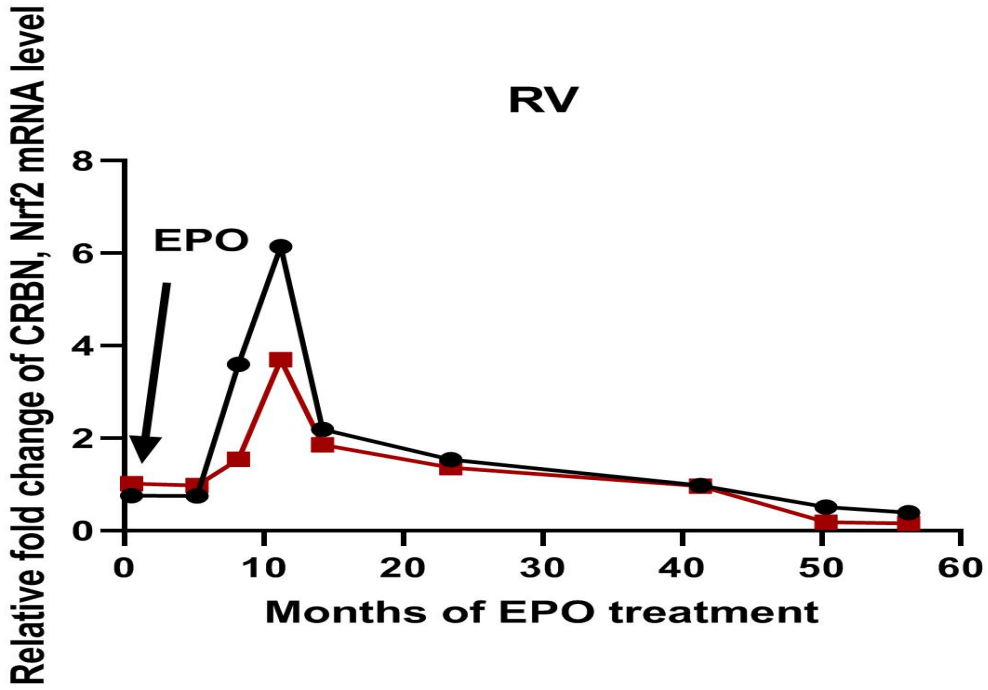


Fig.18: Secondary MDS-RARS, the level of CRBN mRNA and Nrf2 mRNA during EPO. The patient has not completed his treatment yet. Red color presents Nrf2 mRNA expression.

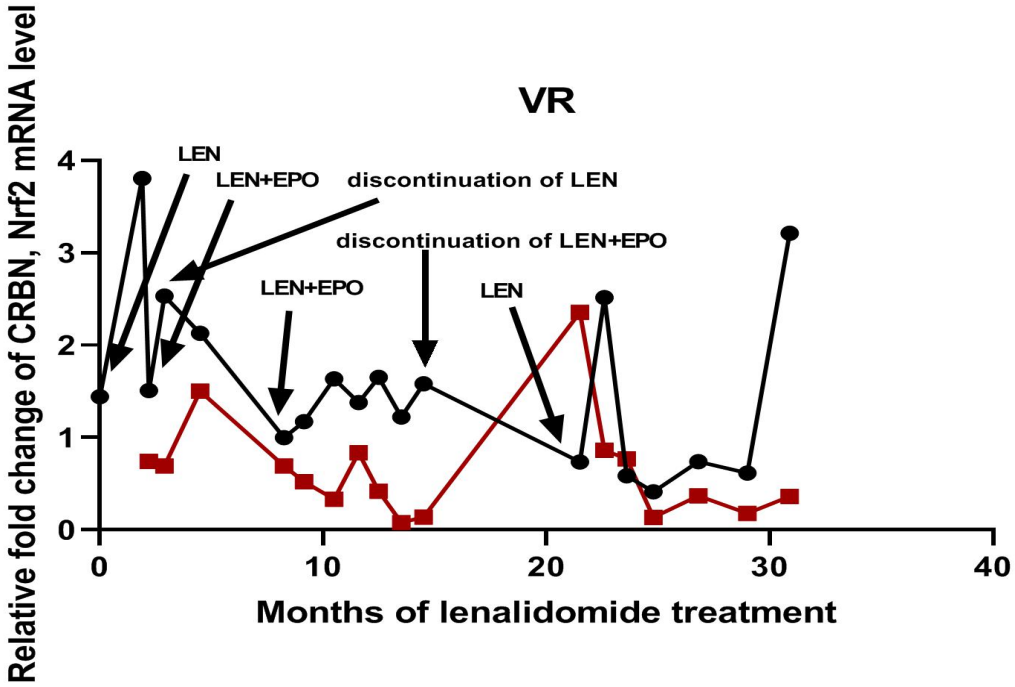


Fig.19: The female 5q- patient (q21.2-q34) temporarily responded to the LEN and its combination of treatment. The patient completed treatment. After five months, her hemoglobin was decreased, and she became TRF dependent. She has started her treatment again and obtained a hematology response. She continues in her treatment. Fig.19 illustrates the course of Nrf2 and CRBN mRNA during LEN+EPO therapy. Red color presents Nrf2 mRNA expression.

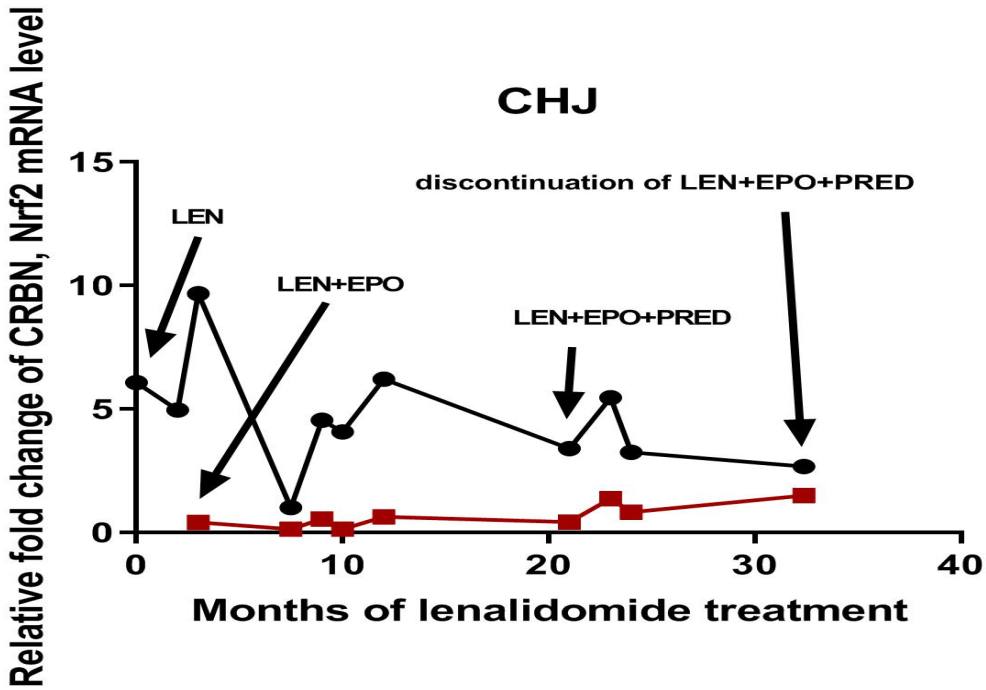


Fig.20: The female patient with MDS/MPD-RARS-T (myelodysplastic/myeloproliferative neoplasm with refractory anemia with ring sideroblasts with thrombocytosis) was treated with LEN+EPO+PRED. The treatment was temporally successful. Fig.20 illustrates the course of Nrf2 and CRBN mRNA during LEN+EPO and LEN+EPO+PRED therapy. Red color presents Nrf2 mRNA expression.

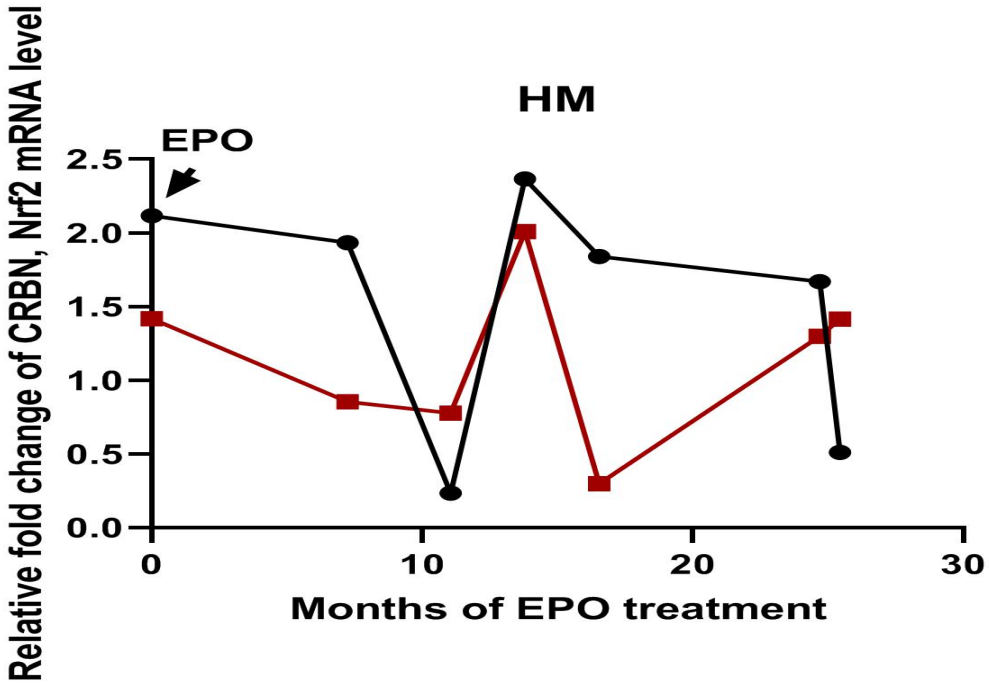


Fig.21: MDS- RCMD with the combination of del(5q) and del(2), the level of CRBN mRNA and Nrf2 following EPO treatment in time. The patient has not responded to EPO treatment, she is waiting on approving LEN by the health insurance. Fig.21 illustrates the course of Nrf2 and CRBN mRNA during EPO therapy. Red color presents Nrf2 mRNA expression.

We also investigated polymorphism A/G located at site 29 nucleotides before the transcriptional start site of the CRBN gene. Our goal was to find if polymorphism A/G can be used as a prognostic factor for the response on LEN treatment in 5q- syndrome, or in its combination. We sequenced MDS patients without treatment (46), during treatment (16), non-5q- (7), and healthy control (10).

Here we show evidence that polymorphism is not a biomarker of LEN response in MDS with 5q- syndrome. In table 1 is an overview of our results. We detected that allele A (81.5%) is predominant compared with allele G (18.5%) in MDS patients with 5q-

syndrome. We obtained a little bit higher percent in samples of 5q- syndrome, which were LEN responders (87.5%) against allele G (12.5%). In contrast, non-5q- patients with no LEN respond had allele A in 85.7% of cases and allele G in 14.3%. We analyzed 75% allele A and 25% allele G in healthy controls. Finally, our results do not correspond to Sardnal's research, where he and his colleagues examined non-5q- samples and LEN sensitivity.

Table 7: Analyzation of the A/G polymorphism located at 29 nucleotides of the 5´untranslated region (chromosome 3 in position 3179746; NC_000003.12; Homo sapiens, GRCh38).

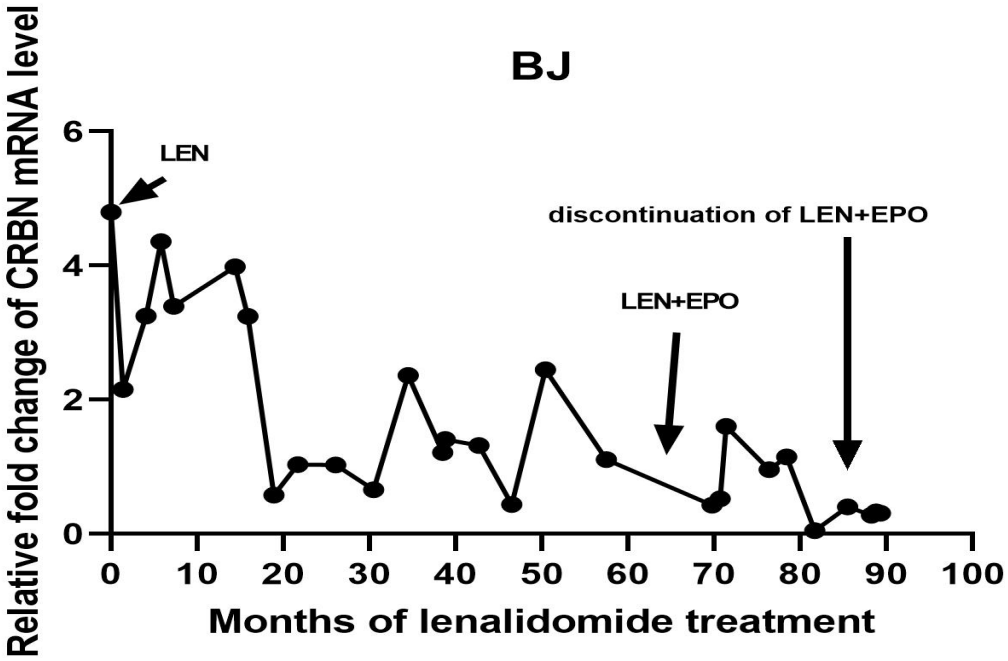
Amount of patients	**Homozygote A**	**Homozygote AG**	**Homozygote G**	**Amount of A allele**	**Amount of G allele**
5q-	31	13	2	75 (81.5%)	17 (18.5%)
5q- patients with respond on LEN treatment	12	4	0	28 (87.5%)	4 (12.5%)
Non-5q- patients with no respond on LEN treatment	5	2	0	12 (85.7%)	2 (14.3%)
Healthy control	6	3	1	15 (75.0%)	5 (25%)

4.4. Effect of combination lenalidomide, erythropoietin, prednisone in MDS patients with del(5q) or combination del(5q) and trisomy 8 in the various clones

Sometimes patients who respond to LEN therapy stop responding. In these cases, doctors added EPO or EPO+PRED to LEN. Our results confirm the additional efficacy of EPO and PRED, but predominantly, it is a temporary effect. Thirty-three patients

stopped responding to LEN and were added EPO to LEN. Fifteen of the thirty-three patients temporarily responded or did not respond to LEN+EPO, and they obtained three-combination of therapy (LEN+EPO+PRED).

A.)



B.)

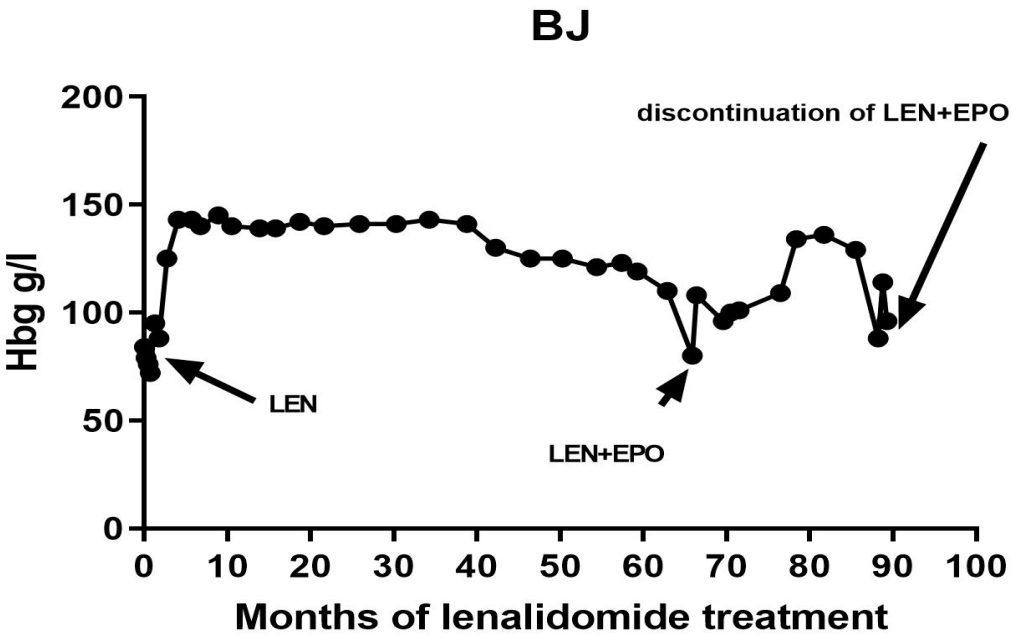
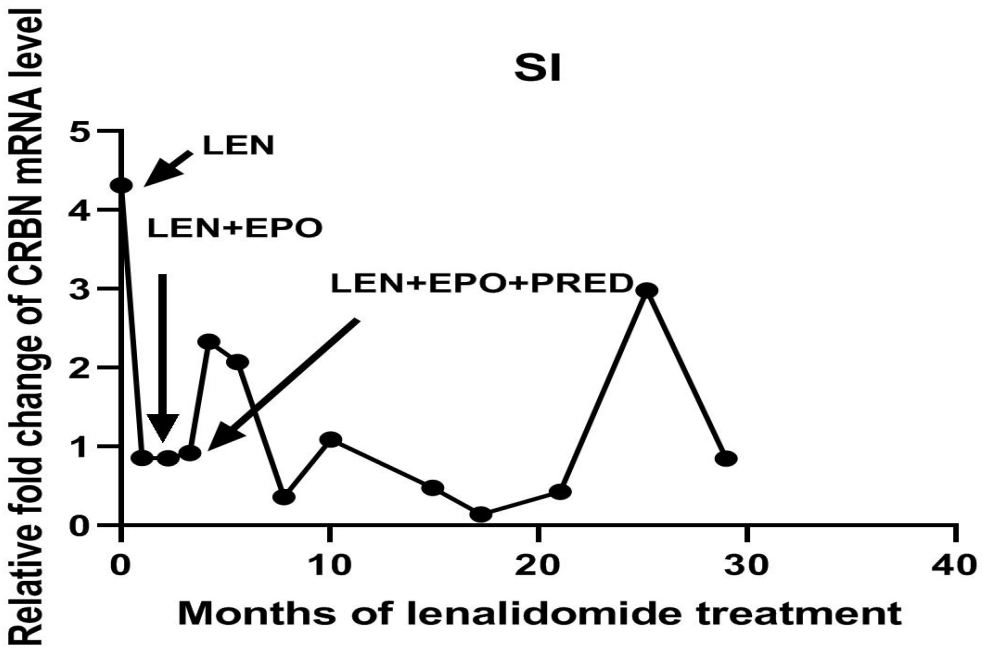


Fig.22: The patient with del(5q) was treated with LEN and EPO after decreasing hemoglobin. In most cases, response EPO after adding to LEN is temporal. She transformed to MDS-EB-1, then to AML. Fig. A illustrates the course of cereblon mRNA during therapy and Fig.B shows the level of hemoglobin.

A.)



B.)

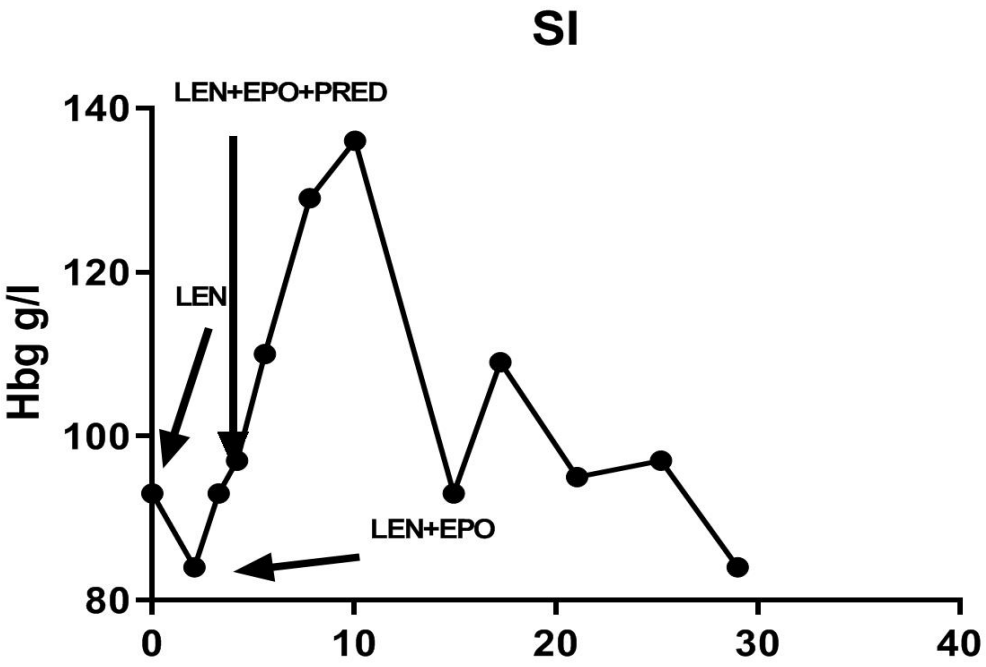
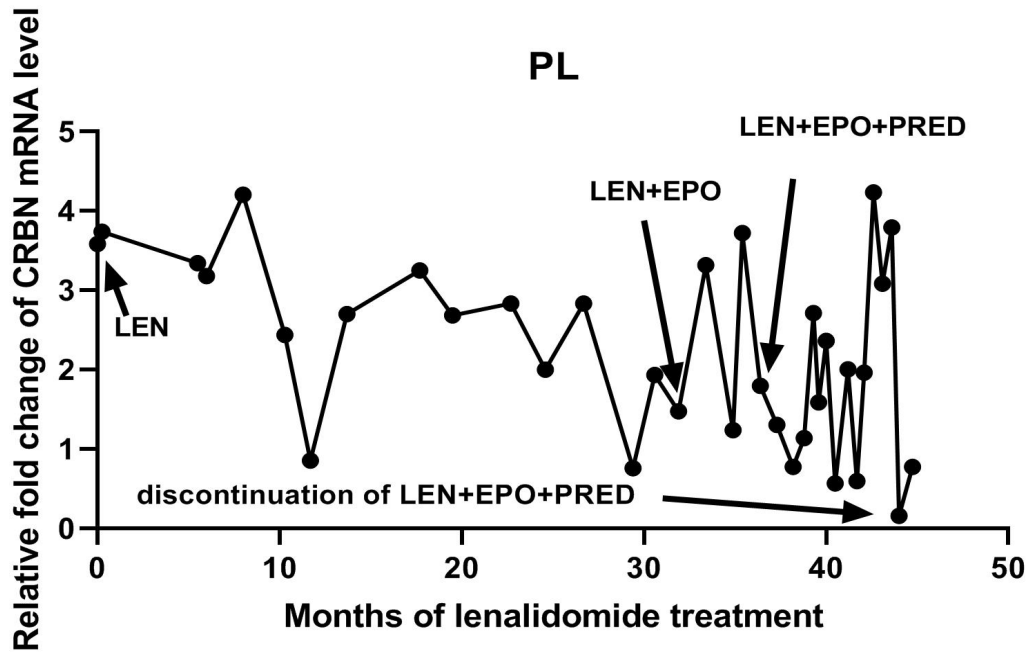


Fig.23: MDS–RCMD with del(5q) with trisomy 8, she has received combination LEN+ EPO+PRED. The effect of treatment is temporary, as in the cause of combination LEN+EPO. Fig. A shows the course of cereblon mRNA during LEN+PO+PRED therapy and Fig.B shows the level of hemoglobin.

A.)



B.)

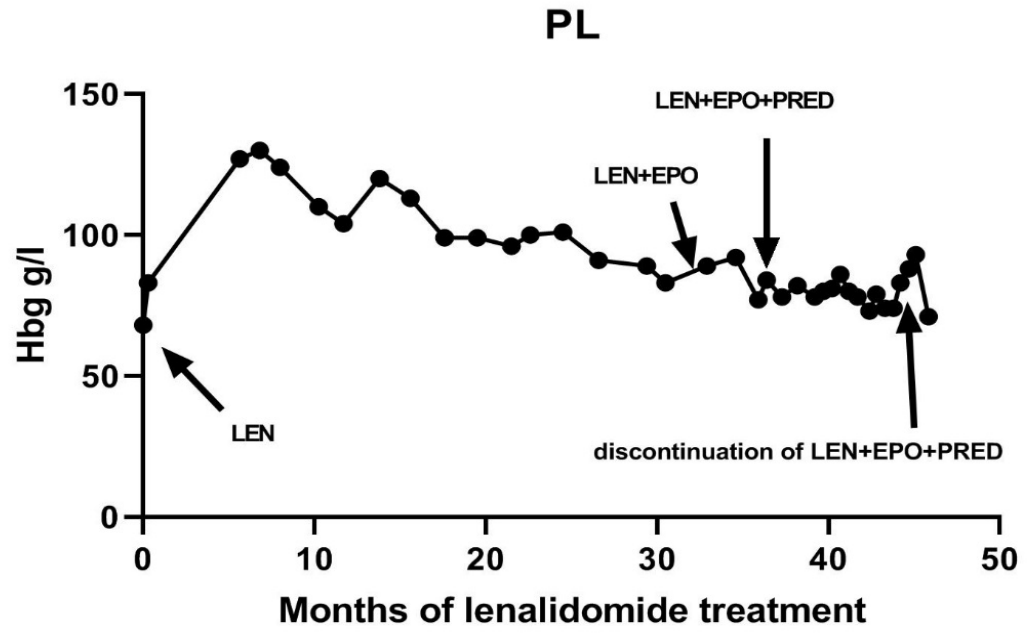
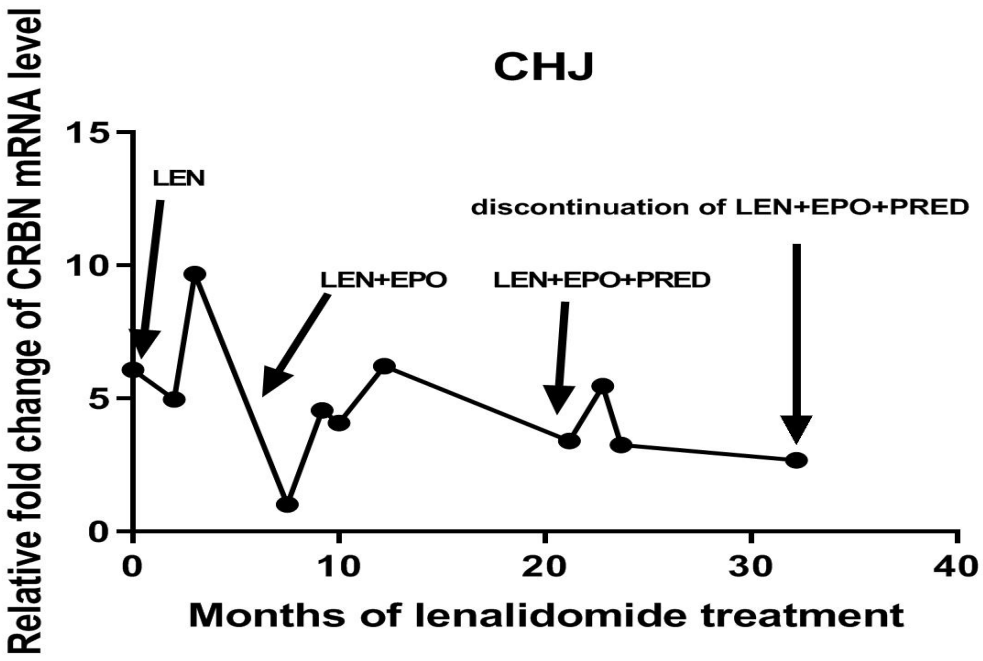


Fig.24: The long-term treated female MDS patient with del(5q) by LEN. The lower level of CRBN mRNA indicated failure of treatment. The patient completed her treatment and she started to be treated by Vidaza. At this time, the patient was dead. Fig. A demonstrates the course of cereblon mRNA during therapy and Fig.B shows the level of hemoglobin.

A.)



B.)

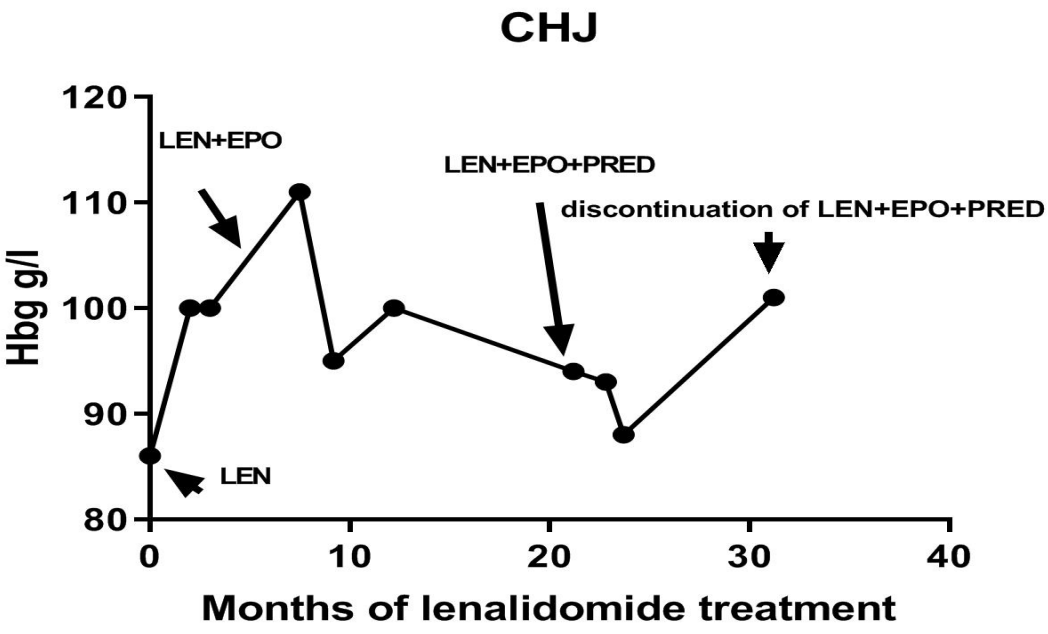
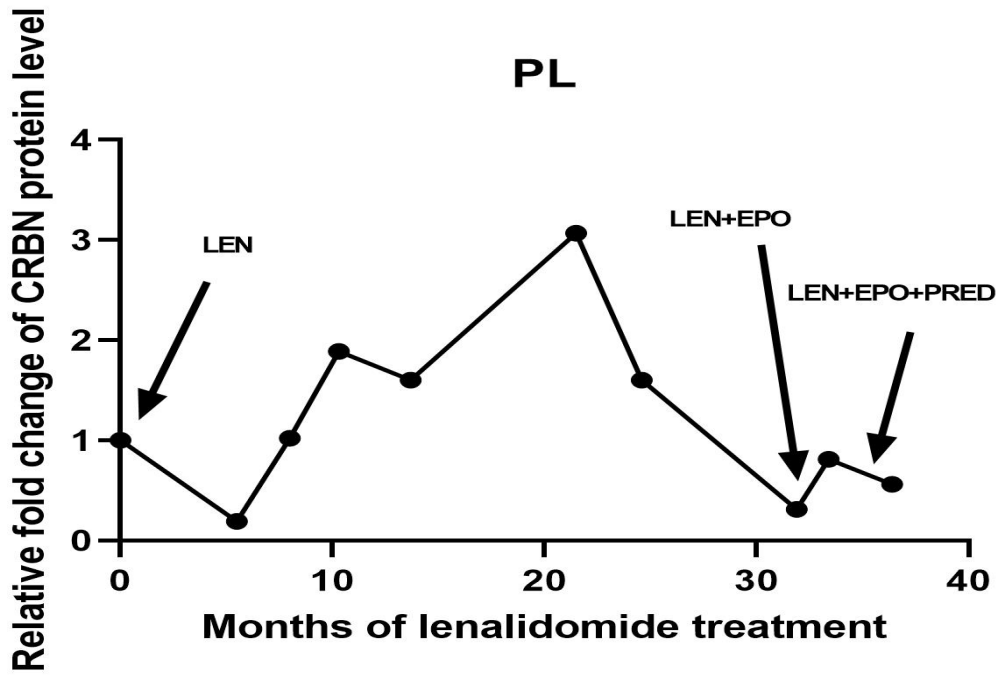


Fig.25: MDS/MPD- RARS-T with normal karyotype, the female patient was treated with LEN+EPO+PRED with partial response. The patient transformed to MDS-EB-2. Fig. A demonstrates the course of cereblon mRNA during therapy and Fig.B shows the level of hemoglobin.

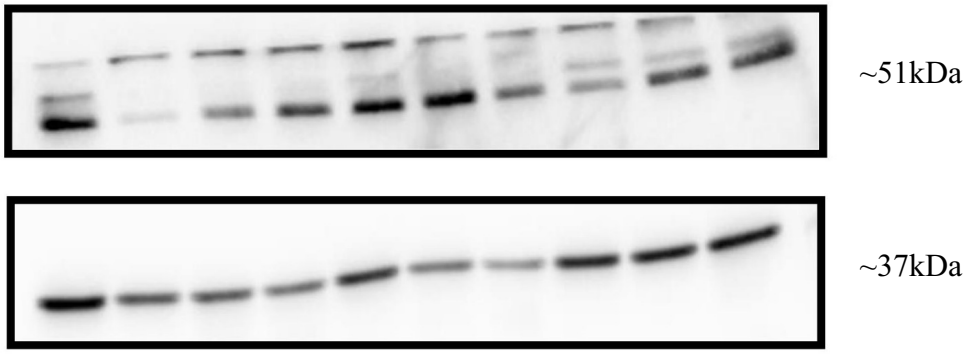
4.5. Comparison of level CRBN mRNA and protein

We analyzed CRBN protein in LEN patients with del(5q) alone (6 patients) or patients with combination del(5q) and trisomy 8 (1 patient) in the various clones before and during LEN therapy or its combinations (LEN+EPO, LEN+EPO+PRED). CRBN protein expression of chosen patients corresponded with response to LEN therapy or its combinations. We analyzed one patient with a normal karyotype during EPO treatment to CRBN protein, Nrf2, and HO 1 protein levels. The level of CRBN protein corresponded with the patient's response to therapy. Nrf2 and HO 1 protein expression correlated too. In patients with combined therapy was the level of Nrf2 and HO 1 protein expression more interesting. Successful treated patients follow the known pattern- Nrf2 up-regulated HO 1. Moreover, the unsuccessfully treated patient has a higher level of Nrf2 than HO 1. Unfortunately, we cannot conclude our outputs as significant because we analyzed only one patient who did not respond to the LEN+EPO+PRED therapy. The measured data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

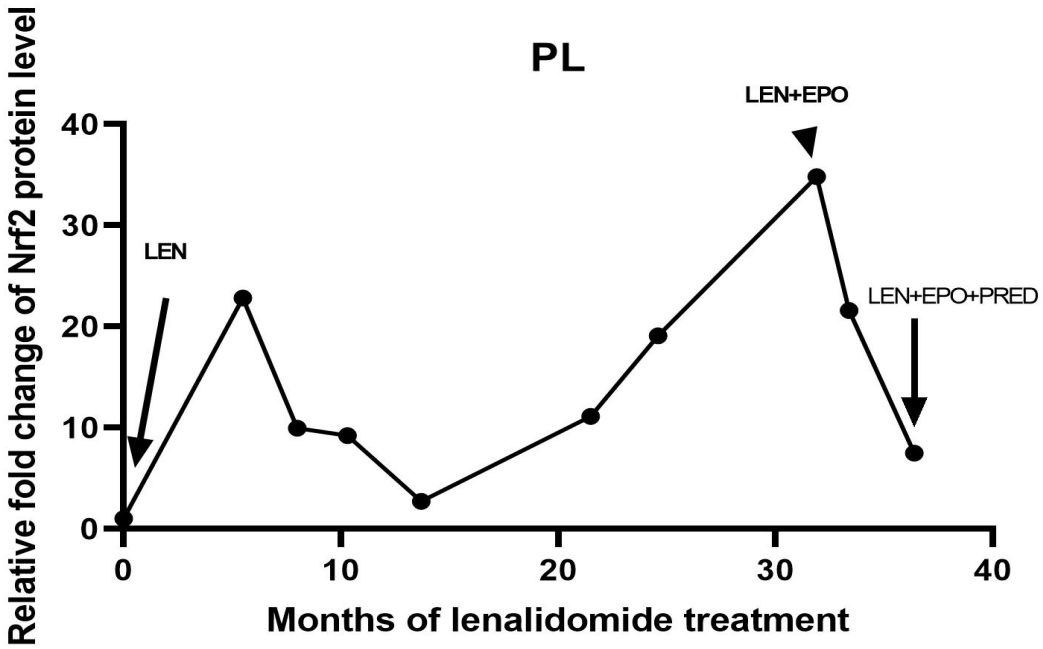
A.)

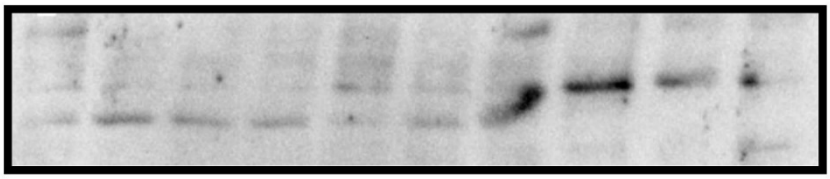

PL
Relative fold change of CRBN protein level
4
3
2
1
0
LEN
LEN+EPO
LEN+EPO+PRED
0
10
20
30
40
Months of lenalidomide treatment

B.)


~51kDa
~37kDa

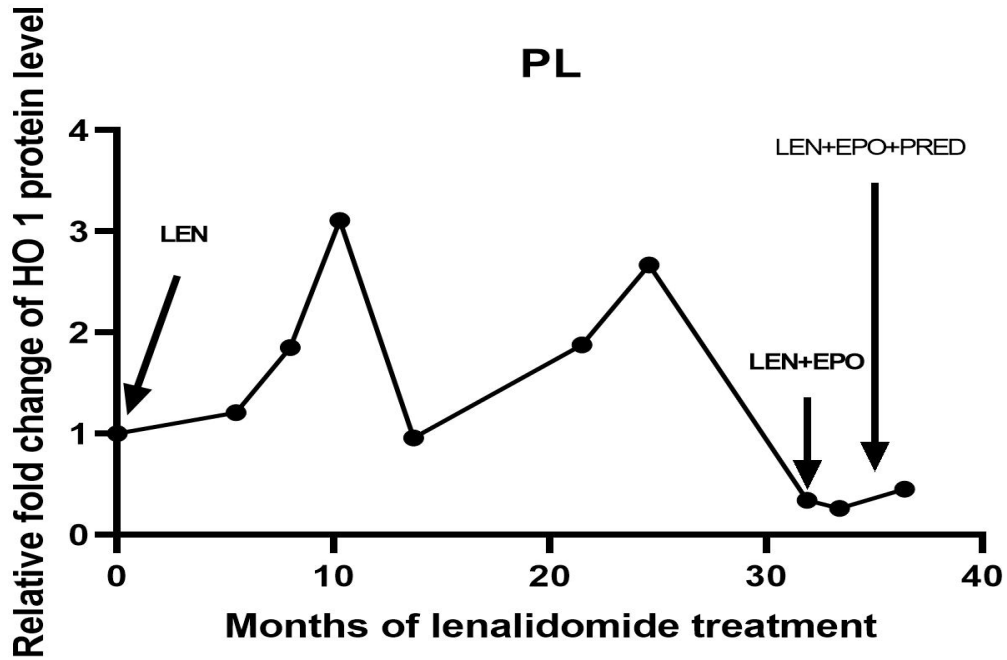
C.)

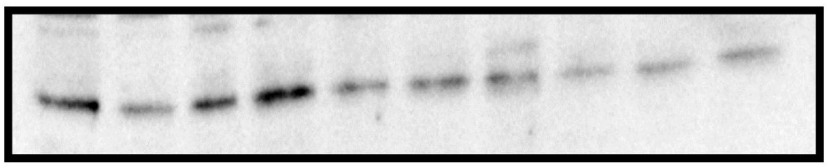




~100kDa
~62kDa

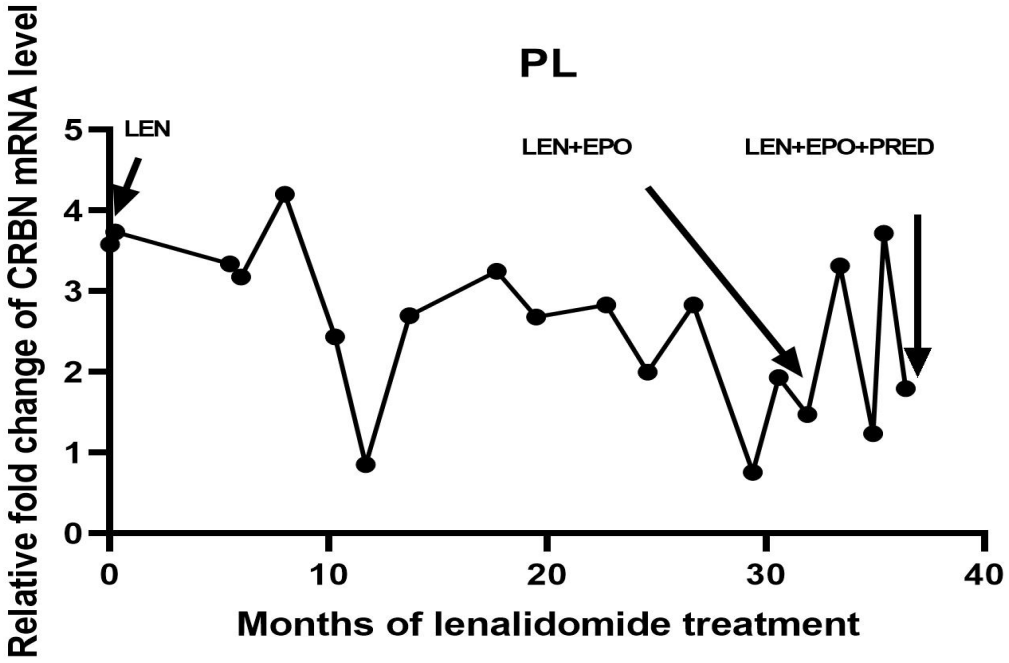
D.)





~33kDa

E.)


PL
LEN
LEN+EPO
LEN+EPO+PRED
Relative fold change of CRBN mRNA level
0
1
2
3
4
5
0
10
20
30
40
Months of lenalidomide treatment

F.)

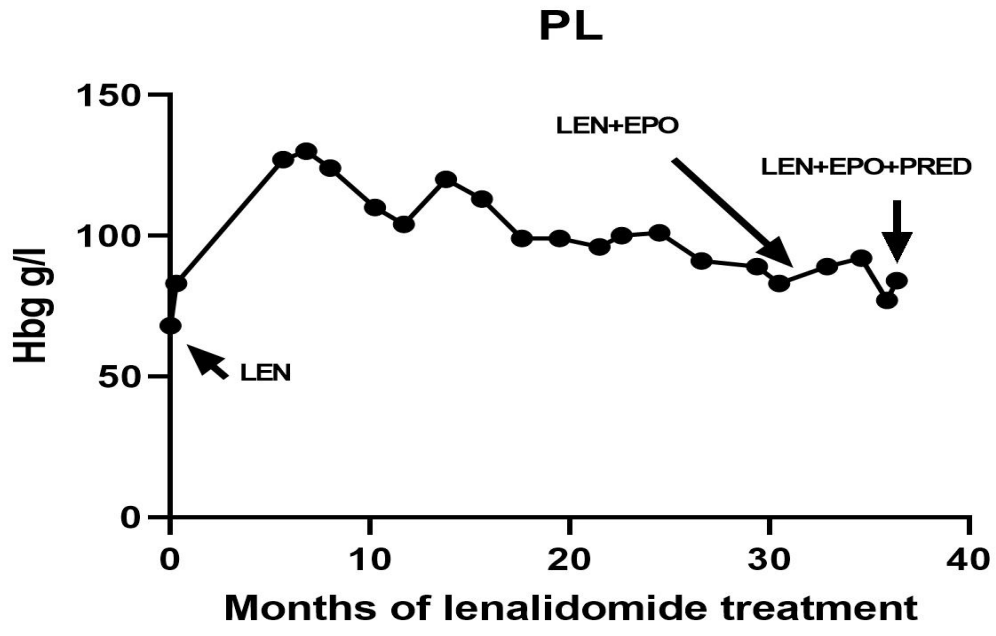

PL
LEN+EPO
LEN+EPO+PRED
LEN
Hbg g/l
0
50
100
150
0
10
20
30
40
Months of lenalidomide treatment

Fig.26: The female patient with 5q- syndrome responded to LEN therapy around two years. Then she progressed to MDS-EB-1. Then to LEN was added EPO, and after a couple of months followed PRED. Her treatment was unsuccessful. Fig. A and B mean CRBN protein expression before and during LEN therapy. The first band is before LEN rest bands are during the treatment. The first picture is CRBN protein with a weight of ~51kDa. The second picture is GAPDH with a weight of ~37kDa. Fig.C illustrates the level of Nrf2 protein (~62kDa), and Fig.D shows HO 1 protein expression (~33kDa). Fig.E is CRBN mRNA expression, and Fig.F is the level of hemoglobin. Fig. C and D are verification of our results.

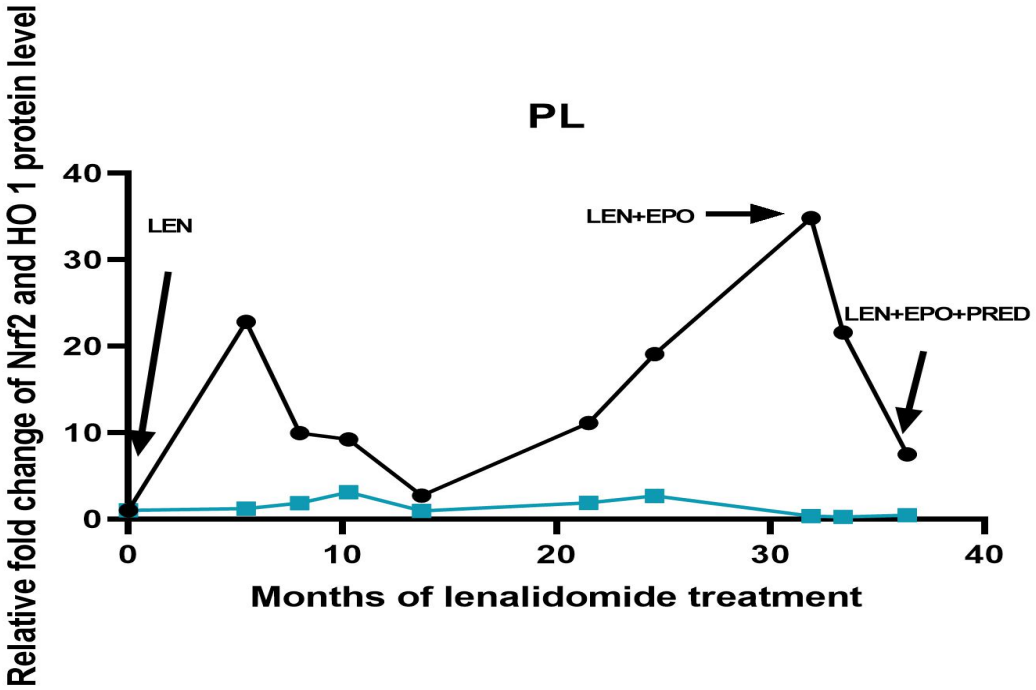
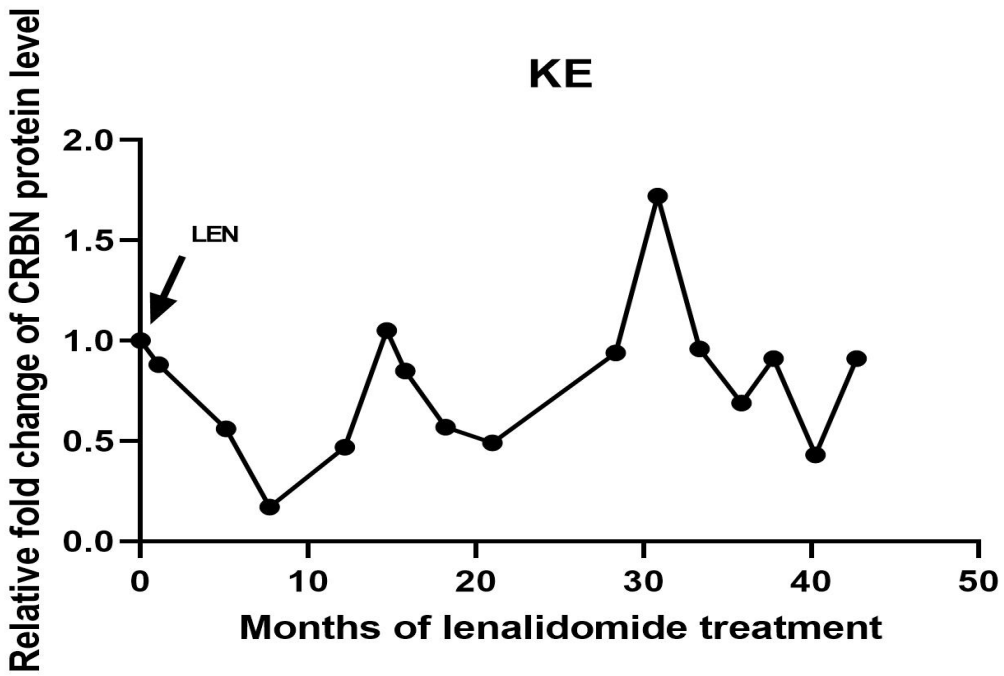
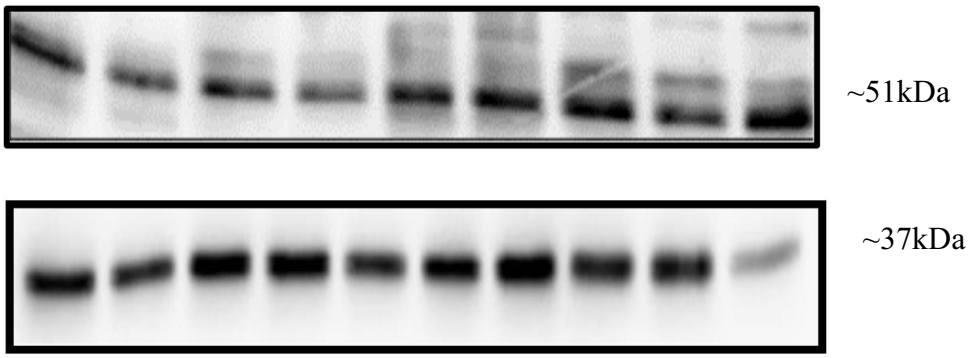


Fig.27: This figure illustrates the comparison of Nr2 and HO 1 proteins levels of previous Fig.26. Blue color presents HO 1 protein expression.

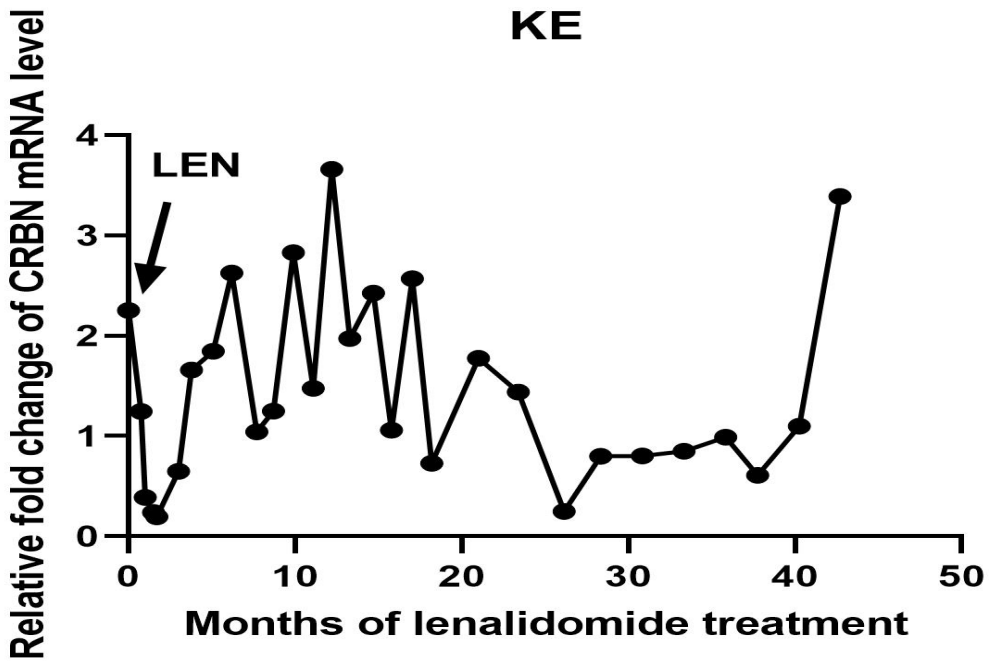
A.)


KE
Relative fold change of CRBN protein level
2.0
1.5
1.0
0.5
0.0
LEN
0
10
20
30
40
50
Months of lenalidomide treatment

B.)


~51kDa
~37kDa

C.)



D.)

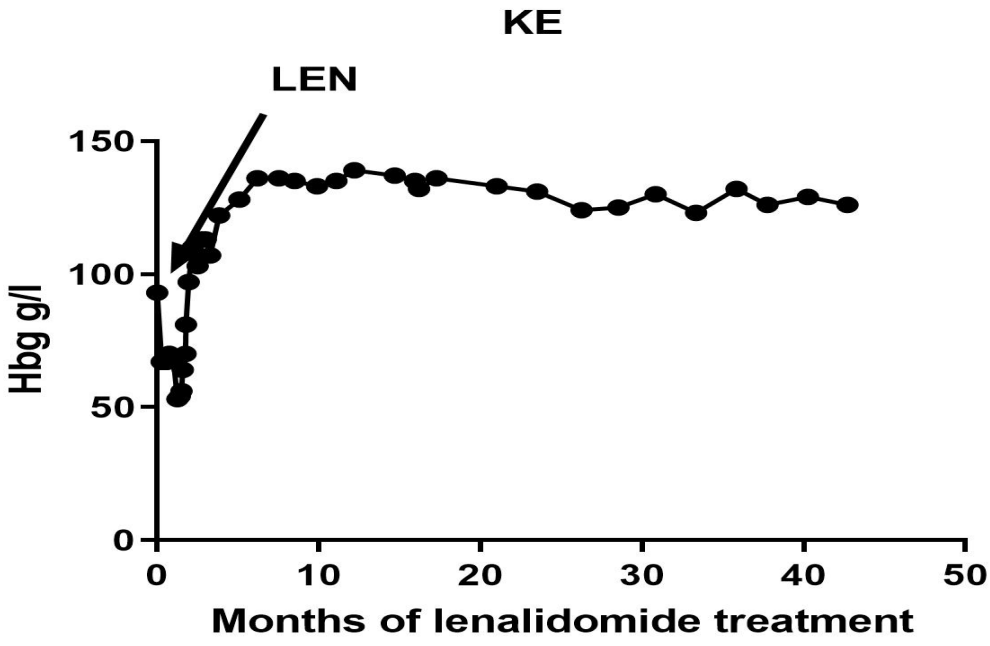
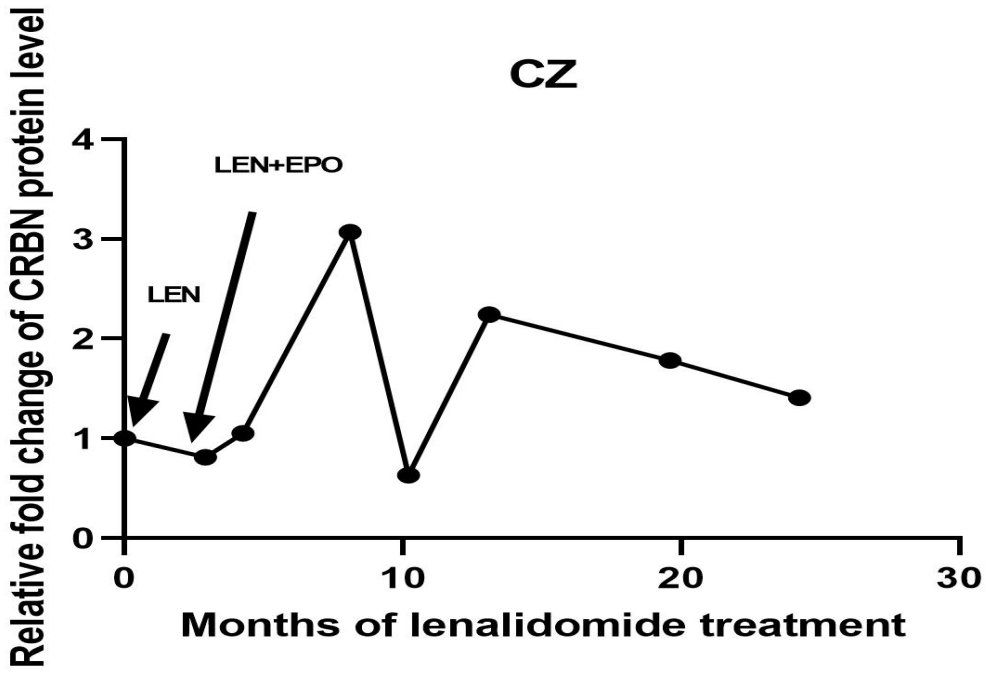


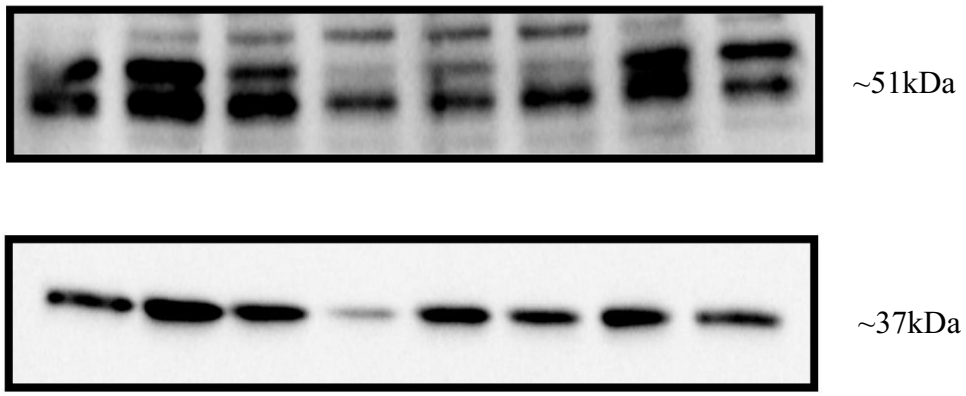
Fig.28: The female patient with del(5q) and trisomy 8 with successful therapy for five years. Fig. A and B mean CRBN protein expression before and during LEN therapy. The first band is before LEN, and the rest bands are during the treatment. The first picture is

CRBN protein with a weight of ~51kDa. The second picture is GAPDH with a weight of ~37kDa. Fig.C is CRBN mRNA expression, and Fig.D is the level of hemoglobin. Fig. C and D are verification of our results.

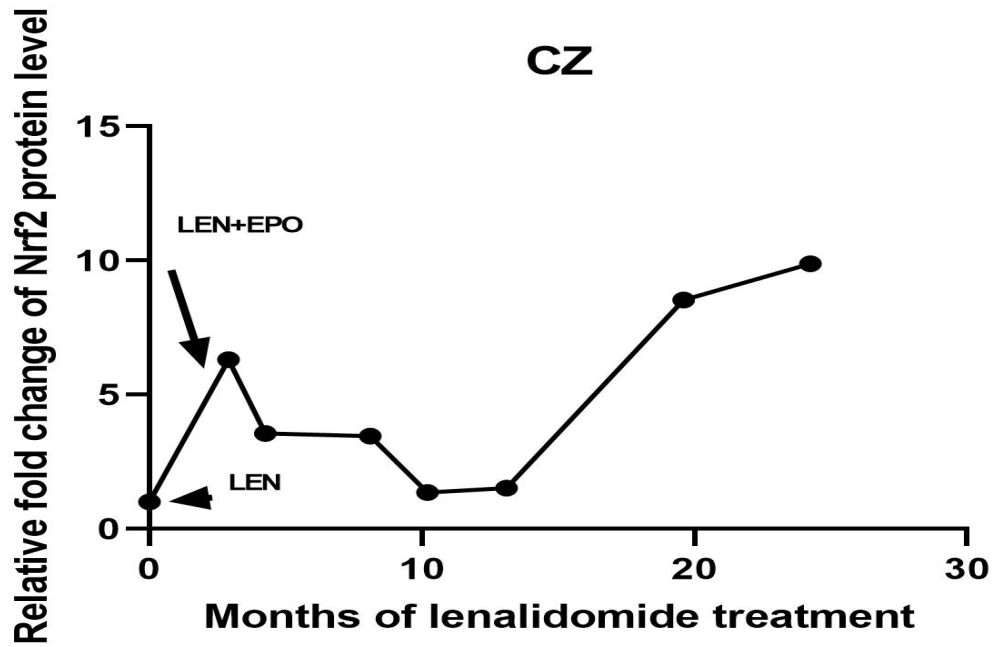
A.)

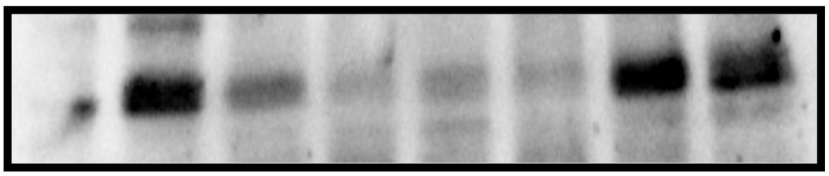


B.)



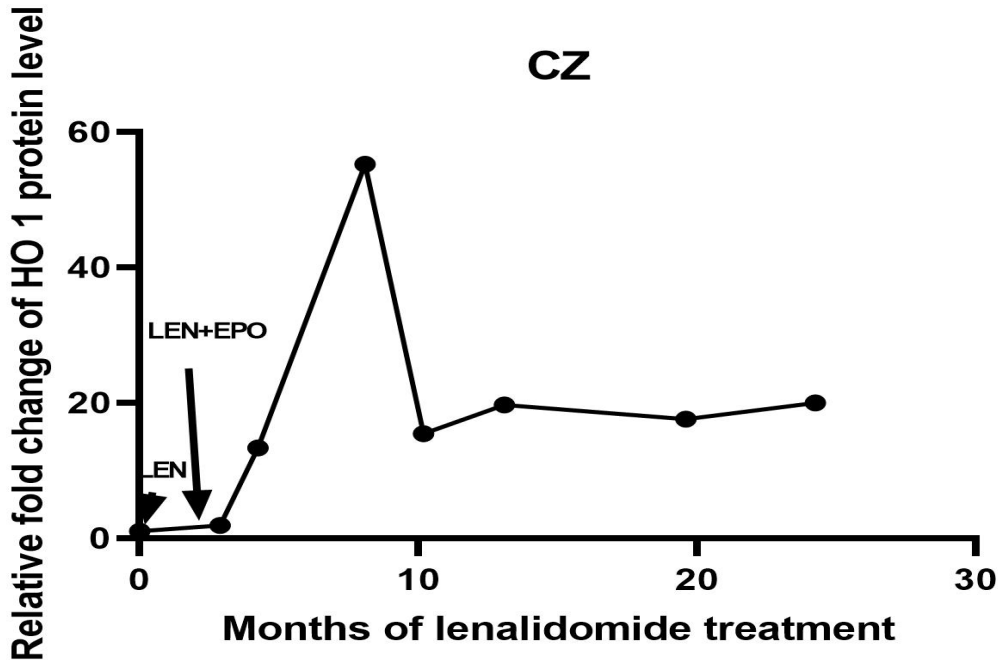
C.)

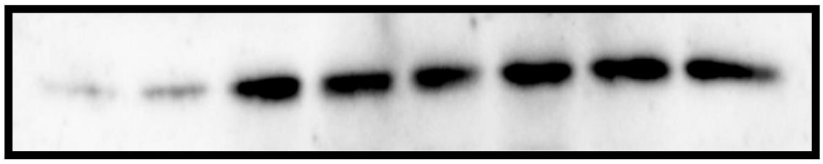

CZ
Relative fold change of Nrf2 protein level
15
10
5
0
LEN+EPO
LEN
0
10
20
30
Months of lenalidomide treatment



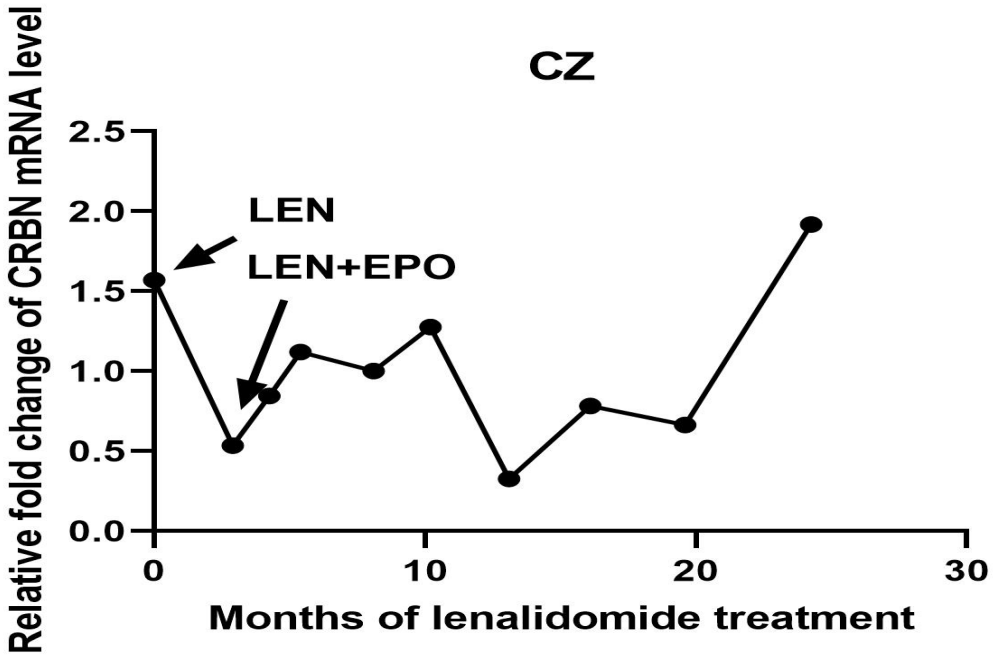
~62kDa

D.)


CZ
Relative fold change of HO 1 protein level
60
40
20
0
LEN+EPO
LEN
0
10
20
30
Months of lenalidomide treatment


~33kDa

E.)


CZ
Relative fold change of CRBN mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
LEN
LEN+EPO
0
10
20
30
Months of lenalidomide treatment

F.)

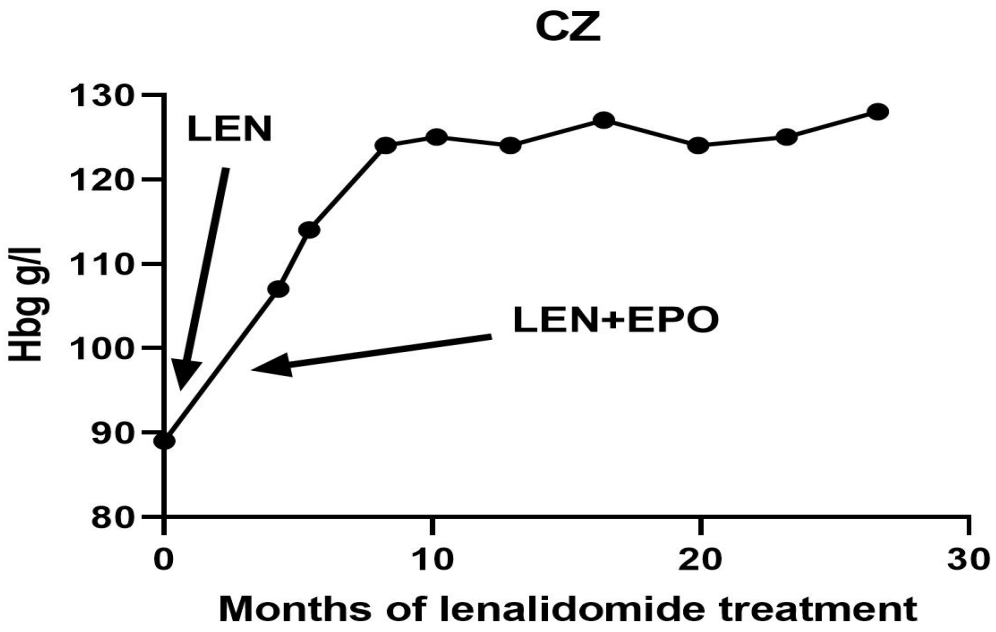

CZ
Hbg g/l
130
120
110
100
90
80
LEN
LEN+EPO
0
10
20
30
Months of lenalidomide treatment

Fig.29: The female patient with del(5q) with successful LEN+EPO therapy. The patient achieved a complete response to combined treatment. Fig. A and B mean CRBN protein expression before and during LEN or LEN+EPO therapy. The first band is before LEN, and the rest bands are during the treatment. The first picture is CRBN protein with a weight of ~51kDa. The second picture is GAPDH with a weight of ~37kDa. Fig.C illustrates the level of Nrf2 protein (~62kDa), and Fig.D shows HO 1 protein expression (~33kDa). Fig.E is CRBN mRNA expression, and Fig.F is the level of hemoglobin. Fig. C and D are verification of our results.

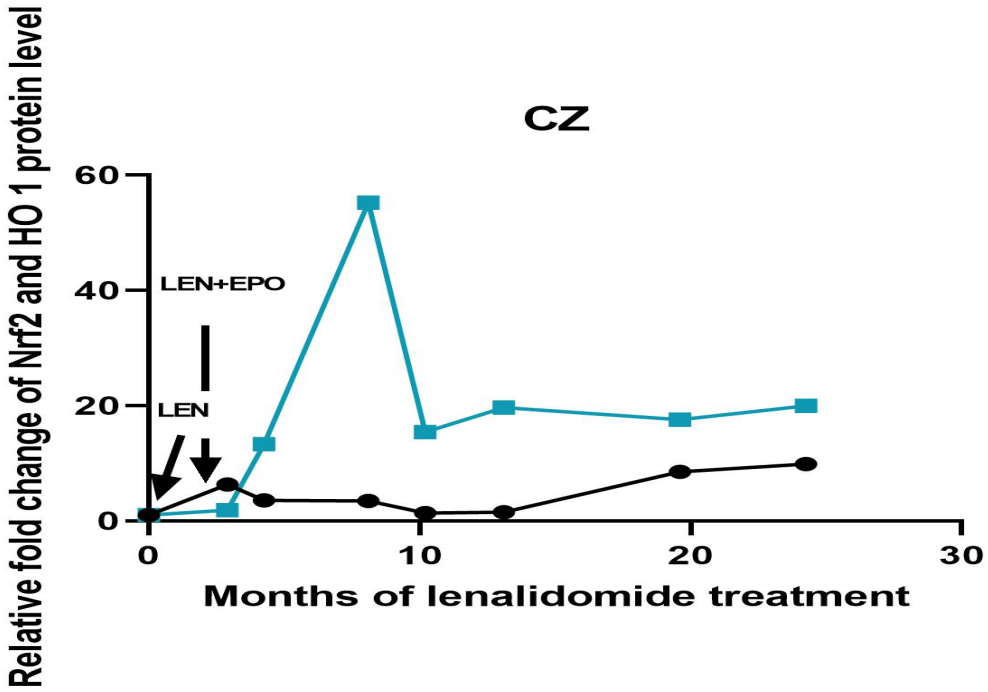
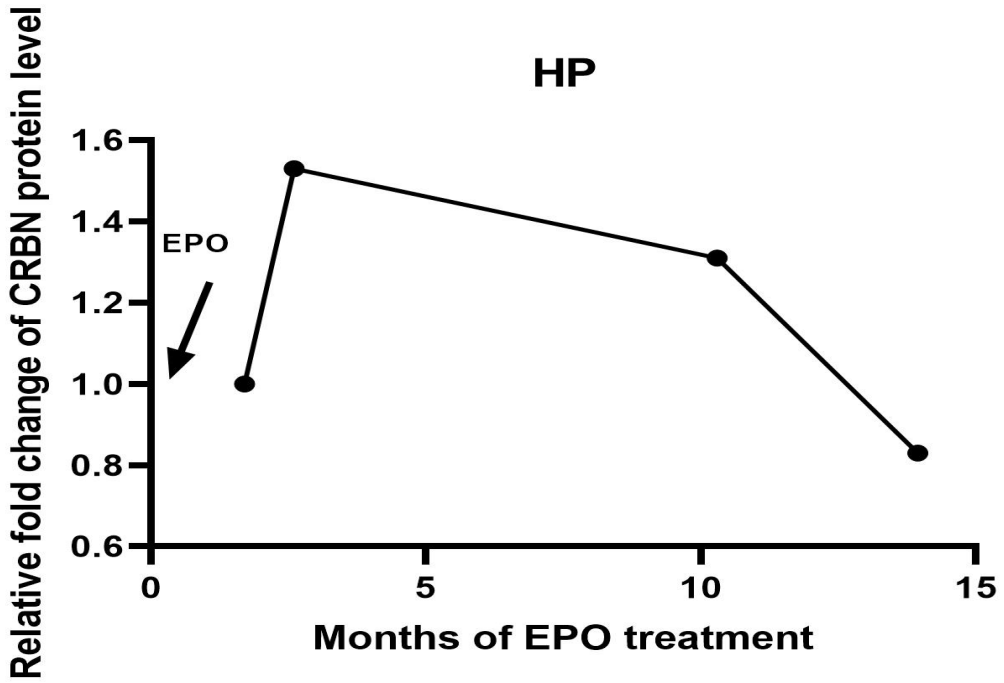
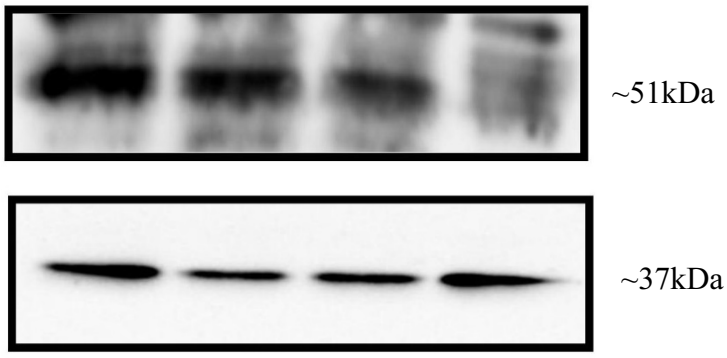


Fig.30: This figure illustrates the comparison of Nr2 and HO 1 proteins levels of previous Fig.29. Blue color presents HO 1 protein expression.

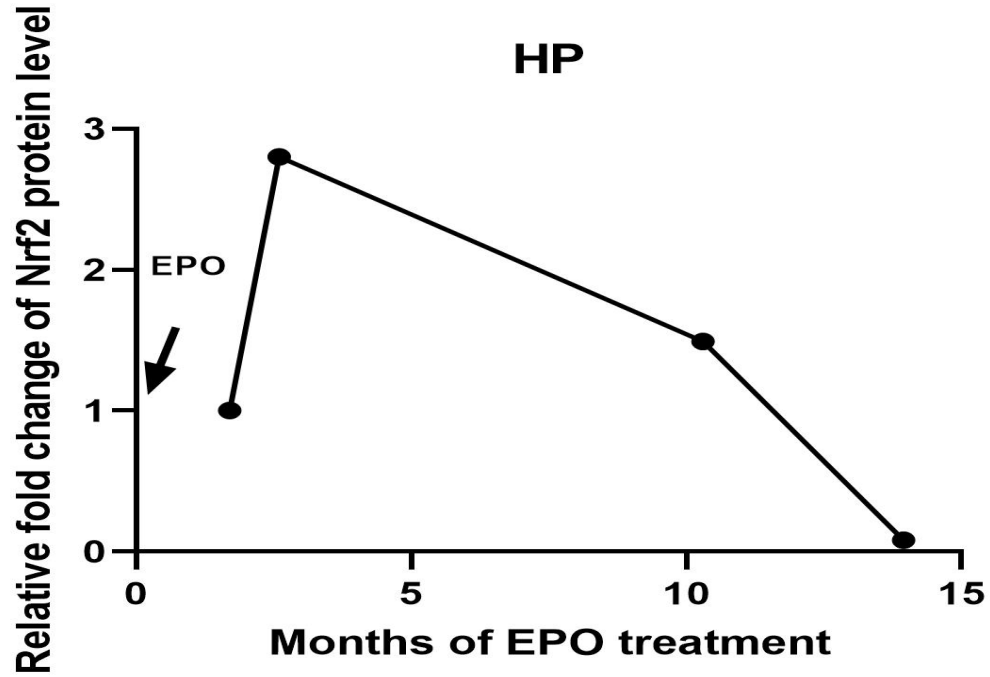
A.)

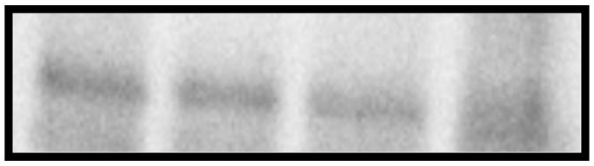

HP
Relative fold change of CRBN protein level
1.6
1.4
1.2
1.0
0.8
0.6
EPO
0
5
10
15
Months of EPO treatment

B.)


~51kDa
~37kDa

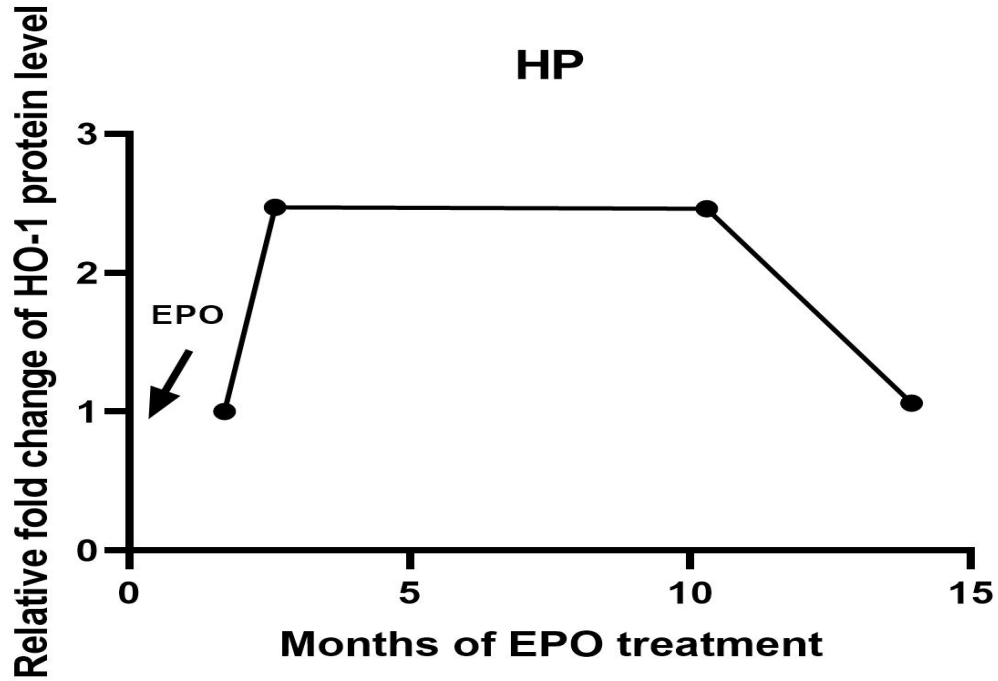
C.)

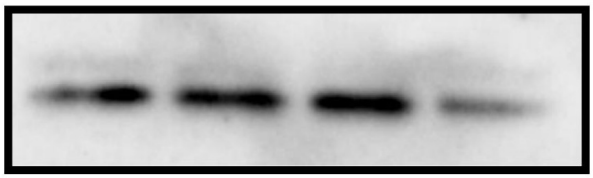

HP
Relative fold change of Nrf2 protein level
EPO
0
1
2
3
0
5
10
15
Months of EPO treatment



~62kDa

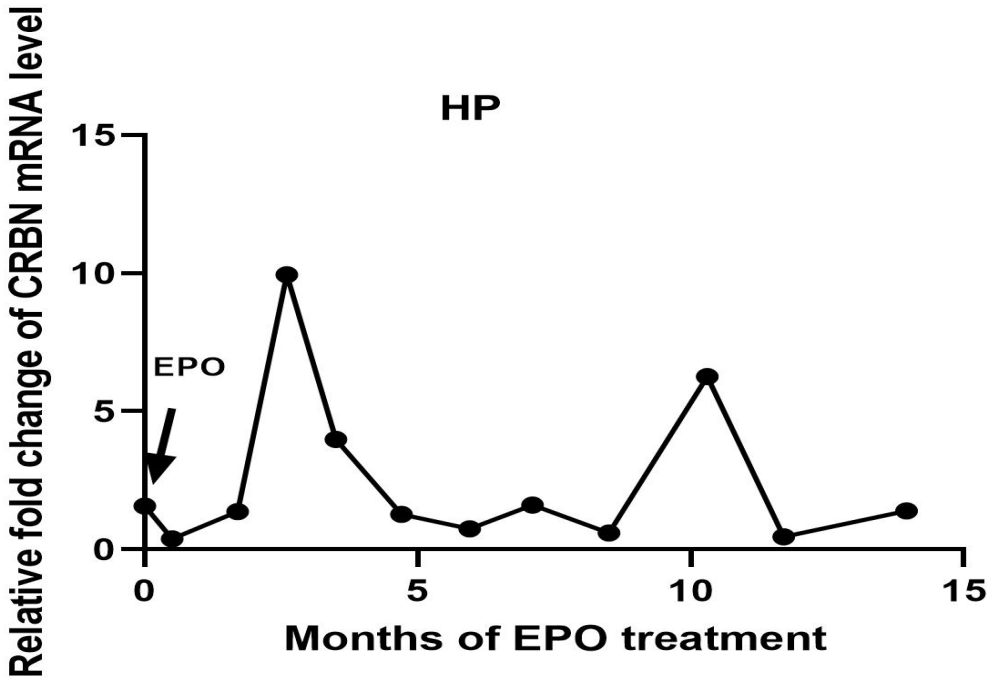
D.)


HP
Relative fold change of HO-1 protein level
EPO
0
1
2
3
0
5
10
15
Months of EPO treatment



~33kDa

E.)


HP
Relative fold change of CRBN mRNA level
15
10
5
0
EPO
0
5
10
15
Months of EPO treatment

F.)

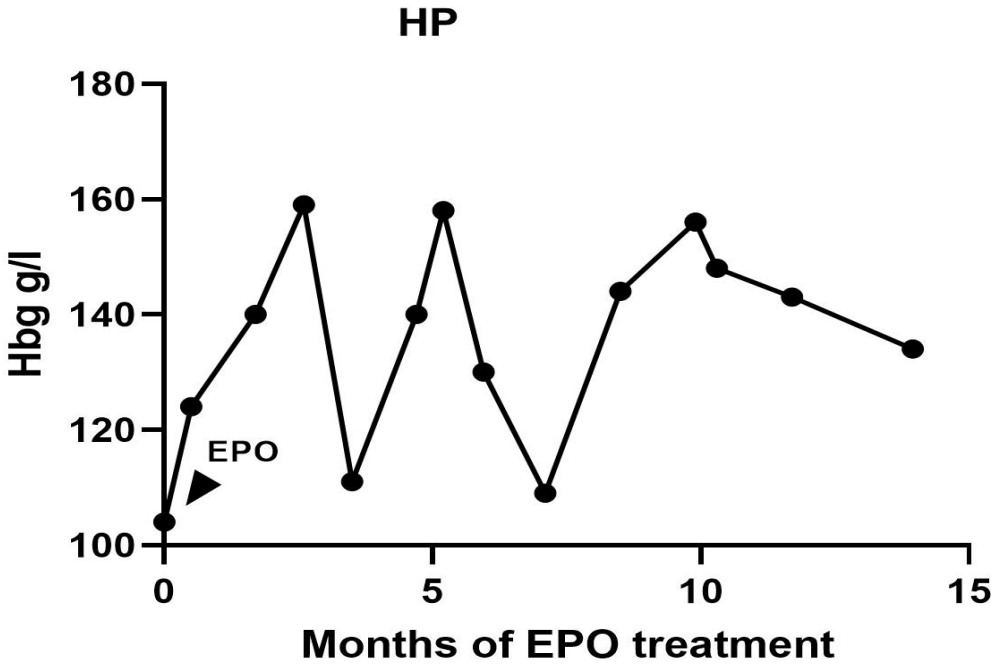

HP
Hbg g/l
180
160
140
120
100
EPO
0
5
10
15
Months of EPO treatment

Fig.31: The female patient with normal karyotype on EPO treatment. Fig. A and B mean CRBN protein expression during EPO therapy. The first picture is CRBN protein with a weight of ~51kDa. The second picture is GAPDH with a weight of ~37kDa. Fig.C illustrates the level of Nrf2 protein (~62kDa), and Fig.D shows HO 1 protein expression (~33kDa). Fig.E is CRBN mRNA expression, and Fig.F is the level of hemoglobin. Fig. C and D are verification of our results.

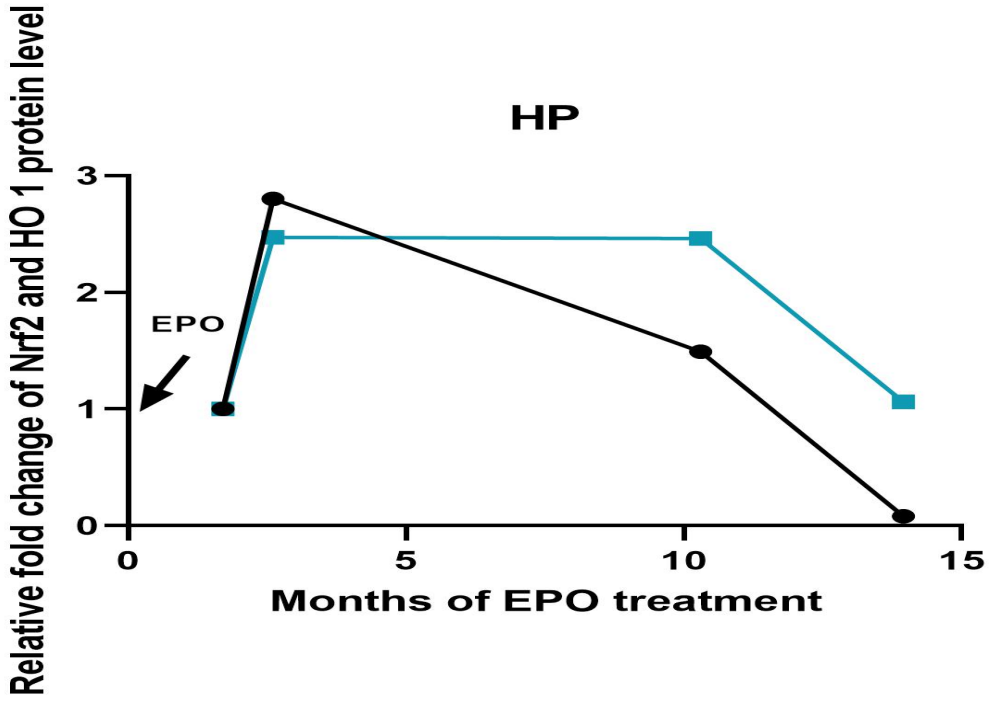


Fig.32: This figure illustrates the comparison of Nr2 and HO 1 proteins level of previous Fig.31. Blue color presents HO 1 protein expression.

4.6. The effect of arsenic trioxide with LEN + EPO +PRED combination in MDS-L and SKM-1 cells

We read about the efficiency of arsenic trioxide in high-risk MDS and promyelocytic leukemia. Our goal was to find out if arsenic trioxide can be used as a treatment in low-risk MDS associated with LEN, EPO, PRED, or in a combination of these agents. For our research, we used SKM-1 and MDS-L cell lines. We created 19 groups, as seen in the picture.

The experiments were repeated three times. At first, we measured the level of CRBN mRNA. ATO, LEN, EPO, PRED alone or their combinations had overexpressed compared to the control group (without treatment). Unexpectedly, we did not expect that

group LEN+EPO be lower level than control or other groups. We found out significant results in combinations of LEN+EPO+ATO, PRED+EPO+ATO, and PRED+EPO+LEN+ ATO.

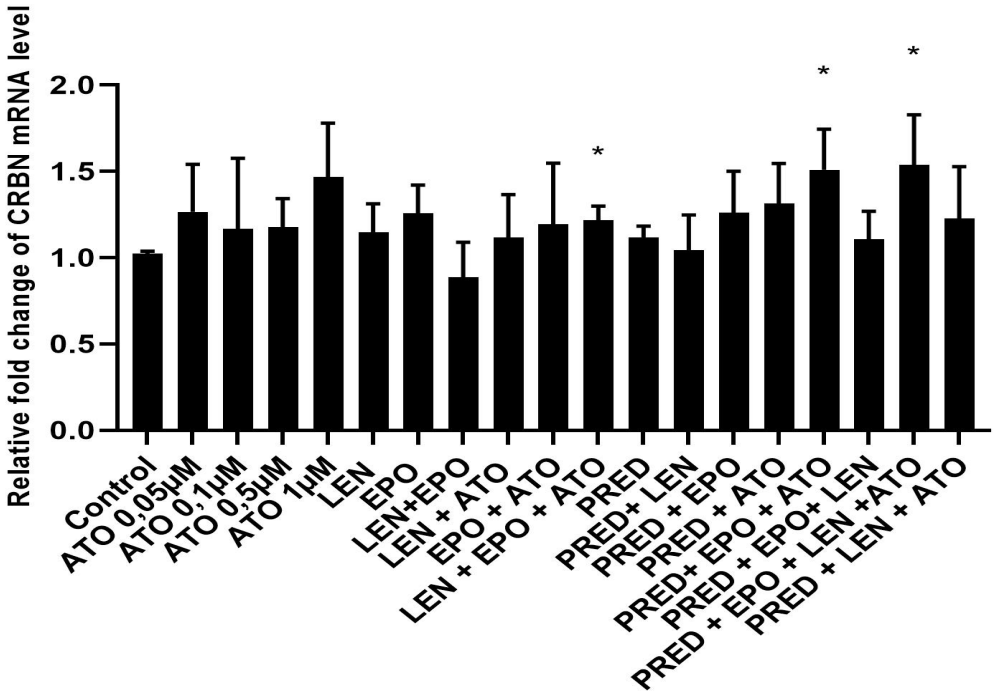


Fig.33: The picture illustrated the level of CRBN mRNA in triplicates of MDS-L cells.

In Nrf2 mRNA were similar results. The level of Nrf2 mRNA was increased in ATO alone or between our chosen groups. Moreover, we observed the same level of Nrf2 mRNA in 0,1μM ATO in contrast with control which was significant and PRED+EPO+ATO. In conclusion, significant results were found in 0,05μM ATO, 0,1μM ATO, and PRED+EPO+LEN+ATO. The same Nrf2 mRNA expression as in control is in group PRED+EPO+ATO.

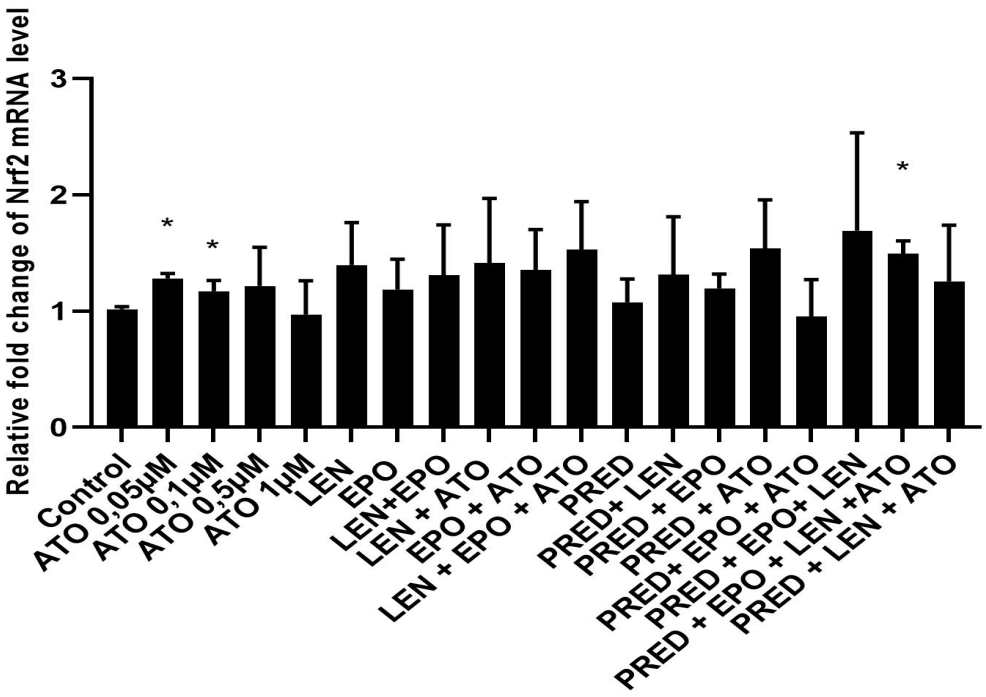


Fig.34: The picture illustrated the level of Nrf2 mRNA in triplicates of MDS-L cells.

The article of Murata [Murata et al., 2015] reported that Nrf2 regulates PINK1 under oxidative stress. We were interested in whether Nrf2 can regulate PINK1 in hematology cases. Our results did not confirm this hypothesis in MDS-L cells. The outcomes from the analyzed samples were different. Significant results we obtained only in underregulated PRED in comparison to the control sample. 1μM ATO was also underregulated. The rest of the groups were upregulated compared with control or were at the same level as the control (EPO, PRED+LEN, PRED+EPO+ATO, PRED+EPO+LEN+ATO).

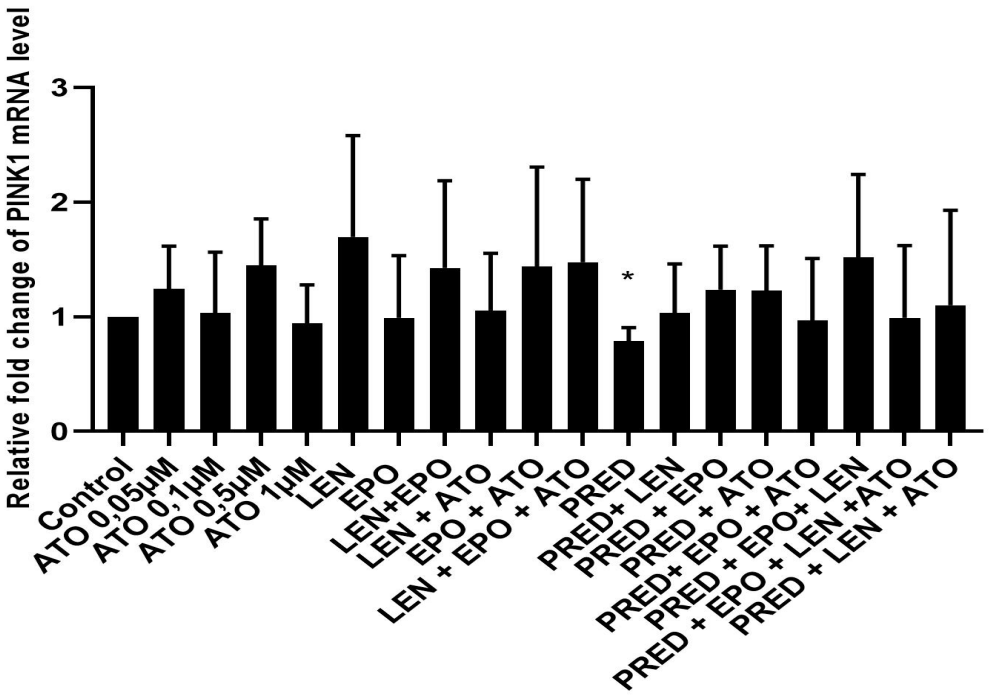
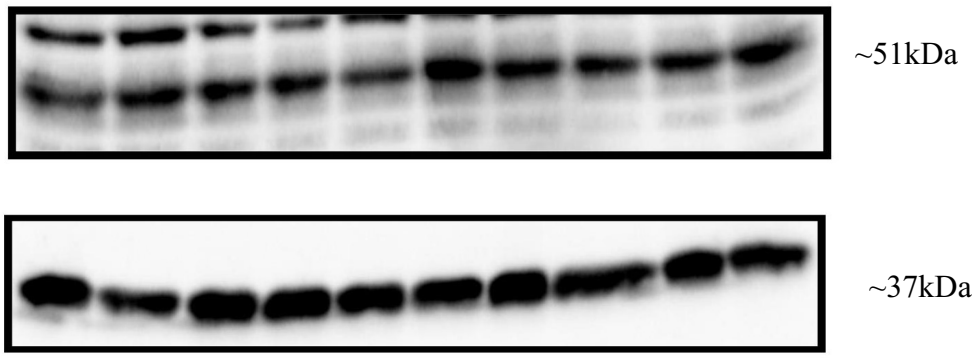


Fig.35: The picture illustrated the level of PINK1 mRNA in triplicates of MDS-L cells.

We measured CRBN protein expression in MDS-L cells. Moreover, our protein results did not correlate with CRBN mRNA. Almost every group had a higher level of expression than the control group. Only 0,05μM ATO, 0,1μM ATO, PRED+EPO, and PRED+ATO were underregulated, thereof cohort of PRED+EPO was significant. Another significant result was found in cohorts of LEN and EPO+ATO which were a higher level of CRBN protein than control.

A.)



B.)

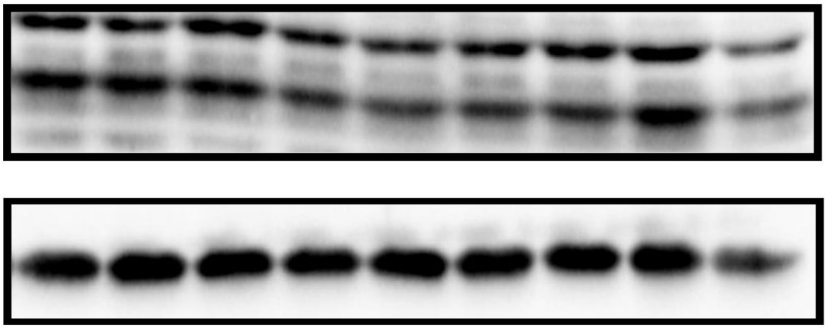


Fig.36: Figures A and B mean the same order of ATO, LEN, EPO, and PRED in MDS-L as set out in the graph in the graph form. The first and third picture is CRBN protein with a weight of ~51kDa. The second and fourth picture is GAPDH with a weight of ~37kDa.

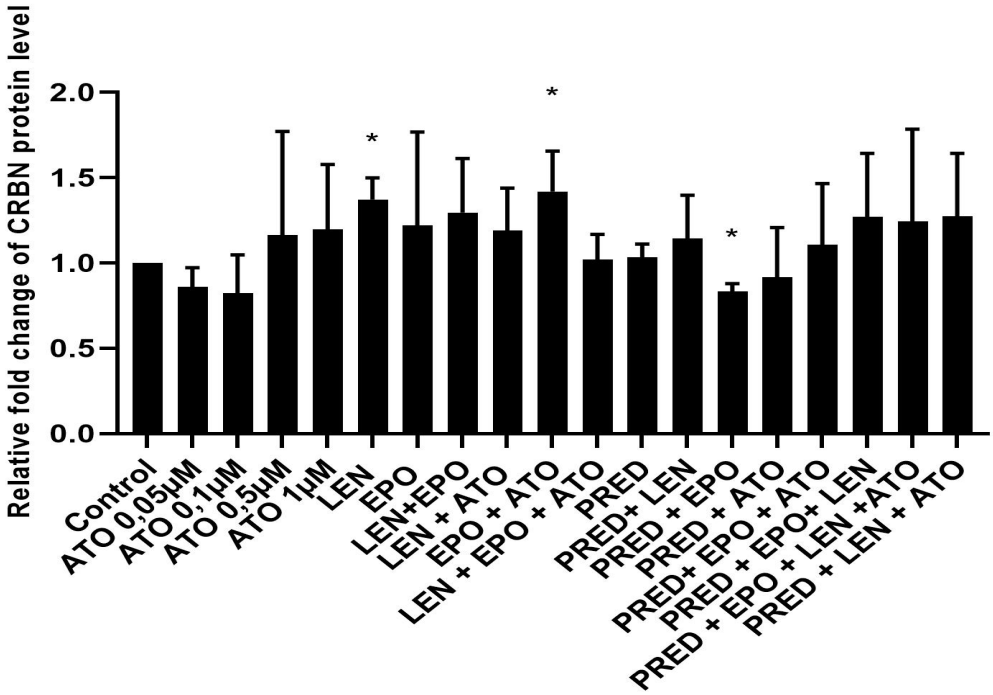


Fig.37: CRBN protein expression in MDS-L cell culture.

We also measured CRBN, Nrf2, and PINK1 mRNA expression in SKM-1 cell lines. We expected the lower CRBN mRNA expression in SKM-1 cell lines because of our experience with patients with normal karyotype. We found significant results in CRBN mRNA in combination with LEN+EPO, LEN+PRED, PRED+EPO+ATO, and

PRED+EPO+LEN that were expected underexpression in comparison to the control cohort. Interestingly, 0,5μM ATO, 1μM ATO, and PRED+LEN+ATO had overexpressed in comparison with the control group.

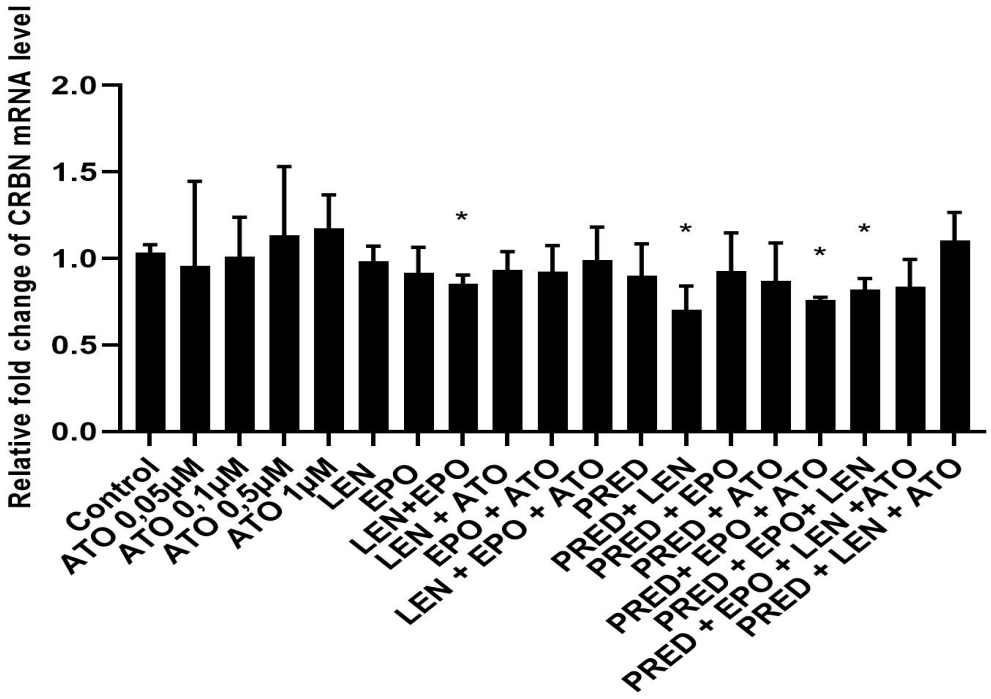


Fig.38: The picture illustrated the level of CRBN mRNA in triplicates of SKM-1 cells.

We analyzed Nrf2 mRNA in SKM-1 cells, too. In comparison with CRBN mRNA, the results were quite different. Upregulated were cohorts LEN, EPO, LEN+EPO, LEN+EPO+ATO, PRED, PRED+LEN, PRED+EPO+ATO, PRED+EPO+LEN+ATO, and LEN+EPO+ATO. Unfortunately, the significant result was in underexpressed 0,1μM ATO.

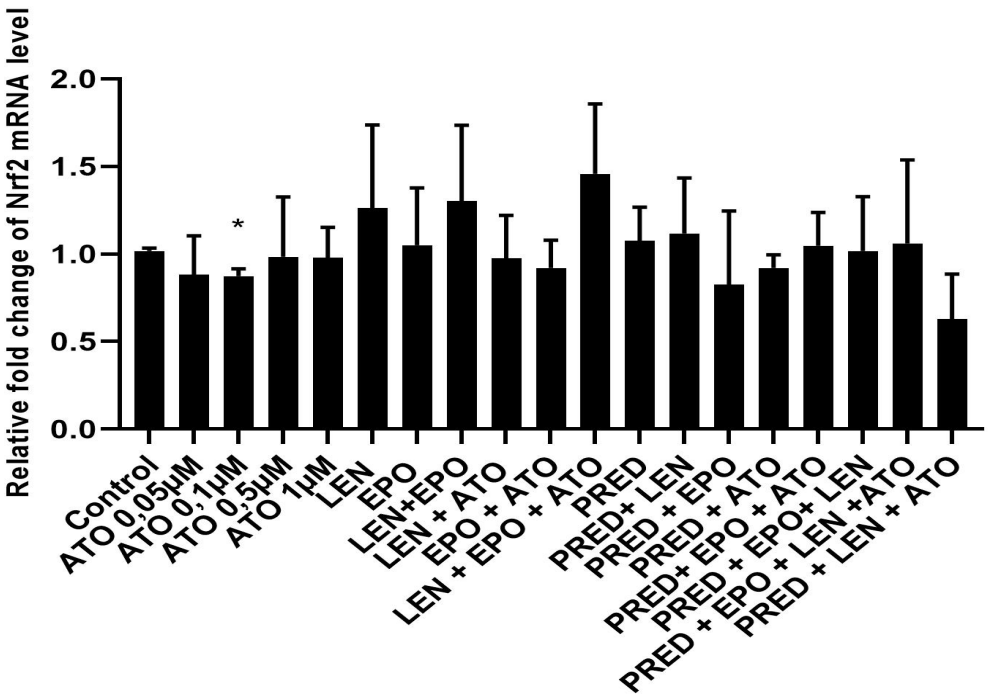


Fig.39: The picture illustrated the level of Nrf2 mRNA in triplicates of SKM-1 cells.

In measured PINK1 mRNA were overregulated only groups of 0,5μM ATO, 1μM ATO, LEN, and PRED+EPO+ATO. Furthermore, the significant result was in underregulated LEN+EPO, LEN+EPO+ATO, and PRED+EPO+LEN. According to the outcomes, we cannot confirm the correlation between Nrf2 mRNA and PINK1 mRNA. In this way, we did not confirm that Nrf2 regulates PINK1 in myelodysplastic syndrome.

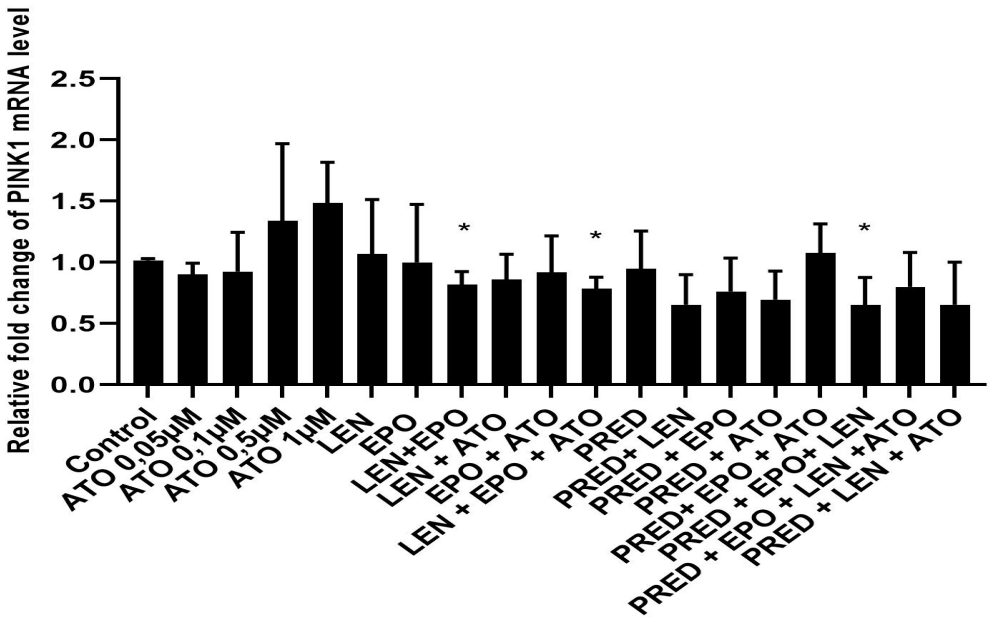
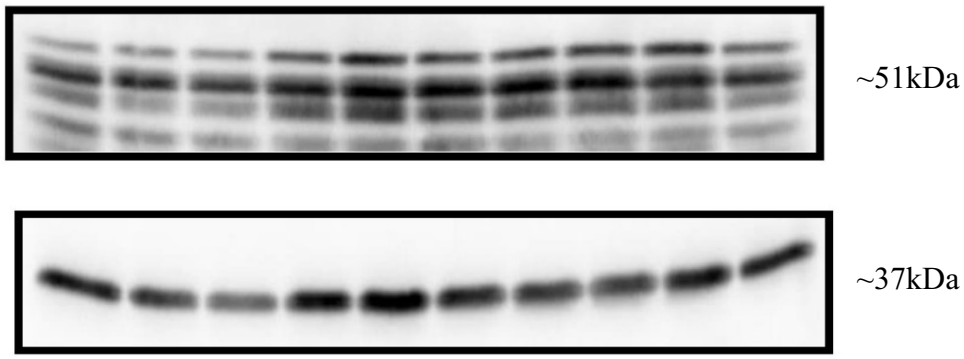


Fig.40: The picture illustrated the level of PINK1 mRNA in triplicates of SKM-1 cells.

We measured CRBN protein expression in SKM-1 cells. Our protein results did not correlate with CRBN mRNA. The group of 0,05μM ATO and LEN+EPO had the same level of CRBN protein as a control. The only group of LEN+ATO and EPO+ATO was up-regulated. We found significant results in the underexpressed group of 0,5μM ATO, PRED, PRED+LEN, PRED+EPO, PRED+LEN+ATO. Interestingly, we found out that PRED+LEN is significant in both CRBN mRNA and protein CRBN.

A.)



B.)

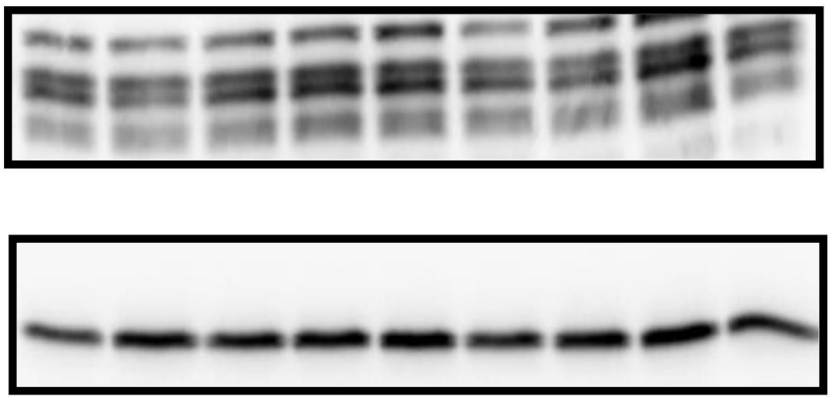


Fig.41: Figures A and B mean the same order of ATO, LEN, EPO, and PRED in MDS-L as set out in the graph in the graph form. The first and third picture is CRBN protein with a weight of ~51kDa. The second and fourth picture is GAPDH with a weight of ~37kDa.

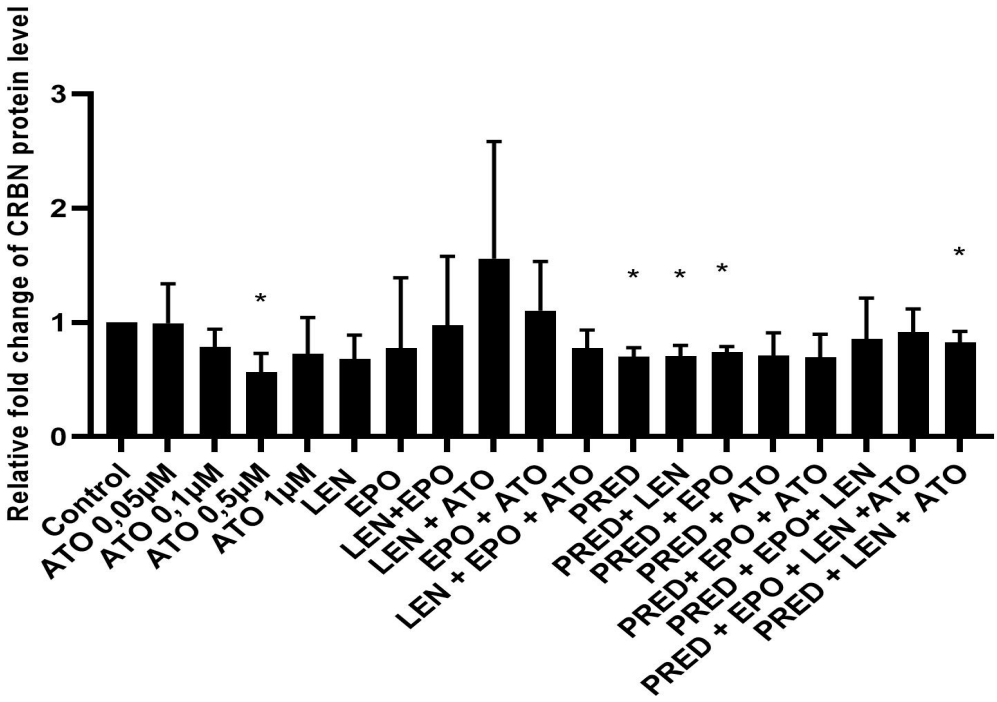


Fig.42: CRBN protein expression in SKM-1 cell culture.

5. DISCUSSION

Anemia is characterized by a lower level of hemoglobin (below 100 g/L) and it is typical for low-risk myelodysplastic syndrome. The first line of treatment of anemic patients is the addition of erythropoiesis-stimulating agents (ESAs) that were approved by the U.S. Food and Drug Administration (FDA), showing to improve erythropoiesis. The most common use of ESAs is Erytropoietin, NeoRecormon (Epoietin beta), or Binocrit (Epoietin alfa).

The response of lower-risk MDS patients on ESA treatment is around 30-60%, with the median duration of response about 24 months. MDS patients who had failed therapy with ESA and became higher transfusion burden (more than 2 units a month) or had higher baseline serum EPO level (> 500 IU/ml) could be considered resistant to ESAs. MDS patients with del(5q) have significantly lower responses to EPO than MDS with normal karyotype [Patnaik et al.,2017; Kelaidi et al., 2008].

Second-line treatments after relapse or resistance to ESAs include immunomodulatory drugs (IMIDs). LEN acts directly on hematopoietic progenitor cells to increase erythropoiesis and selectively inhibit the growth of del(5q) erythroid progenitors in vivo, with no significant effect on the growth of normal CD34+ progenitors, or cytogenetically normal progenitors in MDS with del(5q) clones [Ebert et al.,2008; Duong et al., 2012]. In the Czech Republic is approved lenalidomide treatment in MDS cases with del(5q) or del(5q) with trisomy 8, not in patients with normal karyotype. Lenalidomide has an impressive efficacy in MDS with del(5)(q31-q34). Patients with this abnormality of karyotype achieve in 45% complete cytogenetic response and an even higher rate of hematologic response. However, non-5q- achieved lenalidomide response only in 26% [Tefferi et al.,2007; Ebert et al., 2008].

There is no biomarker, which can predict lenalidomide response and determine who should receive the drug and who not. On based multiple pieces of research in MM, scientists suggested that cereblon could be a prognostic factor in lenalidomide response [Heintel et al., 2013]. We used these investigations as a background to our examinations [Jonasova et al., 2015].

Cereblon is a substrate of the cullin-RING E3 ubiquitin ligase complex 4 (CRL4) consisting of cullin 4 (CUL4), DNA damage- binding protein 1 (DDB1), and ROC1 [Díaz-Rodríguez and Pandiella, 2016]. IMIDs do not inhibit the enzymatic activity of the $CRL4^{CRBN}$ but grant neomorphic activity on CRBN to mediate the selective ubiquitylation

and proteasomal degradation of CK1A1in MDS cells. Interestingly, CRBN mediates ubiquitylation and proteasomal degradation of IKZF1 and IKZF3 in human MM. Moreover, it was described various thalidomide analogs, but if these substrates interact with one another and if they influence IMIDs activity is unclear [Jan et al., 2021; Sperling et al., 2019].

We completely analyzed 119 patients with del(5q) and del(5q) with trisomy 8. The cohort of 104 patients was before or without LEN which was used for comparison of the level of CRBN mRNA. Fifteen patients with isolated del(5q) were not counted in this comparison because we obtained samples after LEN therapy. Of 119 analyzed samples, 83 used LEN treatment.

Gandhi and his colleagues identified various CRBN splice variants in MM and primary cells [Gandhi et al., 2014]. These results led us to standardize an assay for the measurement of CRBN gene expression. The best essay is TaqMan assay-Hs00372271_m1, which measures all CRBN mRNA variants translated to a functional protein. The variant without exon 10 failed to bind to LEN, which leads to IMIDs resistance [Gooding et al., 2021].

We also detected a splice variant in CRBN and confirmed deleted exon 8 and exon 10, or both. We did not detect other splice variants of CRBN mRNA. Thus, LEN is bound to CRBN at exon 10 and 11, we confirm the importance of the exon for LEN binding and its sensitivity in treatment.

We compared the level of CRBN in cohorts (5q-, del(5q) with trisomy 8, trisomy 8, non-5q- and healthy control) in PB and BM that were not treated by LEN. We expected and confirmed the higher level of CRBN mRNA in 5q- and del(5q) with trisomy 8 in PB. Slightly lower levels of CRBN mRNA were found in trisomy 8 in PB but higher levels compared to non-5q- and healthy controls. Trisomy 8 can be linked with autoimmune diseases and inflammatory in 15- 25% of cases with numerous rates in different MDS/MPN subtypes [Wesner et al., 2019]. These features can cause an increased level of CRBN mRNA. As we expected, the lower level of CRBN mRNA were in non- 5q- and healthy controls. The level of CRBN mRNA was different in comparison with PB. The higher level of CRBN mRNA was found out in del(5q) with trisomy 8 and trisomy 8, alone in comparison to 5q- syndrome. Moreover, the lowest level of CRBN remains in non- 5q- and healthy controls.

We analyzed the level of CRBN in PB and BM MDS patients with del(5q) and del(5q) included trisomy 8 before, during, and after LEN treatment, respective in the combination

of LEN+EPO, LEN+EPO+PRED. The level of CRBN was more significant in PBs because we received numerous samples of PBs than BMs. We have found, the high level of CRBN mRNA is connected to a good LEN response. The lower level of CRBN mRNA is linked to poor LEN response and progression low-risk to high-risk MDS (MDS-EB1 and MDS-EB2), or AML. The weak response to LEN is still described in connection with p53 mutations.

However, substrates interact and compete with one another that alternations in the expression of one substrate can affect the degradation of others, which can be influenced CRBN expression and the levels of alternative substrates. These data suggest that there can be another mediator or other mediators of thalidomide analog resistance, which include posttranslational modification of substrates and alternations in downstream targets of CSNK1A1, IKZF1, and IKZF3 [Sperling et al., 2019]. CRBN expression is once up and once down, which is seen in Figures (Chapter Results), and this action of substrates can explain our results in CRBN measurements. The main point of this work last that the level of CRBN is important before LEN, which predicts whole IMID treatment. Unfortunately, no material was available to examine *CRBN* gene expression in isolated $CD34^+$ cells.

Interestingly, mutations in MPN driver genes (JAK2, MPL, CALR-calreticulin) is rare in MDS with del(5q). If LEN is useful in 5q- syndrome with concomitant JAK2 mutations has never been properly examined. Musto et al. described in one case who demonstrated a great response to lenalidomide, where LEN improved anemia and thrombocytosis. Another research group supports the efficiency of LEN in 5q- syndrome and CALR mutations, with the disappearance of 5q clone and CALR mutation [Alshaban et al.,2018]. In 5q- patients are unique with myelofibrosis, according to data it is only 0,8% [Takahashi et al., 2015]. Teferi et al. reported three patients with del(5q) linked to primary or post-polycythemia vera/essential thrombocythemia myelofibrosis who had a positive experience with LEN. These three patients were JAK2 (V617F) positive. It seems that LEN may benefit patients with del(5q) and myelofibrosis when karyotype shows either 5q- syndrome or non-complex type [Tefferi et al., 2007; Takahashi et al., 2015]. We did not obtain samples from patients with del(5q) associated with myelofibrosis, thus we cannot collate our examinations or CRBN expression.

Various causes have reported in patients with RARS-T (ring sideroblast refractory anemia including thrombocytosis) who were treated with LEN and achieved the desired effect. MDS- RARS-T is typically associated with JAK2 (V617F) mutations, much less

commonly, the myeloproliferative leukemia virus oncogene (MPL) W515K/L mutation. In two causes of MDS-RARS-T with JAK2 (V617F) mutations were successfully treated by LEN, in one patient disappeared of disease clone (JAK2). Similar results in MDS/MPN-RS-T were described by Divoux and Keen. Divoux reminded that these examinations are needed to confirm, but because of the rarity of the disease, the trial is very difficult to perform [Huls et al., 2010; Divoux et al., 2020; Keen et al., 2016].

We obtained a few samples from the MDS cohort with normal karyotype thanks to the international clinical trial (NCT01029262). We analyzed seven patients with a diagnosis of MDS with refractory cytopenia with multilineage dysplasia (RCMD), in addition, one of the patients had myelofibrosis. Unfortunately, none of them responded to LEN treatment. Initial CRBN expression was lower, supporting our study that CRBN is also a biomarker for patients without 5q.

Outside of the clinical trial, there were four people whom SÚKL approved LEN treatment. Two of them were MDS-RARS-T, another sample was MDS-MLD (MDS with multiline dysplasia), who was at the time of writing the discussion at the beginning of therapy. One MDS-RARS-T was negative on JAK2 did respond partial, after adding EPO and then following PRED. After three combinations of LEN, the patient responded for a while and then stopped responding to treatment. Second MDS-RARS-T was positive on JAK2 mutation and did not respond on LEN from the beginning of treatment, even after the addition of EPO and PRED (information about JAK2 mutation were obtained from the report of patients). Both of them progress to MDS-EB-1. These two cases could not correlate with previous studies about the efficiency of LEN in MDS- RARS-T. The third patient did not also respond on LEN.

We cannot claim that CRBN mRNA can be a biomarker in non-5q- who are treated by LEN. We received a small amount of cohort of these patients. The main problem remains that a lot of these samples were obtained after the first dose of LEN.

The possible prognostic factor for non-5q- patients can be dual pyroptotic biomarkers (apoptosis-associated speck-like protein containing a CARD /ASC/ and erythropoietin) which can predict erythroid response to LEN and recombinant EPO. The study suggests that patients with greater activation of medullary inflammation could benefit more from LEN and EPO treatment. Results were provided by dual pyroptotic biomarkers. Importantly, LEN reduces S100A9 production in non-5q- patients and reduces the suppression of EPO elaboration by S100A9. In addition, it can stabilize erythropoietin

receptors by inhibiting RNF41. These results have to be confirmed in clinically applicable to recognize non-5q- patients with inflammasome activation [Wang et al., 2021].

It was mentioned above, numerous articles reported CRBN as the potential biomarker in MM patients. Unfortunately, there is a small amount of data that opponents these results. Qian and his group did not found out a difference in the overall response rate in pomalidomide therapy. Moreover, they observed a high level of CRBN is connected to the trend in longer progression-free survival and overall survival in MM []. Another study suggests CRBN has not been used as a predictive biomarker to IMIDs sensitivity in MM according to standard immunohistochemistry [Dimopoulos et al., 2019].

Every case is individual, so there is no timeline for prediction when LEN loses its effectiveness. It was shown that the combination of EPO and LEN can increase response in non-5q- MDS patients. LEN can improve EPO receptor signaling in MDS cells without del(5q) by inhibiting the protein tyrosine phosphate CD45, this way led to the reversal of CD45-induced inhibition of EPO/STAT5 signaling. Moreover, LEN stabilizes the erythropoietin receptor by inhibiting RNF41, promoting plasma membrane accumulation of signaling component JAK2 and the erythropoietin receptor complex to enhance response to EPO. [Toma et al., 2016; List et al., 2021]. After adding EPO to LEN, there is a possibility that the hematologic response to this therapy represents a delayed response to LEN. Patients on LEN therapy respond to treatment within 2-4 cycles, indicating the effect of the addition of epoetin alfa. [Komrokji et al., 2012]. Also, MDS with del(5q) response to the combination of LEN+EPO where response rates exceed 70% and the median is greater than 2 years [Kelaidi et al., 2007]. S100A9 directly suppresses erythropoietin transcription and elaboration in HepG2 hepatoma cells and induces the secretion of TNF alpha, IL-6, IL-8 (interleukin 8), IL-1 beta through TLR4-dependent activation of NF-kappa B. These inflammatory cytokines also suppress erythropoietin production in low-risk MDS. Moreover, LEN suppresses the nuclear transport of NF-kappa B to mitigate the suppression of erythropoietin production in hepatoma cells by both S100A9 and TNF-alpha. Furthermore, LEN modulates activation of cytokines may reduce cell injury, but may also relieve repression of renal erythropoietin and the endocrine response to anemia in MDS [Cluzeau et al., 2017]. The combination of thalidomide and prednisone has a little higher response than thalidomide alone and prolongs response in primary fibrosis and anemia with or without transfusion dependence [Luo et al., 2018]. The effect of the combination of LEN + EPO + PRED was described by Jonasova in low-risk MDS [Jonasova et al., 2018].

Sardnal and his group described that the A/G polymorphism located at 29 nucleotides of the 5´untranslated region (chromosome 3 in position 3179746; NC_000003.12; Homo sapiens, GRCh38) has a prognostic significance in non-5q- MDS patients and LEN sensitivity. On this basis, we analyzed the A/G polymorphism in 5q- patients. We detected that allele A (81.5%) is predominant compared with allele G (18.5%) in MDS patients with 5q- syndrome. We obtained a slightly higher percent in samples of 5q- syndrome, which were LEN responders (87.5%) against allele G (12.5%). In contrast, non-5q- patients with no LEN respond had allele A in 85.7% of cases and allele G in 14.3%. We analyzed 75% allele A and 25% allele G in healthy controls. Moreover, we did not confirm that the A/G polymorphism has the prognostic factor in our group of MDS patients. In addition, Toma et al. confirmed G polymorphism in the 5' untranslated region of *CRBN* gene as the biomarker for erythroid response. This possible impact of CRBN (rs1672753) to LEN response with or without EPO in MDS has not to be identified [Toma et al., 2016].

Gandhi pointed out that the level of CRBN mRNA and protein is different. Interestingly, a dual-color immunohistochemistry assay also did not observe any correlation between CRBN mRNA and protein levels. They used a highly specific CRBN65 antibody with an anti-CD 138 antibody in MM patients [Ren et al., 2016]. It seems that commercially available CRBN antibodies are non-specific. So far the best antibody is from Celgene. Unfortunately, we did not obtain this antibody for our research, and it's not commercially available. Chang and his colleagues from Mayo clinic have developed three specific monoclonal antibodies against human CRBN and determined their epitopes. These antibodies can be used for western blott, immunoprecipitation, and immunohistochemistry. Their results showed highly specific antibodies against human CRBN 50- 63 peptide (2B11G10's epitope) or 62-75 peptide (2f11G5 or 4B9D3's epitope). They indicated that these antibodies can be used to confirm the relationship between CRBN expression and IMIDs sensitivity [Chang et al., 2017]. As we know, these antibodies are not commercially available too and we were also unsuccessful in receiving them. We used commercially available mouse polyclonal antibody for the determination of CRBN protein. According to a ladder, we determined CRBN, and we confirmed that CRBN protein predicts a quicker response to LEN therapy. The main problem remains in the amount of protein in a small patient´s sample, which may not be enough to determine CRBN levels. In this way, we suggest relying on the determination of CRBN mRNA that

shows prognostic signature too. Advantages of this selection are quicker results, more sensitivity, and a less expensive method.

Nrf2 is not fully examined in the hematology field especially in Hodgkin lymphoma, non-Hodgkin lymphoma, multiple myeloma, and myelodysplastic syndrome. However, some studies show the effectiveness of Nrf2 regulating survival of cancer cells in AML, chronic myeloid, and lymphocytic leukemia [Rushworth and MacEwan, 2011]. Nrf2 as a transcription factor mediates the anti-oxidative response of enzymes and regulates cytoprotective genes as HO 1, NAD(P)H: quinone oxidoreductase 1 (NQO1), and glutathione S- transferase. Also, Nrf2 can be responsible for chemoresistance and protect normal cells from transforming into cancer cells, but also promotes survival of cancer cells in a deleterious environment [Zhang et al., 2012]. In addition, Nrf2 mediated oxidative stress response pathway is linked with tumor resistance to arsenic-induced cytotoxicity [Liu et al., 2010]. EPO upregulated HO 1 expression in a rat model of renal injury and SH-SY5Y cell lines. In the same cell line, EPO induced the nuclear transcription of Nrf2, and it seems to mediate the cytoprotective responses against oxidative stress [Jin et al., 2011].

We monitored MDS non-5q- patients on EPO treatment and patients with del(5q) alone or in combination del(5q) and trisomy 8 during EPO or LEN+EPO therapy. In our analysis, we confirm our prediction, in 70.27%, the level of Nrf2 mRNA follows or overtakes the level of CRBN mRNA. In cases (the cohort of 5q- syndrome and del(5q) with trisomy 8 when patients stop to respond to LEN+EPO therapy, Nrf2 mRNA does not correspond with CRBN mRNA expression. We analyzed Nrf2 mRNA expression in MDS/MPD neoplasm with RARS-T. This patient was treated by a combination of LEN+EPO+PRED. We measured Nrf2 mRNA expression only in this one sample. Out of 14 people on EPO treatment, we registered only two cases (14,29%) when Nrf2 mRNA expression did not follow CRBN mRNA expression. 5q- syndrome and del(5q) with trisomy 8 group on EPO respond to our prediction in 66,67%, and a cohort of LEN+EPO responded to 71,43% cases. Furthermore, these results have to be confirmed to bigger groups than we obtained. Our results confirm that Nrf2 is bound to CRBN promotor because of Nrf2 mRNA following or forgoing CRBN mRNA. Our outcomes showed the importance of Nrf2. We analyzed Nrf2 protein and HO 1 protein in chosen patients with isolated del(5q) and del(5q) with trisomy 8. We found out that the level of Nrf2 protein is higher than of HO 1 in patients who did not respond to LEN+EPO, LEN+EPO+PRED therapy. On the contrary, patients with a lower level of Nrf2 protein and a higher level of

HO 1 protein respond to treatment. It can suggest that successfully treated patients with LEN+EPO or LEN+EPO+PRED are protected against oxidative stress by activating Nrf2/HO 1 pathway. These results correlated with known knowledge that the level of Nrf2 increases the level of HO 1. We cannot conclude from these results because it is not enough analyzed samples. We can only suggest it works this way.

At this moment, there is no better model of MDS than human SKM-1 and MDS-L cell lines, unfortunately, both of these cells have a complex karyotype. The best model for MDS appears human mice for examining LEN sensitivity or other IMIDs.

MDS-L cell line was established from MDS92 and used to study the mechanism of LEN because cells have del(5q). This cell line exhibits two major clones, MDS-L-2007 needing a higher concentration of IL-3 and in 82% was observed del(5q), whereas MDS-LGF cell line can proliferate without IL-3, del(5q) was observed below cut off. Furthermore, both clones have several mutations in NRAS, CEBPA, TP53, and TET2. The differences between these clones are MDS-L-2007 cells overexpressed CCL2 and CD7 in contrast to MDS-LGF that can be used as a model in high-risk MDS [Shafiee et al., 2021 and Kida et al.,2018].

We worked with MDS-L-2007 in the belief that contained del(5q). Unfortunately, we received information from Dr. Dostalova- Merkerova that our cell culture has been transformed by karyotyping and fluorescence in situ hybridization (FISH). The FISH probe XL 5q31/q33/5p confirmed isochromosome i(5)(p10) and derivate chromosome 4 that rise by translocation of the long arm of chromosome 5 (covering area 5q31 and 5q33) to the short arm of chromosome 4. According to multi-color fluorescence in situ hybridization, the presence karyotype of our MDS-L is 49,XY,der(4)t(4;5)(p?16;q11.2),i(5)(p10),der(6)(p?),-7,+8,i(8)(q10), der(10)t(7;10)(q11.2;p11.2),der(12)t(12;14)(p10;q10),der(13)t(7;13)(p12;p11.2),-14, i(15)(q10),+18,der(18)t(1;18)(q?12;q?11.2),+19,der(19)t(6;19)(?;p13),+20,+21,der(21) t(15;21)(q?;p12)x2,i(22)(q10),der(22)t(11;22)(?;p12).

ATO is used in the treatment of promyelocytic leukemia, MM, MDS, and non-Hodgkin lymphoma [Dawood et al., 2018, Xu et al., 2014]. ATO targets the mitochondrial function decreasing the mitochondrial membrane potential through multiple specific targets as Bcl-2 and permeability transition pore complex releasing cytochrome C, which activates the caspase. Another result is the increased release of reactive oxygen species (ROS) from mitochondria [Li and Sun, 2015]. However, the

clinical efficacy of ATO in MM is low, but other studies suggest a promising effect of ATO in combination with other agents [Rölling and Illmer, 2009].

The combinations of LEN and ATO were also investigated in primary effusion lymphoma (PEL). These combinations decreased organ infiltration and enhanced survival. Interestingly, the low doses of ATO sensitized MM cells to LEN through upregulation of CRBN expression resulted in apoptosis. It also confirmed that combination therapy induced the increased percentage of cells in the G0/G1 phase and decrease in proliferative phases in S and G2/M, which can increase inhibition of MM cells viability of this combination. Furthermore, ATO can downregulate Cdc25 enhancing LEN's cytostatic effects and also can be linked to the induction of TRAIL. ATO alone does not modulate IL-6 but helps LEN's inhibitory effects on IL-6. [Moodad et al., 2020; Jian et al., 2017; Wang et al., 2013]. ATO in combination with prednisone, ifosfamide, and ascorbic acid can be used as a treatment in relapsed MM patients, originally treated by LEN or other new agents. MM patients with minimal response can achieve a better response by increasing ATO [Li et al., 2015].

ATO resistance in a lung cancer line (A549R) was mediated through miRNA 155, whereas suppress cellular apoptotic activities and upregulated the expression of antioxidants NQO1 and HO 1 via activation of the Nrf2 signaling pathway. This way was A549R cells were protected from ATO- induced apoptotic cell death [Gu et al., 2017]. Some studies showed that silencing Nrf2 did not affect ATO-mediated apoptosis. ATO directly active Keap1 and among its cysteine residues (Cys273 and Cys288) are important in the suppression of Nrf2 by Keap1. Furthermore, Cys 151 is important in ATO responsiveness. There are hypotheses that ATO binds different sets of cysteine residues of Keap1 to regulate function in the Nrf2 signaling pathway [Zhang et al., 2012].

We prepared 19 groups (ATO, LEN, EPO, PRED alone or its combination) in MDS-L and SKM-1 cell lines, where we monitored CRBN mRNA and protein. We found out that almost every group of the experiment was overexpressed in CRBN mRNA in MDS-L line. The only group of LEN+EPO was downregulated. Moreover, the significant results were LEN+EPO+ATO, PRED+EPO+ATO, and PRED+EPO+LEN+ATO. CRBN protein expression had slightly different results than CRBN mRNA. Interestingly, 0,05μM ATO, 0,1μM ATO, PRED+EPO, and PRED+ATO had a lower CRBN protein expression than other groups, where PRED+EPO was significant. The significant results were observed in overexpressed groups of LEN and EPO+ATO. Unfortunately, we cannot compare our results with known or similar results which do not exist. Moreover,

we confirmed Dr. Tohyama, that MDS-L line is unstable and can change karyotype. We have a new abnormality in MDS-L line (translocation of the long arm of chromosome 5 to chromosome 4) with parallel loss of 5q deletion. We can make a hypothesis that the patients with this translocation would respond to LEN, EPO, PRED, ATO, and its combination, too. In SKM-1 cells, we obtained significant results in CRBN mRNA in combination with LEN+EPO, LEN+PRED, PRED+EPO+ATO, and PRED+EPO+LEN that were expected underexpression in comparison to the control cohort. Interestingly, 0,5μM ATO, 1μM ATO, and PRED+LEN+ATO had overexpressed in comparison with the control group. As we expected, CRBN protein expression was underregulated almost in every group in SKM-1 cells. Experimental outcomes suggest that LEN, EPO, PRED, ATO alone, or its combination are not suitable choices of agents in myelodysplastic syndrome with normal karyotype, alternatively without isolated del(5q). Obtained output correlate from SKM-1 cells correlate with our records from MDS- patients with normal karyotype with LEN or LEN+EPO, LEN+EPO+PRED.

The function of Nrf2 is a critical transcriptional up-regulator of the PINK1 (PTEN-induced putative kinase 1) gene in human neuroblastoma SH-SY5Y cells in response to various stresses. Upregulated PINK1 by Nrf2 has a protective effect on mitochondria and therein advantage for cell survival. Overexpressed Nrf2 stimulates metastatic potential and enhances the survival of cancer cells [Murata et al., 2015]. According to this publication, we measured in 19 groups PINK1 mRNA and compared with results Nrf2 mRNA. In MDS-L cell line, PINK1 mRNA was overexpressed in almost every group, only PRED was significantly underexpressed. When we compare results from the measurement of Nrf2 mRNA, outcomes were different. PRED was not underexpressed, contrariwise, the lower level was found out in groups of 0,1μM ATO (significant) and PRED+EPO+ATO. The higher level of significant results was obtained in groups 0,05μM ATO, 0,1μM ATO, and PRED+EPO+LEN+ ATO. However, in SKM-1 cell line, PINK1 mRNA was overexpressed in 0,5μM ATO, 1μM ATO, LEN, and PRED+EPO+ATO. The significant result was in underexpressed LEN+EPO, LEN+EPO+ATO, and PRED+EPO+LEN. Nrf2 mRNA was overexpressed in LEN, LEN+EPO, and LEN+EPO+ATO. Unfortunately, the significant result was in underexpressed 0,1μM ATO. These results indicate that Nrf2 mRNA does not regulate PINK1 mRNA in myelodysplastic syndrome.

6. CONCLUSIONS

1. We compared CRBN mRNA expression in MDS patients with isolated del(5q), del(5q) with trisomy 8, trisomy 8, normal karyotype, and heath control. We showed the higher level of CRBN mRNA is in samples with 5q- syndrome, del(5q) with trisomy 8 compared with other low-risk MDS and healthy control. We also correlated CRBN protein expression with the same groups. Interestingly, we found out almost the same level of expression in these groups. The cohort of 5q- syndrome had slightly reduced expression.
2. We confirmed the up-expression of CRBN mRNA and protein leads to a better response to LEN therapy. However, the underexpression CRBN mRNA and protein lead to failure LEN therapy and progress to MDS- EB-1, MDS- EB-2, or AML.
3. We proved that exons of 8 and 10 are important for LEN binding to CRBN. Without these exons, LEN does not bind to CRBN, and it leads to failure therapy.
4. We identified that A/G polymorphism is not a prognostic factor in MDS patients with isolated del(5q) and del(5q) with trisomy 8.
5. After LEN failure, it is added EPO or EPO+PRED to LEN. It is clear from our results that the addition of these drugs can help patients temporarily improve their therapy.
6. We have shown the importance of the role of Nrf2 in low-risk MDS. Nrf2 is bound to CRBN promotor, and thereby the level of Nrf2 mRNA follows or forgoes CRBN mRNA. We found out that for successfully treated patients is need to be activated Nrf2/HO 1 pathway. In unsuccessfully treated patients with LEN+EPO, or LEN+EPO+PRED is activated only Nrf2 pathway, HO 1 has a lower level of expression.
7. Finally, results of ATO show effectiveness in combination with other drugs in MDS-L cell line. Unfortunately, we cannot say that ATO and its combination will be working in patients with isolated del(5q) because of our translocation of the long arm of chromosome 5 to chromosome 4. ATO, PRED, LEN, EPO seem not to be suitable choices in the therapy of patients with normal karyotype/alternatively without isolated del(5q). Moreover, it seems that Nrf2 does not regulate PINK1 mRNA in our cell lines.

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8. LIST OF PUBLICATIONS IN WHICH AUTHOR PARTICIPATED

1. **High level of full-length cereblon mRNA in lower risk myelodysplastic syndrome with isolated 5q deletion is implicated in the efficacy of lenalidomide**

   Jonasova, A., **Bokorova, R.,** Polak, J., Vostry, M., Kostecka, A., Hajkova, H., Neuwirtova, R., Siskova, M., Sponerova, D., Cermak, J., Mikulenkova, D., Cervinek, L., Brezinova, J., Michalova, K., & Fuchs, O. (2015). European journal of haematology, 95(1), 27–34.

2. **Rationale of targeting protein cereblon as a potential strategy for cancer treatment**

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3. **Cereblon and Its Role in the Treatment of Multiple Myeloma by Lenalidomide or Pomalidomide**

   Fuchs, O., **Bokorová, R.,** Vostrý M., Kostečka, A., Polák, J. (2014) International Journal of Hematological Disorders,Vol. 1, No. 1A, 13-20

4. **Clonal Hematopoiesis as a Precursor of Myeloid Cancers**

   Fuchs, O. and **Bokorova, R.** (2020), Recent Advances in Hematology research: Hematologic Malignancies: An Overview, Copland, B., (editor), Nova Science Publishers, Inc., pp. 189-217, ISBN: 978-1-53618-124-1.

5. **Toll-Like Receptors Signaling Pathways in Hematologic Malignancies**

   Fuchs, O. and **Bokorova, R.** (2020), Recent Advances in Hematology research: Hematologic Malignancies: An Overview, Copland, B., (editor), Nova Science Publishers, Inc., pp. 263-299, ISBN: 978-1-53618-124-1.

6. **Targeted Protein Degradation Using Proteolysis Targeting Chimeras (PROTACs) in Hematologic Malignancies**

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7. **LENALIDOMIDE AS AN IMPORTANT DRUG IN HEMATOLOGIC MALIGNANCIES**

   Fuchs, O. and **Bokorova, R.,** (2019), Advances in Medicine and Biology. Volume 150. Ed. Berhardt L.V., Nova Science Publishers, Inc., pp. 35-89, ISBN: 978-1-53616-223-3.

8. **MOLECULAR MECHANISMS OF LENALIDOMIDE ACTION IN HEMATOLOGIC MALIGNANCIES**

   Fuchs, O. and **Bokorova, R.,** (2019), R. Advances in Medicine and Biology. Volume 150. Ed. Berhardt L.V., Nova Science Publishers, Inc., pp. 35-89, ISBN: 978-1-53616-223-3.

9. **Cereblon as the Cellular Target Responsible for the Anti-Multiple Myeloma Activity of Immunomodulatory Drugs (Thalidomide, Lenalidomide and Pomalidomide).**

   Bokorová, R., Polák, J., Vostrý, M., Kostečka, A., Fuchs, O., (2014), Multiple myeloma: risk factors, diagnosis and treatments, 81-100, New York: Nova Biomedical, ISBN 978-1-63321-514-6

10. **Preclinic Studies of PROTACs in Hematological Malignancies.**

    Fuchs O, **Bokorova R.** Cardiovasc Hematol Disord Drug Targets. 2021;21(1):7-22.

11. **Aberrantly elevated suprabasin in the bone marrow as a candidate biomarker of advanced disease state in myelodysplastic syndromes**

    Pribyl, M., Hubackova, S., Moudra, A., Vancurova, M., Polackova, H., Stopka, T., Jonasova, A., **Bokorova, R**., Fuchs, O., Stritesky, J., Salovska, B., Bartek, J., Hodný, Z., (2020). Molecular oncology, 14(10), 2403-2419

12. **Úloha signální dráhy transformačního růstového faktoru-β v hemopoéze a studie nových inhibitorů této dráhy pro léčbu nízkorizikového myelodysplastického** syndromu

    Fuchs O., **Bokorová R**., Transfuze a hematologie dnes, 2019; 25 (4): 310-318

13. **Cílený rozklad obtížně inhibovatelných proteinů způsobujících maligní hematologická onemocnění pomocí dvoufunkčních molekul využívajících polyubikvitinaci a rozklad**

    Fuchs O., **Bokorová R.,** Transfuze a hematologie dnes, 2020; 26(4):292–301.

9. CONTRIBUTION OF RADKA BOKOROVA TO PRESENTED PUBLICATIONS

Research paper #1:

Radka Bokorova as the first co-author contributed to the creation and finalization of the publication (35%). She sequenced splicing variants of the cereblon mRNA and analyzed A/G polymorphism. Also, she contributed to sample processing. On the first page of this publication is declared that both first authors identically contributed to this publication.

Review paper #2:

Radka Bokorova participated in the manuscript preparation and submission by sharing 40% input. She also prepared Fig.2.

Review paper #3:

Radka Bokorova as a co-author participated in the manuscript preparation and submission in 30%. She processed parts about proteins bindings to cereblon in multiple myeloma and regulation of its levels by lenalidomide and the possible role of mRNA levels for cereblon and cereblon as a biomarker for lenalidomide efficacy or resistance in multiple myeloma patients treated with lenalidomide or pomalidomide.

Chapter in book #4:

Radka Bokorova as a co-author participated in the manuscript preparation and submission in 5%. She contributed to the writing of the introduction.

Chapter in book #5:

Radka Bokorova as a co-author participated in the manuscript preparation and submission in 10%. She contributed to the writing of the TLR signaling pathway in myelodysplastic syndrome.

Chapter in book #6:

Radka Bokorova as a co-author participated in the manuscript preparation and submission in 5%. She contributed to the writing paragraphs about MDM2 and PROTACs.

Chapter in book #7:

Radka Bokorova participated in the manuscript preparation and submission in 25%. She contributed to the writing of paragraphs about del(5q) and lenalidomide treatment.

Chapter in book #8:

Radka Bokorova as a co-author participated in the manuscript preparation and submission in 30%. She contributed to the writing of paragraphs about lenalidomide and its mechanism of efficacy through cereblon.

Chapter in book #9:

Radka Bokorova participated in the manuscript preparation and submission in 90%.

Review paper #10:

Radka Bokorova as a co-author participated in the manuscript preparation and submission in 30%. She prepared pictures, a table, and part of PROTACs in casein kinase 1A1 and MDM2

Research paper #11:

Radka Bokorova as a co-author prepared samples that were used for analyses in this publication (5%).

Review paper #12:

Radka Bokorova helped with the manuscript preparation (20%). She prepared pictures.

Review paper #13:

Radka Bokorova helped with the manuscript preparation (15%). She prepared pictures.

10. PUBLICATIONS