

## ORIGINAL ARTICLE

# Oxidative and nitrosative stress in myeloproliferative neoplasms: the impact on the AKT/mTOR signaling pathway

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## Summary

**Purpose:** A common feature of malignancies is increased reactive oxygen species (ROS) and reactive nitrogen species (RNS). We analyzed the influence of oxidative and nitrosative stress on the activation of AKT/mTOR signaling pathway in myeloproliferative neoplasms (MPN).

**Methods:** Oxidative stress-induced gene expression in circulatory CD34<sup>+</sup> cells of MPN patients was studied by microarray analysis. Biomarkers of oxidative and nitrosative stress were determined using spectrophotometry in plasma and erythrocyte lysate. The levels of nitrotyrosine, inducible NO synthase (iNOS) and AKT/mTOR/p70S6K phosphorylation were determined by immunocytochemistry and immunoblotting in granulocytes of MPN patients.

**Results:** Antioxidants superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPx1) gene expression were increased in circulatory CD34<sup>+</sup> cells, while SOD1 and GPx enzymes were reduced in the erythrocytes of MPN. Plasma malonyl-dialdehyde and protein carbonyl levels were elevated in MPN. The total antioxidant capacity in plasma and

erythrocyte catalase (CT) activities was the most prominent in primary myelofibrosis (PMF) with JAK2V617F heterozygosity. The total nitrite/nitrate (NOx) level was augmented in the plasma of PMF patients ( $p < 0.001$ ), while nitrotyrosine and iNOS were generally increased in the granulocytes of MPN patients. Activation of AKT/mTOR signaling was the most significant in PMF ( $p < 0.01$ ), but demonstrated JAK2V617F dependence and consequent p70S6K phosphorylation in the granulocytes of essential thrombocythemia (ET) and polycythemia vera (PV) patients. Hydrogen peroxide stimulated mTOR pathway, iNOS and nitrotyrosine quantities, the last one prevented by the antioxidant *N*-acetyl-cysteine (NAC) in the granulocytes of MPN.

**Conclusion:** Our study showed increased levels of oxidative and nitrosative stress parameters in MPN with JAK2V617F dependence. The ROS enhanced the constitutive activation of AKT/mTOR signaling and nitrosative parameters in MPN.

**Key words:** AKT/mTOR signaling, myeloproliferative neoplasm, nitrosative stress, oxidative stress

## Introduction

MPN are diseases of hematopoietic stem cells characterized by clonal proliferation of the myeloid progenitors with increased quantity of mature cells [1]. The most common JAK2V617F mutation leads to a ligand-independent activation of downstream signaling pathways by constitutive phosphorylation resulting to uncontrolled cell proliferation and excessive production of ROS [2,3]. The

signaling pathways affected by JAK2V617F mutation include the Janus kinase - signal transducers and activators of transcription (JAK-STAT) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) [4]. One of the downstream targets of AKT is the mammalian target of rapamycin (mTOR) that serves as a central regulator of cell growth, proliferation and survival [5-7]. Therefore, AKT/mTOR

signaling is under the influence of constitutive activation of JAK-STAT pathway in MPN.

The JAK2V617F mutation induces accumulation of ROS in the hematopoietic stem cells, where ROS act as a mediator of oxidative stress and genomic instability [8,9]. Although previous reports showed an increased production of ROS in MPN, the role of nitric oxide (NO)-related parameters were not sufficiently revealed [10,11]. AKT/mTOR signaling pathway is potential target of both ROS and NO-related activity for malignant myeloproliferation [9,12]. Oxidative and nitrosative stress have mutagenic capacities for initiation and development of MPN pathogenesis, as well as activation of AKT/mTOR signaling.

We hypothesized that increased endogenous ROS and reactive nitrogen species (RNS) support the AKT/mTOR signaling activation, in parallel with reduced antioxidant properties, and impact myeloproliferation in MPN. In addition to oxidative stress-induced gene expression in circulatory CD34<sup>+</sup> cells, we analyzed biomarkers of oxidative and nitrosative stress in plasma, granulocytes and erythrocytes of MPN patients according to JAK2V617F allele burden. Further on, we examined the activation of AKT/mTOR signaling and its target S6K in granulocytes of MPN according to JAK2V617F allele burden. Using *in vitro* studies, we studied ROS activation of mTOR signaling and interference with NO-related activity.

## Methods

### *Plasma collection and separation of erythrocytes*

This study included 73 *de novo* MPN patients (24 cases of PV, 24 cases of ET and 25 cases of PMF) and 10 age-matched healthy controls. The median age of patients was 59 years (range: 40-75) at the time of diagnosis, while the median age of healthy donors was 54 years (range: 38-67). All patients had signed informed consent, approved by the Local Ethics Committee, in accordance with the Declaration of Helsinki. The diagnosis of MPN was based on the criteria of the World Health Organization from 2016. Blood samples of MPN patients were collected in tubes with ethylenediamine-tetraacetic acid (EDTA). The plasma was separated by centrifugation and hemolysate was obtained by mixing erythrocytes and cold distilled water. The supernatant was obtained by centrifugation and stored at -70°C until analysis.

### *Biochemical assays*

The ferric reducing ability/anti-oxidant power (FRAP) assay used anti-oxidants as reductants in a redox-linked colorimetric method [13]. Colorimetric method for determination of SOD1 activity was based on the superoxide-anion dependent autoxidation of adrenaline

in alkaline environment [14]. Biochemical analysis of CAT activity was based on the decomposition rate of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in the presence of the enzyme [15]. To determine GPx activity, an indirect method was used to monitor a decrease in the absorption of NADPH at 340 nm in the presence of glutathione reductase (GR). The spectrophotometric method for GR activity was based on the monitoring of NADPH oxidation in the reduction reaction of oxidized glutathione [16]. The hemoglobin concentration was determined spectrophotometrically after it was treated with Drapkin's solution [potassium ferricyanide (K<sub>3</sub>Fe(CN)<sub>6</sub>) and potassium cyanide (KCN)]. The activity of antioxidant enzymes in the erythrocytes was expressed in relation to the amount of hemoglobin (U/mg Hb).

Lipid peroxidation was estimated by a spectrophotometric assay based on the maximum absorption of the MDA complex with thiobarbituric acid, using tetrametoxyp propane as a standard [17]. Determination of protein carbonyl (PC) content in oxidatively modified plasma proteins was based on the method of Levin and colleagues [18]. The amount of PC was expressed in relation to the amount of total proteins in the sample.

The Griess method is a colorimetric method for the determination of nitrite concentration which is based on their reaction with Griess's reagent (2% sulfanilamide and 0.1% n-(1 naphthyl) Ethylenediamine dihydrochloride) to form purple AZO compound whose absorbance was measured spectrophotometrically [19]. This method was used in the present study to determine the concentration of nitrite and nitrate after their conversion into nitrites by vanadium chloride (VACL3).

### *Protein isolation and immunoblotting*

Granulocytes were isolated after separation of cell fractions by Lymphocyte Separation Medium (LSM, Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and hypotonic lysis of the precipitated erythrocytes. For protein isolation, granulocytes were lysed in chilled RIPA buffer (50mM Tris-HCl, pH 7.6, 150mM sodium chloride, 1% Triton x-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 2mM EDTA, 1mM DTT, 50 mM sodium fluoride) with inhibitor cocktail (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and sodium-orthovanadate. The protein aliquots were stored at -70°C until analysis. For Western blotting, equal amounts of protein samples were run on polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (AppliChem GmbH, Germany). Membranes were probed with primary antibodies to AKT, pAKT, p70S6K, p-p70S6K and ERK1 (Santa Cruz Biotechnology, Dallas, TX, USA). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-mouse immunoglobulin (Thermo Scientific Pierce, USA) were used as secondary antibodies. Western blots were developed using the enhanced chemiluminescence reagent system (Amersham, GE Healthcare, UK) according to the manufacturer's instructions and densitometric analysis of scanned immunoblot bands with Image Master Total Lab (GE Healthcare) software.

### Immunocytochemistry

The MPN and control granulocytes were resuspended in RPMI-1640 medium (Capricorn, Scientific, Germany) and the cell suspension ( $0.4 \times 10^6$  cell/ml) was used to obtain cytopins by centrifugation. Slides were fixed in acetone and stored at  $-20^\circ\text{C}$  until use. Before proceeding with immunocytochemistry, the microscopic slides were washed with phosphate buffered saline (PBS). Endogenous peroxidase was blocked with 3%  $\text{H}_2\text{O}_2$  solution. Incubation with primary antibody, 3-nitrotyrosine (RND, Minneapolis, MN, USA), iNOS, mTOR and pmTOR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) took place at  $+4^\circ\text{C}$ , overnight. After incubation with biotinized anti-rabbit immunoglobulins the cells were treated with streptavidin conjugated to the horseradish peroxidase (Ultraspec Detection System, HRP, ThermoScientific, UK). Finally, the slides were incubated in a solution of substrate-chromogen (Liquid DAB+Substrate Chromogen System, Dako, USA).

### Immunohistochemistry

Immunohistochemistry antigen detection by mTOR and pmTOR (Santa Cruz Biotechnology) antibodies in the MPN bone marrow sections had three more initial steps compared to immunocytochemistry: deparaffinization in xylol, rehydration through a series of alcohol and antigen retrieval. For this purpose, the tissue sections were immersed in citrate buffer (pH=6) and exposed to microwave radiation (560 W) for 21 min.

### In vitro study

To determine if ROS can alter the pmTOR/mTOR ratio we treated MPN granulocytes with 0.25 mM and 0.5 mM  $\text{H}_2\text{O}_2$  for 30 min. After isolation, the cells were resuspended in RPMI-1640 medium (Capricorn Scientific, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma Diagnostics, USA), L-glutamine and 1% PenStrep (100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin, Capricorn Scientific, Germany). The cells were incubated for 2 hrs at  $37^\circ\text{C}/5\%$   $\text{CO}_2$ . Three groups were pretreated with the antioxidant N-acetyl-cysteine (NAC) (Sigma Aldrich, USA) at a final concentration of 3mM, 30 min before adding the  $\text{H}_2\text{O}_2$ . After treatment, granulocytes suspension was used for preparation of cytopins.

### DNA sequencing

Genomic DNA was extracted from the granulocytes of MPN using the proteinase K and phenol-chloroform technique. Single nucleotide mutation JAK2V617F was characterized by DNA sequencing after PCR amplification performed with wild-type JAK2-specific forward primer 5'-TGGCAGAGAGAATTTCTGAACT-3' and reverse primer 5'-TTCATTGCTTTCTTTTCACA-3'. The PCR amplified samples were analyzed by sequencing on an automated ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA) with AB DNA Sequencing Analysis Software (v 5.2) by the Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit.

### Microarray analysis

The  $\text{CD}34^+$  cells were isolated from the collected mononuclear cells using a magnetic separation column (Super Macs II, Miltenyl Biotec, Bergisch Gladbach, Germany) and a mixture of magnetic microbeads conjugated with antibody against  $\text{CD}34$  (Miltenyl Biotec). In the microarray study, for determination of gene expression in  $\text{CD}34^+$  cells we used biological replicates of 9 healthy donors, 7 PV patients, 9 ET patients and 4 PMF patients. The human oligo probe set was from Operon Human Genome Array-Ready Oligo Set Version 4.0 (Eurofins MWG Operon, Huntsville, AL, USA) and the RNA samples were processed and analyzed as already described, while total human universal RNA (HuURNA, BD Biosciences, Palo Alto, CA, USA) served as a universal reference control in the competitive hybridization [20]. The microarray data were available from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE55976).

### Statistics

One way ANOVA and Dunnett's posttest were applied using Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as the mean  $\pm$  SEM, and differences at  $p < 0.05$  were accepted as the level of significance.

## Results

### Oxidative stress in myeloproliferative neoplasms

Using microarray analysis we studied the oxidative stress-induced gene expression in circulatory  $\text{CD}34^+$  cells of MPN patients. SOD2 gene expression was increased in PV and PMF cases (Table 1). GPX1 gene expression was significantly increased in ET, while peroxiredoxin 1/2 (PRDX1/2) was increased in PMF cases (Table 1). Increased SOD2 gene expression was more prominent in ET patients with no JAK2V617F (similarly to GPX1) and PV homozygous for JAK2V617F mutation (Table 2). PRDX2 gene expression was decreased in JAK2V617F heterozygous forms of ET and PV cases (Table 2). As clinical biomarkers for oxidative stress, the MDA and PC levels were significantly higher in the plasma of MPN than in healthy subjects (Table 3). SOD activity was decreased by 30% ( $p < 0.01$ ) and GPx activity by 14% ( $p < 0.05$ ) compared to controls in the lysate of ET erythrocytes (Table 3). GPx showed 20% weaker activity in PV patients compared to controls ( $p < 0.01$ ). In PMF patients, we detected significantly decreased activity of SOD ( $p < 0.05$ ). The erythrocytes activity of CAT was significantly increased in PMF compared to controls ( $p < 0.01$ ), as well as FRAP plasma values ( $p < 0.05$ , Table 3). There was a negative correlation between SOD activity in erythrocytes and the concentration of MDA ( $r = -0.406$ ,  $p < 0.01$ ) and PC

**Table 1.** Oxidative stress induced gene expression in myeloproliferative neoplasms

Groups		A -Control	B - ET	C - PV	D - PMF	BG
Genes	CD34 <sup>+</sup>	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
Superoxide dismutase 1	SOD1	-1.31±0.5	-1.64±0.39	-1.2±0.45	-0.79±0.47	
Superoxide dismutase 2	SOD2	1.79±0.49	3.33±0.43	3.02±0.71**	3.43±0.76**	A-C,D
Prostaglandin E synthase 3	PTGES3	-0.82±0.4	-0.48±0.88	0.01±0.65	0.26±0.55	
Glutathione peroxidase 1	GPX1	1.38±0.46	2.5±0.62**	1.84±0.57	2.31±0.54	A-B
Peroxiredoxin 1	PRDX1	-0.76±0.5	-0.91±0.33	-0.41±0.38	0.06±0.56**	B-D
Peroxiredoxin 2	PRDX2	-1.4±0.44	-2.03±0.39	-1.85±0.23	-1.32±0.46**	B-D

BG: between groups, Control: healthy subjects (n=9), ET: essential thrombocythemia (n=9), PV: polycythemia vera (n=7), PMF: primary myelofibrosis (n=4), SD: standard deviation. \*\*p<0.01.

**Table 2.** Oxidative stress induced gene expression in myeloproliferative neoplasms according to JAK2V617F allele burden

Groups	JAK2V617F	A -Control	B-ET no mut	C - ET htz	D - PV htz	E-PV homo
Genes	CD34 <sup>+</sup>	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Superoxide dismutase 1	SOD1	-1.31±0.5	-1.45±0.24	-1.76±0.42	-1.24±0.47	-1.04±0.35
Superoxide dismutase 2	SOD2	1.79±0.49	3.35±0.22**	3.32±0.52	2.72±0.14	3.3±0.9**
Prostaglandin E Synthase 3	PTGES3	-0.82±0.4	-0.06±0.32	-0.62±0.96	-2.78±0.49	-2.87±0.2*
Glutathione Peroxidase 1	GPX1	1.38±0.46	2.72±0.7 **	2.35±0.51	1.72±0.77	1.8±0.28
Peroxiredoxin 1	PRDX1	-0.76±0.5	-0.96±0.38	-0.89±0.29	-0.58±0.48	-0.23±0.21
Peroxiredoxin 2	PRDX2	-1.4±0.44	-1.74±0.2	-2.21±0.4*	-2.03±0.23*	-1.69±0.13

Control: healthy subjects (n=9), ET: essential thrombocythemia (n=9), PV: polycythemia vera (n=7). SD: standard deviation, homo: homozygous (n=4), htz: heterozygous (n=3 for PV and 6 for ET), no mut: no mutation (n=3). \*p<0.05, \*\*p<0.01 vs.control.

**Table 3.** Biomarkers for oxidative stress and antioxidants in patients with myeloproliferative neoplasms

Oxidative stress markers	Control	PV	ET	PMF
	AV±SD	AV±SD	AV±SD	AV±SD
MDA (nmol/ml)	2.43±0.08	3.18±0.17**	3.78±0.34***	3.31±0.33*
	n=10	n=22	n=24	n=23
PC (nmol/mg protein)	0.9±0.09	1.36±0.12*	1.38±0.15*	1.35±0.12*
	n=8	n=17	n=16	n=18
Antioxidant enzymes				
CAT (U/gHb)	68.44±3.03	48.75±6.82	69.14±4.32	82.88±3.28**
	n=10	n=14	n=12	n=11
SOD (kU/gHb)	7.72±0.23	6.11±0.9	5.54±0.52**	6.33±0.46*
	n=7	n=12	n=11	n=12
GPx (U/gHb)	0.71±0.04	0.57±0.03**	0.61±0.02*	0.74±0.04
	n=9	n=20	n=21	n=25
GR (U/gHb)	5.16±0.36	6.5±0.52	5.67±0.35	5.55±0.3
	n=9	n=24	n=24	n=33
Total antioxidant capacity				
FRAP (μmol/ml)	1.24±0.1	1.38±0.07	1.18±0.06	1.48±0.07*
	n=8	n=14	n=15	n=16

MDA: malondialdehyde, PC: protein carbonyl, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, GR: glutathione reductase, FRAP: ferric reducing antioxidant power, ET: essential thrombocythemia, PV: polycythemia vera, PMF: primary myelofibrosis, n: number of patients, AV: average, SD: standard deviation. p<0.05\*, p<0.01\*\*, p<0.001\*\*\* vs. control.



( $r=-0.371$ ,  $p<0.05$ ) in the plasma of MPN. In PV patients with JAK2V617F homozygosity, GPx and CAT activities were the lowest in comparison to controls ( $p<0.01$ ) and other MPN subgroups ( $p<0.05$ , Table 4). JAK2V617F heterozygosity significantly increased SOD activity in PMF and reduced SOD and GPx activities in ET and PV patients (Table 4). The intracellular level of ROS was increased 2.86-fold in the granulocytes of MPN patients compared to controls ( $p<0.001$ , data not shown). Markers of

oxidative stress were increased in MPN, while antioxidant enzymes were mostly reduced with partial JAK2V617F dependence.

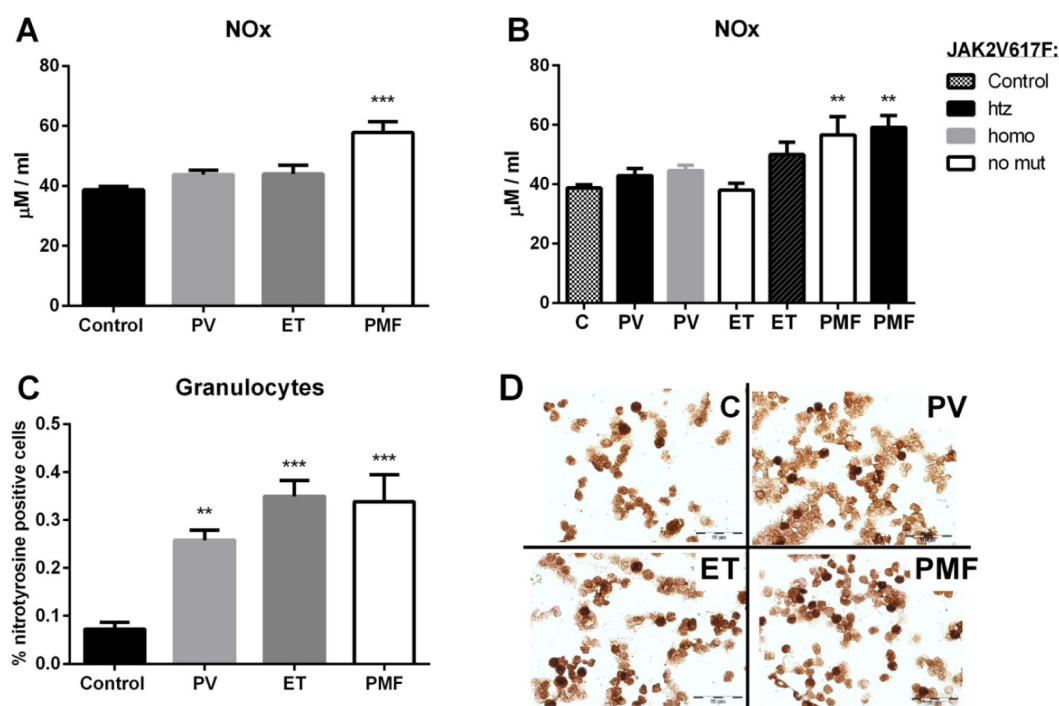
#### *The NO derivatives and NO-producing enzyme iNOS in myeloproliferative neoplasms*

Besides oxidative stress, we evaluated RNS as inducer of nitrosative stress. The concentration of total nitrite/nitrate (NOx) was the highest in plasma of PMF patients (Figure 1A). This NOx

**Table 4.** Antioxidants in myeloproliferative neoplasms according to JAK2V617F allele burden

	Control		PV		ET		PMF	
JAK2V617F		htz	homo	0	htz	0	htz	
CAT (U/gHb)	68.43±3.03 n=10	70.32±4.92 n=7	27.17±4.68* n=7	63.88±5.2 n=7	76.51±6.57 n=5	77.24±4.57 n=6	89.7±2.6** n=5	
SOD (kU/gHb)	7.72±0.23 n=7	5.37±0.12* n=7	7.14±0.14 n=5	6.39±0.3* n=6	4.51±0.94** n=5	6.21±0.48* n=6	6.33±0.75* n=6	
GPx (U/gHb)	0.71±0.03 n=9	0.59±0.04* n=10	0.47±0.01** n=5	0.66±0.03 n=9	0.54±0.03* n=6	0.82±0.05 n=6	0.65±0.12 n=6	
FRAP (μmol/ml)	1.24±0.09 n=8	1.51±0.13 n=7	1.33±0.08 n=7	1.24±0.08 n=7	1.11±0.08 n=7	1.42±0.13 n=7	1.58±0.08* n=7	

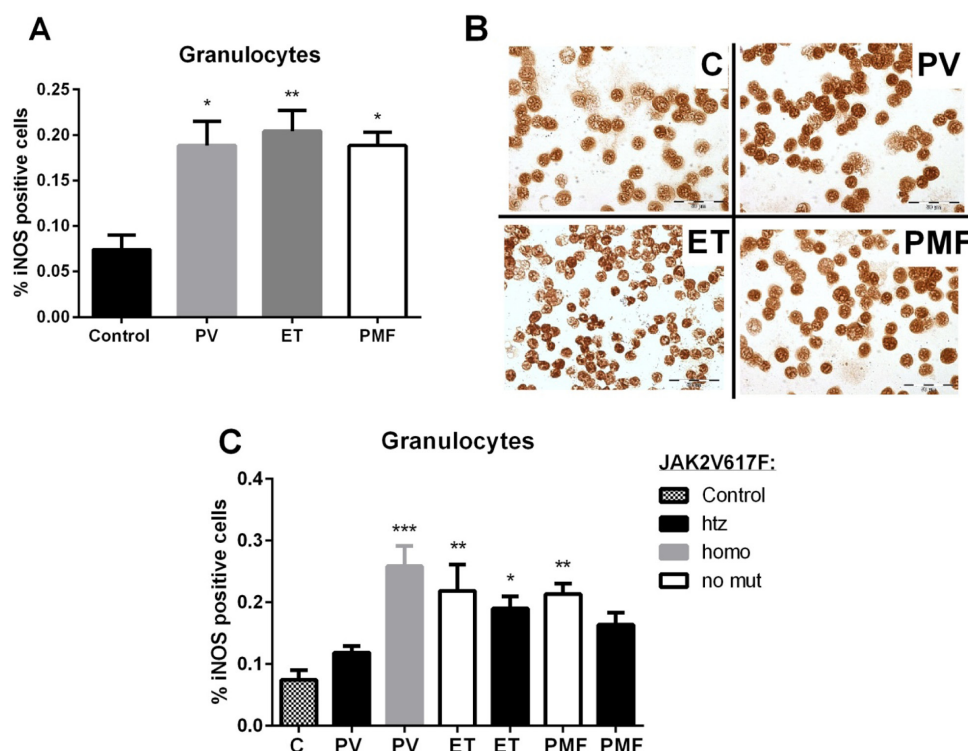
SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, FRAP: ferric reducing antioxidant power, ET: essential thrombocythemia, PV: polycythemia vera, PMF: primary myelofibrosis, homo: homozygous, htz: heterozygous, 0: no mutation.  $p<0.05^*$ ,  $p<0.01^{**}$  vs. control.



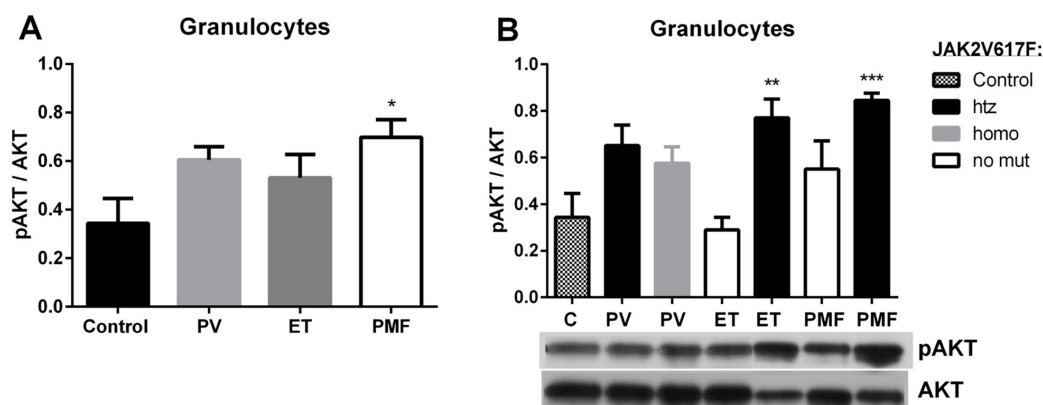
**Figure 1.** Total nitrite and nitrate (NOx) levels in plasma of patients with myeloproliferative neoplasms (MPN). **A)** NOx levels in plasma of polycythemia vera (PV,  $n=12$ ), essential thrombocythemia (ET,  $n=12$ ), primary myelofibrosis (PMF,  $n=12$ ) and healthy subjects (control,  $n=6$ ); **B)** NOx levels in plasma of healthy subjects and MPN according to JAK2V617F allele burden: homozygous (homo), heterozygous (htz) and no mutation (no mut) forms ( $n=6$ ); **C)** Nitrotyrosine levels in granulocytes of PV, ET, PMF and control ( $n=6$ ). **D)** Immunocytochemistry of nitrotyrosine-positive granulocytes in PV, ET, PMF and control (C). Values are mean  $\pm$  SEM. \*\* $p<0.01$  and \*\*\* $p<0.001$  vs. control.

value was also significantly different from PV and ET patients ( $p < 0.01$ ), and was not influenced by JAK2V617F mutation (Figure 1B). All MPN entities had significantly higher quantity of nitrotyrosine positive granulocytes compared to controls (Figure 1C,D). There was a positive correlation of nitrotyrosine levels ( $r = 0.369$ ,  $p = 0.045$ ) and NOx plasma concentration ( $r = 0.358$ ,  $p < 0.05$ ) with iNOS

frequency, significantly increased in granulocytes of MPN (Figure 2A,B). JAK2V617F homozygous form of PV had significantly larger number of iNOS positive cells than the heterozygous form ( $p < 0.01$ , Figure 2C). In addition, only PMF with JAK2 wild type had significantly increased iNOS quantity ( $p < 0.01$ , Figure 2C). NOx was increased preferentially in the plasma of PMF, while



**Figure 2.** Inducible nitric oxide synthase (iNOS) quantity in granulocytes of patients with myeloproliferative neoplasms (MPN). **A)** iNOS levels in granulocytes of polycythemia vera (PV,  $n = 12$ ), essential thrombocythemia (ET,  $n = 12$ ), primary myelofibrosis (PMF,  $n = 12$ ) and healthy subjects (control,  $n = 6$ ); **B)** Immunocytochemistry of iNOS-positive granulocytes of PV ( $n = 12$ ), iNOS ET ( $n = 12$ ), PMF ( $n = 12$ ) and control (C,  $n = 6$ ). **C)** iNOS levels in granulocytes of controls (C), PV, ET, PMF according to JAK2V617F allele burden: homozygous (homo), heterozygous (htz) and no mutation (no mut) forms ( $n = 6$ ). Values are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control.



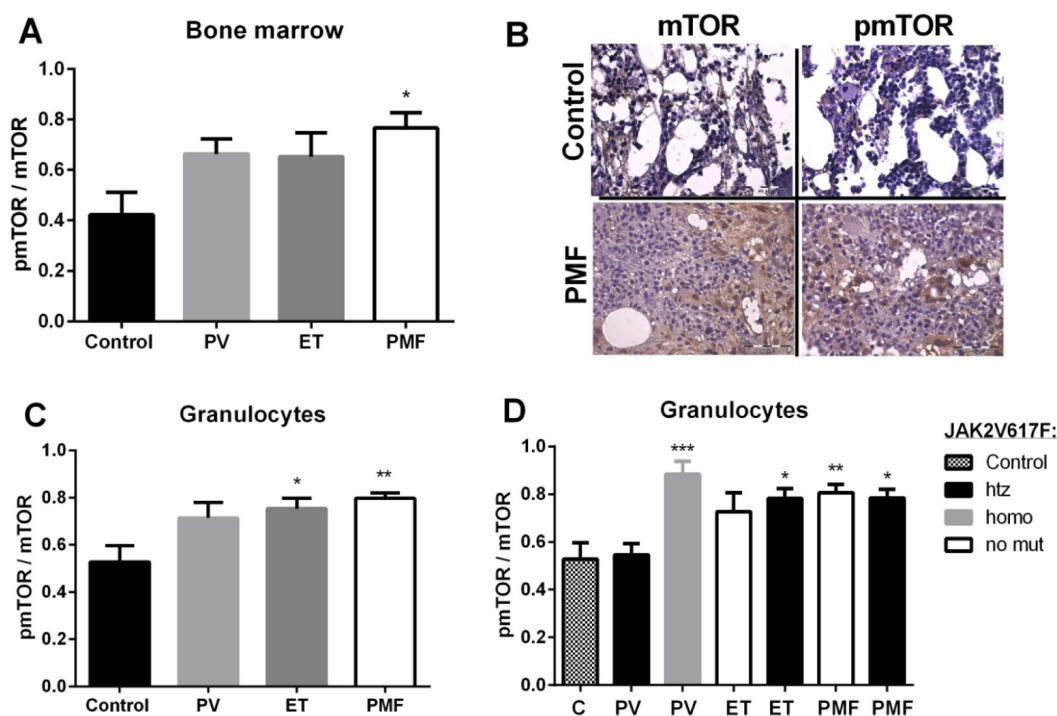
**Figure 3.** Activation of AKT signaling in granulocytes of patients with myeloproliferative neoplasms. **A)** pAKT / AKT ratio in granulocytes of polycythemia vera (PV,  $n = 16$ ), essential thrombocythemia (ET,  $n = 12$ ), primary myelofibrosis (PMF,  $n = 15$ ) and healthy subjects (control,  $n = 6$ ); **B)** pAKT / AKT ratio in granulocytes of controls (C), PV, ET, PMF according to JAK2V617F allele burden: homozygous (homo,  $n = 8$ ), heterozygous (htz,  $n = 6-8$ ) and no mutation (no mut,  $n = 6-7$ ) forms. Values are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control.

nitrotyrosine and iNOS quantity were augmented in the granulocytes of MPN demonstrating JAK2V617F dependence in PV and PMF cases.

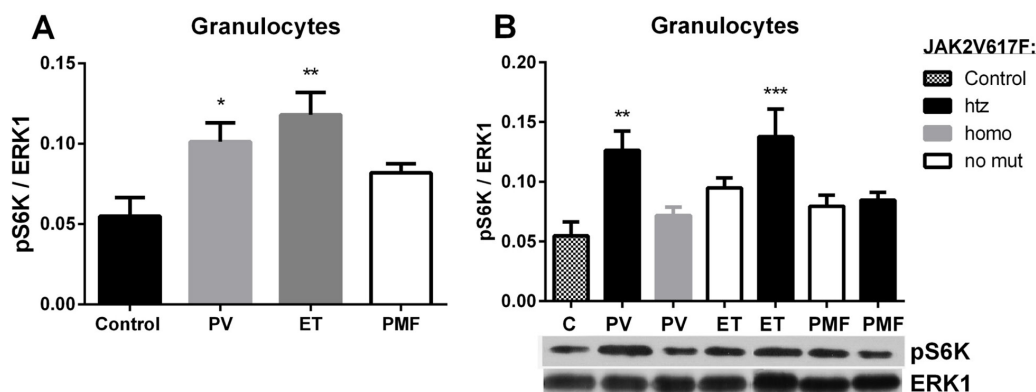
*The AKT/mTOR survival signaling pathway in granulocytes of myeloproliferative neoplasms*

The PI3K/AKT/mTOR pathway regulates various cellular processes such as survival, prolifera-

tion, and neoplastic transformation [21]. Activation of AKT signaling was increased in PMF patients (Figure 3A), but only JAK2V617F heterozygous forms of ET and PMF showed a statistical significance compared to controls (Figure 3B). There was a significant difference between ET patients with and without JAK2V617F mutation ( $p < 0.01$ , Figure 3B). The activation of mTOR pathway was most



**Figure 4.** Activation of mTOR signaling in granulocytes of patients with myeloproliferative neoplasms (MPN). **A)** pmTOR / mTOR ratio in bone marrow stroma cells of polycythemia vera (PV, n=7), essential thrombocythemia (ET, n=7), primary myelofibrosis (PMF, n=7) and healthy subjects (control, n=4); **B)** Immunohistochemistry of mTOR and pmTOR positive cells in bone marrow of PMF and control; **C)** pmTOR / mTOR ratio in granulocytes of PV, ET, PMF (n=10) and control (n=6); **D)** pAKT/AKT ratio in granulocytes of controls (C), PV, ET, PMF according to JAK2V617F allele burden: homozygous (homo), heterozygous (htz) and no mutation (no mut) forms (n=5). Values are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control.



**Figure 5.** Phosphorylation of p70S6K in granulocytes of patients with myeloproliferative neoplasms (MPN). **A)** pp70S6K in granulocytes of polycythemia vera (PV, n=13), essential thrombocythemia (ET, n=13), primary myelofibrosis (PMF, n=14) and healthy subjects (control, n=8); **B)** pS6K in granulocytes of controls (C), PV, ET, PMF according to JAK2V617F allele burden: homozygous (homo, n=6), heterozygous (htz, n=6-7) and no mutation (no mut, n=6-8) forms. Values are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control.

prominent in the bone marrow of PMF patients and granulocytes of PMF and ET cases (Figure 4A and C). We found a positive correlation of mTOR activation in bone marrow cells and granulocytes of PMF patients ( $r=0.464$ ,  $p=0.029$ ). *JAK2V617F* homozygous form of PV had significantly more activated mTOR signaling in granulocytes than the heterozygous form of PV ( $p<0.01$ , Figure 4D). The mTOR activity led to S6K1/2 phosphorylation [22]. The phosphorylation of p70S6K was significantly increased in the granulocytes of PV and ET patients, while only *JAK2V617F* heterozygous forms of PV and ET sustained significance (Figure 5A and B). Activation of AKT/mTOR signaling in PMF and in ET/PV patients with *JAK2V617F* dependence was observed. The phosphorylation of p70S6K was positively correlated with phosphorylation of mTOR ( $r=0.325$ ,  $p=0.046$ ) and AKT ( $r=0.359$ ,  $p=0.044$ ) in MPN granulocytes.

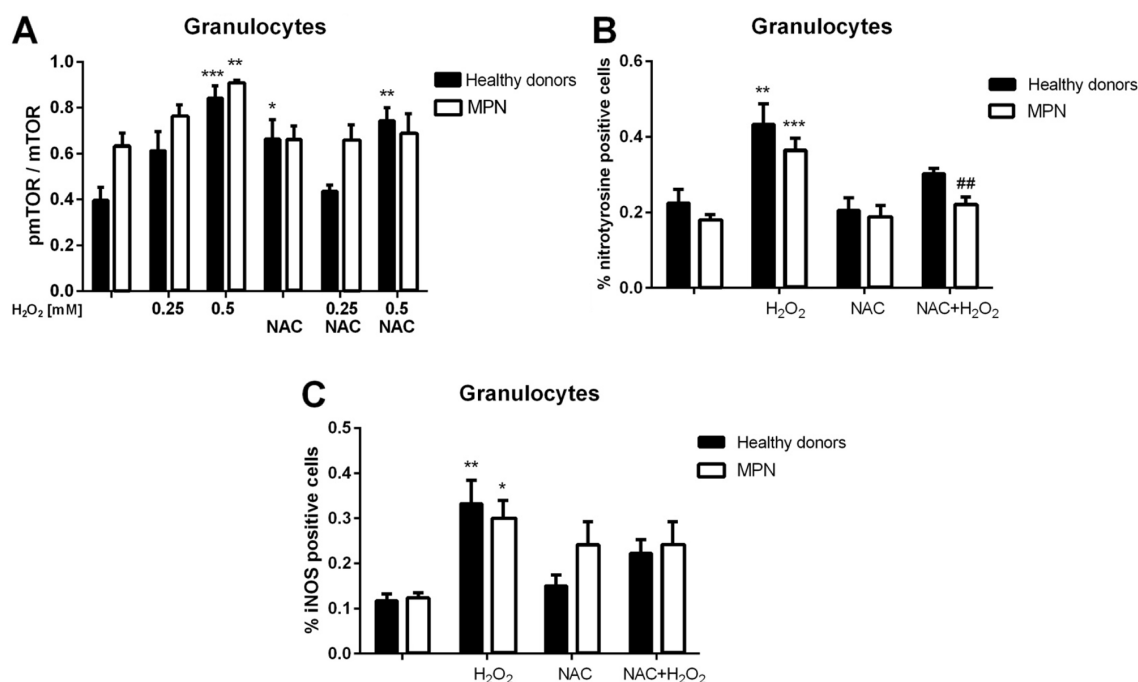
*Reactive oxygen species induction of mTOR signaling, tyrosine nitration and iNOS in granulocytes of myeloproliferative neoplasms*

Antioxidant SOD decreased superoxide anion and produced  $H_2O_2$  [23].  $H_2O_2$  treatment dose dependently increased the phosphorylation of mTOR in the granulocytes of MPN patients and healthy controls (Figure 6A), while antioxidant NAC pre-

vented the phosphorylation of mTOR in MPN cases (Figure 6A). The increased levels of nitrotyrosine in the granulocytes of MPN patients ( $p<0.001$ ) and healthy subjects ( $p<0.01$ ) was observed after treatment with  $H_2O_2$  (Figure 6B). Pre-treatment with antioxidant NAC significantly reduced the stimulation by  $H_2O_2$  in MPN ( $p<0.01$ , Figure 6B). Treatment with  $H_2O_2$  caused an increase of the iNOS-positive granulocytes in MPN patients ( $p<0.05$ ) and healthy controls ( $p<0.01$ , Figure 6C). ROS activated mTOR signaling and increased nitrotyrosine and iNOS levels in the granulocytes of MPN cases. The mTOR inhibitor rapamycin (100 and 500 nM) significantly reduced the proliferation of human erythroleukemia (HEL) cells with *JAK2V617F* mutation (decreased percentage of cells in S and G2/M phase,  $p<0.05$ , with accumulation in G0/G1 phase). The same trend was observed in HEL cells treated with  $H_2O_2$  (range 50-1000  $\mu M$ , not shown). This mTOR inhibitor also reduced the viability of  $H_2O_2$  treated and untreated HEL cells (not shown), which indicates the importance of mTOR pathway for cell survival.

## Discussion

The ROS network would not be complete without a free radical NO [24]. Chemical interaction



**Figure 6.** Oxidative stress induction of nitrotyrosine and iNOS levels, and mTOR signalling in granulocytes of myeloproliferative neoplasms (MPN). **A)** Induction of mTOR signalling by  $H_2O_2$  and regulation by antioxidant N-acetylcysteine (NAC, 3mM) in healthy donors ( $n=5$ ) and MPN ( $n=4$  PV, 2 PMF, 1 ET); **B)** Levels of nitrotyrosine during  $H_2O_2$  (0.5 mM) and NAC (3mM) treatment of healthy donors ( $n=6$ ) and MPN ( $n=3$  PV, 2 PMF, 1 ET); **C)** Levels of iNOS during  $H_2O_2$  (0.5 mM) and NAC (3mM) treatment of healthy donors ( $n=4$ ) and MPN ( $n=3$  PV, 2 PMF, 1 ET). Values are mean  $\pm$  SEM. \* $p<0.05$ , \*\* $p<0.01$  and \*\*\* $p<0.001$  vs. non treated granulocytes. ## $p<0.01$  vs.  $H_2O_2$  treated granulocytes.



of NO with ROS forms RNS and constitutes the basis for the formation of additional oxidative signaling elements [25]. Oxidative stress index and MDA were increased in MPN patients, reduced by therapy, while the total antioxidant status was lower compared to the controls [11,26]. Oxidation protein products and s-nitrosylated proteins were significantly increased in PV and ET patients [10]. The present study showed an altered oxidant/antioxidant balance in MPN. We observed significantly higher plasma MDA (a by-product of lipid peroxidation) and PC levels (product of oxidized proteins) in MPN. However, there was a significant difference between MPN subtypes in terms of antioxidative enzymes activities in erythrocytes. We detected decreased antioxidant protection in the erythrocytes of MPN, but only of SOD in PMF cases. In a previous study with MPN, the SOD activity of polymorphonuclear leucocytes was decreased, of erythrocytes was increased (in contrast to our results), of thrombocytes was not changed compared to healthy volunteers, suggesting that SOD enzyme activity and ROS production may be cell-dependent [27]. Therefore, the oxidative stress evident in MPN is not compensated by all antioxidants.

The aberrant accumulation of ROS and RNS can result in oxidative/nitrosative stress which could modify DNA [28]. *JAK2V617F* positive cells induced paracrine DNA damage to themselves and neighboring normal cells via increased ROS production [29]. It has been found that many proteins involved in antioxidant defense were aberrantly expressed in MPN, according to *JAK2V617F* mutation, such as downregulated antioxidant CAT [30]. In addition, *JAK2V617F* dependent ROS elevation in CD34<sup>+</sup> cells was partially mediated by AKT-induced decrease in CAT expression accompanied by DNA damage [3]. We observed upregulated CAT enzyme activity only in the PMF erythrocytes.

We determined the significantly increased level of intracellular ROS production, revealing the increased oxidative stress and granulocyte activation that produced ROS and RNS. ROS production by neutrophils from MPN patients with *JAK2V617F* mutation has already been reported [2]. The main source of ROS in neutrophilic granulocytes is the membrane-bound multicomponent enzyme NADPH oxidase that is dormant in resting cells, but when activated, generates large quantities of ROS. The major end products of NADPH oxidase activity are superoxide and H<sub>2</sub>O<sub>2</sub> [31]. H<sub>2</sub>O<sub>2</sub> activated myeloperoxidase significantly contributed to the nitrotyrosine formation via oxidation of nitrite (NO<sub>2</sub><sup>-</sup>) to nitrogen dioxide (·NO<sub>2</sub>) that participated in the nitrotyrosine formation

in granulocytes [32]. Nitrotyrosine is a hallmark of chronic inflammation formed by reaction of ONOO<sup>-</sup> with proteins containing tyrosine residues and by peroxidase-mediated reactions between protein tyrosines and nitrites [33]. All MPN entities had significantly higher levels of nitrotyrosine in granulocytes, increased by H<sub>2</sub>O<sub>2</sub> and reverted by antioxidant NAC. In addition, iNOS expression was induced by H<sub>2</sub>O<sub>2</sub>, confirmed in the observed MPN granulocytes [34]. We found significantly increased NO<sub>x</sub> in PMF patients compared to healthy volunteers, PV and ET patients. A previous study showed reduced plasma levels of NO in patients with ET, while PV patients presented elevated plasma levels of NO [35]. The present study revealed a positive correlation between iNOS quantity and nitrotyrosine levels in the granulocytes of MPN. Activated JAK-STAT signaling pathway increased iNOS induction and NO production [36,37]. Constitutive activation of JAK-STAT signaling supports the enhanced iNOS frequency and NO-derivatives level in MPN patients.

The MPN-like mutant hematopoietic progenitors increased ROS through the AKT/mTOR signaling pathway [38,39]. In a murine bone marrow transplantation model, MPN was developed by activated AKT signaling [40]. Dual PI3K/mTOR and JAK1/2 inhibition induced apoptosis of primary MPN CD34<sup>+</sup> cells, inhibited red cell production and reduced splenomegaly in conditional *JAK2V617F* knock-in mice [7,41]. *JAK2V617F* was associated with upregulated phosphorylation of AKT in the MPN megakaryocytes, and to a lesser extent in other hematopoietic cells [4,42]. Principally, ET megakaryocytes revealed elevated p70S6k expression [42]. We revealed activation of AKT/mTOR signaling (increased by ROS) and its downstream effector p70S6K in the granulocytes of MPN, with *JAK2V617F* dependence, as well as in the bone marrow of PMF.

Oxidative/nitrosative stress is active in MPN, not limited by complete antioxidant systems in circulation and influenced by *JAK2V617F*. Moreover, ROS supported the activation of AKT/mTOR signaling pathway and enhanced iNOS frequency and nitrotyrosine levels in MPN. As a final stage of MPN development, PMF had more prominent antioxidants and NO<sub>x</sub> levels, as well as general AKT/mTOR activation. This observation supports a role of oxidative/nitrosative stress in the development of MPN, not just in the initiation of MPN.

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## Conflict of interests

The authors declare no conflict of interests.

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