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Analysis of mitochondrial function and antioxidant capacity in JAK2 V617F-positive chronic myeloproliferative neoplasms

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Abbreviations:
ACK – ammonium-chloride-potassium
APC – allophycocyanin
CML – chronic myeloid leukemia
ET – essential thrombocythemia
FL1 – fluorescence channel 1
HSC – hematopoietic stem cell
IMF – idiopathic myelofibrosis
MF – myelofibrosis
MFI – mean fluorescence intensity
MPN – myeloproliferative neoplasms
MPN-SAF TSS – myeloproliferative neoplasm symptom assessment form total symptom score
MT – MitoTracker™
NAO – nonyl acridine orange
PV – polycythemia vera
TSS – total symptom score

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Abstract

Background and purpose: *The JAK2 V617F mutation, found in the majority of chronic myeloproliferative neoplasms (MPNs), leads to constitutive JAK-STAT pathway activation, driving cell proliferation and increased production of reactive oxygen species (ROS). Mitochondrial dysfunction and oxidative stress are implicated in MPN pathogenesis. In the present study, we aimed to elucidate mitochondrial functionality and patterns of antioxidant and electron transport chain gene expression in patients with JAK2 V617F-positive myeloproliferative neoplasms.*

Materials and methods: *The study included 66 JAK2 V617F-positive MPN patients and 30 healthy controls. All participants completed the Myeloproliferative Neoplasm Symptom Assessment Form Total Symptom Score (MPN-SAF TSS) to assess symptom burden. MitoTracker™ (MT) and Nonyl Acridine Orange (NAO) probes were used for flow cytometric assessment of mitochondrial membrane potential and mass, based on mean fluorescence intensity (MFI). Total RNA was isolated, and expression of* SOD2, CAT, SIRT3 *and* MT-ND1 *was quantified by qPCR using TaqMan™ probes and normalized to* ACTB *using the ΔΔCt method.*

Results: *The MT/NAO MFI ratio, reflecting mitochondrial membrane potential relative to mass, was significantly reduced in JAK2 V617F-positive patients compared to controls ($p = 0.003$). Antioxidant gene expression (*SOD2, SIRT3, CAT*) and* MT-ND1*, a key regulator of oxidative phosphorylation, were markedly downregulated in patients ($p < 0.001$).*

Conclusions: *We show for the first time that patients with the JAK2 V617F mutation exhibit mitochondrial dysfunction, characterized by increased mitochondrial mass, decreased mitochondrial membrane potential, and impaired oxidative phosphorylation and antioxidant defense. To elucidate the potential genetic background of the observed mitochondrial dysfunction, mitochondrial genes should be assessed by sequencing.*

INTRODUCTION

Specific somatic mutations in hematopoietic stem cells (HSCs) of the bone marrow lead to the excessive production of certain myeloid blood cell lineages, resulting in elevated numbers of erythrocytes, platelets, or leukocytes *(1, 2)*. The molecular basis of myeloproliferative neoplasms (MPNs) is well understood in over 95% of cases, with somatic mutations in the *JAK2*, *MPL*, and *CALR* genes contributing to disease phenotype *(3)*. These mutations result in constitutive activation of the

Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway, which is crucial for the proliferation of myeloid cells. The term "chronic myeloproliferative disorder" was first introduced by William Dameshek in 1951, recognizing shared features among polycythemia vera (PV), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF), and chronic myeloid leukemia (CML), all of which involve altered proliferative activity in the bone marrow *(1)*. Recent studies suggest that somatic mutations such as JAK2 V617F may contribute not only to aberrant signaling pathways like JAK-STAT, but also to broader metabolic alterations, including mitochondrial dysfunction *(1,3)*.

Mitochondria are double-membraned cytoplasmic organelles responsible for producing cellular energy in the form of ATP through oxidative phosphorylation (OXPHOS). The inner membrane houses five enzymatic complexes: the first four constitute the electron transport chain, while the fifth, ATP synthase, catalyzes ATP production *(4)*. These complexes function together in OXPHOS and regulate metabolite exchange between the cytosol and mitochondria. The inner membrane's impermeability to ions maintains the proton gradient required for OXPHOS *(5–7)*. In normoxic conditions, healthy cells predominantly rely on OXPHOS for efficient ATP production, whereas cancer cells preferentially engage glycolysis even in the presence of oxygen—a phenomenon known as the Warburg effect *(8, 9)*. This metabolic reprogramming results in markedly lower ATP yield compared to OXPHOS, but provides biosynthetic intermediates that support rapid cell growth and proliferation. Many tumor cells can flexibly shift between glycolysis and OXPHOS, enabling survival and proliferation under varying metabolic conditions *(10)*.

Mutations in mitochondrial DNA (mtDNA) may act both as drivers and consequences of tumorigenesis. They can disrupt metabolic pathways or emerge as a response to metabolic stress in cancer cells *(11)*. In murine models, the D257A mutation in the *POLG* gene, which impairs mtDNA repair, has been associated with increased leukemia incidence *(12)*. Although the Warburg effect was originally considered a hallmark of solid tumors, recent findings suggest its presence in PV, where it correlates with increased activity of hypoxia-inducible factor (HIF) *(13)*. Increased expression of *RUNX1* and *NF-E2* has been observed in PV subtypes with elevated HIF signaling, suggesting a potential link between hypoxia-related pathways and transcriptional regulation in MPNs. While murine models have provided valuable insights, emerging human studies also implicate mtDNA alterations in the pathogenesis of MPNs, underscoring the need for comprehensive mitochondrial genomic analysis across disease subtypes *(12)*. We hypothesized that the JAK2 V617F mutation in MPN patients is associated with altered mitochondrial function, reflected by changes in mitochondrial membrane potential, mitochondrial mass, and dysregulation of genes involved in oxidative stress response. To test this hypothesis, we evaluated mitochondrial features, including *MT-ND1* expression, and the expression of antioxidant genes (*SOD2*, *CAT*, *SIRT3*) in JAK2 V617F-positive MPN patients compared to healthy controls.

MATERIALS AND METHODS

Study design and participants

The study was approved by the Ethics Committee of University Hospital Dubrava, Zagreb, on February 6, 2024 (approval number: 2024/0206-01), and by the Ethics Committee of the University of Zagreb School of Medicine on May 23, 2024 (approval number: 251-59-10106-24-111/50). All 66 MPN patients included in the study were confirmed to carry the JAK2 V617F mutation, while none of the 30 age- and sex-matched healthy controls harbored this mutation. Mutation status was determined by allele-specific PCR and confirmed using the Pillar® MPN NGS panel (Illumina®, San Diego, CA, USA), which also excluded the presence of other driver mutations (e.g., *CALR*, *MPL*). From each participant, two 5 mL EDTA-anticoagulated peripheral blood samples were collected at the Department of Hematology, University Hospital Dubrava, Zagreb, between May 2023 and December 2024. Blood was drawn from patients during routine diagnostic procedures. All participants were informed about the study and signed written consent forms. Hematological analysis was performed for all participants, and clinical symptom assessment was conducted in MPN patients. After granulocyte separation, DNA and RNA were isolated, and sample purity and concentration were measured. Detailed protocols for granulocyte separation and nucleic acid isolation are described below. Mitochondrial parameters were analyzed using flow cytometry. Gene expression of *SIRT3*, *MT-ND1*, *SOD2*, and *CAT* was quantified by real-time quantitative PCR (qPCR), with *ACTB* used as the endogenous control.

Clinical Symptom Assessment in MPN Patients

As a part of data collection, all participants completed the Myeloproliferative Neoplasm Symptom Assessment Form Total Symptom Score (MPN-SAF TSS), also known as the MPN-10 questionnaire. This validated instrument is widely used to assess symptom burden in patients with MPNs. The questionnaire includes ten disease-specific symptoms, each scored on a numeric scale from 0 (no symptoms) to 10 (most severe possible). The assessed symptoms were: fatigue, early satiety, abdominal discomfort, inactivity, problems with concentration, night sweats, pruritus, bone pain, fever (>37.8 °C), and unintentional weight loss over the past six months. The Total Symptom Score (TSS) was calculated by summing

the individual symptom scores and served as an indicator of overall symptom burden. Collected data were analyzed descriptively and used for further statistical correlation with laboratory and genetic parameters of the disease.

Granulocyte Isolation and Nucleic Acid Extraction

Granulocytes were isolated from peripheral blood samples using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. After density gradient centrifugation, granulocytes were collected, washed twice with PBS, and resuspended for subsequent DNA and RNA extraction. Total RNA was isolated from granulocytes using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. After erythrocyte lysis and centrifugation, the cell pellet was lysed in RLT buffer, homogenized using QIAshredder columns, and purified through spin columns with on-column DNase digestion. RNA purity and concentration were assessed using NanoDrop™ UV-Vis spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit™ RNA Broad Range Assay Kit (Thermo Fisher Scientific), according to standard protocols. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Granulocytes were lysed with AL buffer and proteinase K, and the lysate was applied to QIAamp spin columns. Following wash steps with AW1 and AW2 buffers, DNA was eluted in AE buffer. DNA quality and concentration were determined using NanoDrop™ and the Qubit™ dsDNA Broad Range Assay Kit (Thermo Fisher Scientific).

Analysis of Mitochondrial Parameters by Flow Cytometry

To assess mitochondrial status, both mitochondrial membrane potential (via MT) and mass (via NAO) were analyzed in peripheral blood granulocytes using flow cytometry. Erythrocyte lysis was performed with ACK buffer, followed by centrifugation and resuspension in phosphate-buffered saline (PBS). Cells were divided into three tubes labeled as MitoTracker™ Deep Red (MT), Nonyl Acridine Orange (NAO), and negative control. MT and NAO (Thermo Fisher Scientific, Waltham, MA, USA) were added to the corresponding tubes, while PBS was added to the control. After a 30-minute incubation at room temperature in the dark, cells were washed, centrifuged, and resuspended in PBS. Samples were analyzed using a BD FACSLyric™ 3-Laser Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). MT fluorescence was detected in the APC channel ($\lambda\_exc = 644$ nm; $\lambda\_em = 665$ nm), and NAO in the FL1 channel ($\lambda\_exc = 488$ nm; $\lambda\_em = 535$ nm). Differences in mean fluorescence intensity (MFI) were compared between JAK2 V617F-positive patients and healthy controls.

Quantitative PCR (qPCR)

Complementary DNA (cDNA) was used for quantifying gene expression by qPCR on a QuantStudio 5 instrument (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were run in triplicate using TaqMan™ Fast Advanced Master Mix, following the manufacturer's instructions. Each 20 µL reaction contained 10.0 µL of TaqMan™ Fast Advanced Master Mix (2X), 1.0 µL of a 20X TaqMan™ Gene Expression Assay, 2.0 µL of cDNA, and 7.0 µL of nuclease-free water. The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Gene expression was measured for *SIRT3* (Hs00953477_m1), *MT-ND1* (Hs02596873_s1), *SOD2* (Hs00167309_m1), *CAT* (Hs00156308_m1), and the endogenous control *ACTB* (Hs01060665_g1). Cycle threshold (Ct) values were determined for each gene and analyzed using QuantStudio™ 3/5 software (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical analysis was conducted in Python (v3.x) using the pandas, scipy, and seaborn libraries. Descriptive statistics included median, interquartile range (IQR), mean, and standard deviation (SD). Due to non-normal data distribution and unequal group sizes, non-parametric tests were applied: the Mann–Whitney U test for two-group comparisons and the Kruskal–Wallis test for multiple groups. Results were visualized using box plots. Statistical significance was set at $p < 0.05$.

RESULTS

Mitochondrial parameters, including membrane potential and mitochondrial mass, as well as the expression of selected antioxidant genes (*SOD2, SIRT3, CAT*) and the mitochondrial gene *MT-ND1*, were analyzed in JAK2 V617F-positive MPN patients and compared to healthy controls. These analyses were designed to evaluate mitochondrial function and oxidative stress status in the context of MPN pathogenesis. The analysis revealed altered mitochondrial characteristics, reduced expression of key antioxidant genes, and significant downregulation of the mitochondrial gene *MT-ND1* in the patient group, supporting the hypothesis of mitochondrial dysfunction in MPNs.

Clinical Symptoms in MPN Patients

More than 50% of JAK2 V617F-positive patients reported experiencing at least seven of the ten core symptoms assessed by the MPN-SAF TSS questionnaire. The most frequently reported symptom was fatigue (87.9%), followed by inactivity (72.7%), difficulty concentrating (65.2%), bone pain (63.6%), night sweats (59.1%), pruritus (56.1%) and early satiety (50.0%). Symptoms typi-

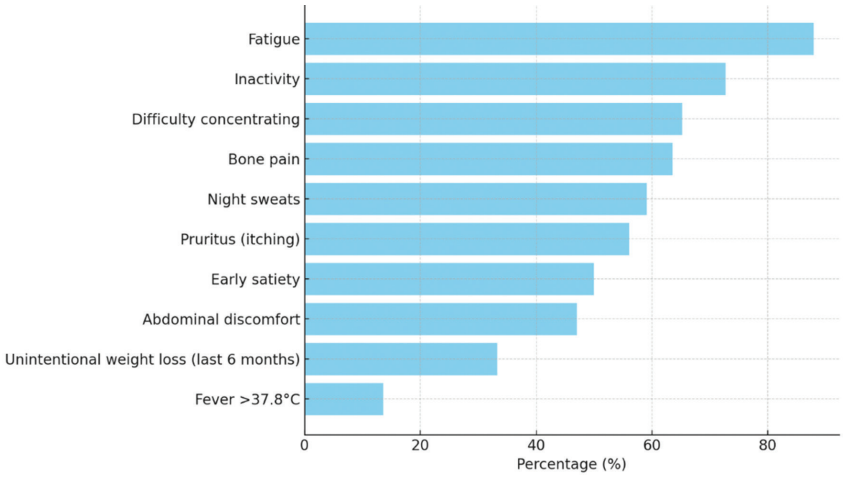

Figure 1. ***Prevalence of symptoms reported by MPN patients via the MPN-SAF TSS questionnaire.*** *Prevalence represents the percentage of patients who reported a score greater than zero for each of the ten listed symptoms.*

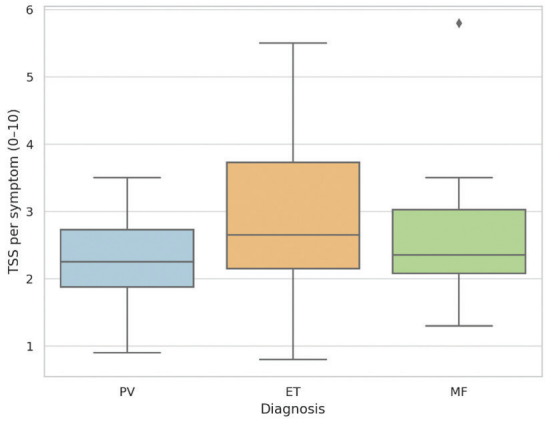

Figure 2. ***Total Symptom Score (TSS) per symptom by myeloproliferative neoplasm diagnosis (polycythemia vera, essential thrombocythemia, myelofibrosis).*** *Higher symptom burden was observed in patients with myelofibrosis and essential thrombocythemia compared to polycythemia vera, but the difference was not statistically significant (Kruskal–Wallis H(2) = 1.77; p = 0.412).*

cally associated with more advanced diseases such as unintentional weight loss, fever and abdominal discomfort - were less frequently reported (<50%) (Figure 1). Although patients with MF and ET had slightly higher average TSS scores per symptom (MF: M = 2.77; ET: M = 2.62) compared to patients with polycythemia vera (M = 2.17), the difference was not statistically significant (Kruskal–Wallis H(2) = 1.77; p = 0.412) (Figure 2).

Analysis of Mitochondrial Parameters by Flow Cytometry

The MFI of MT, which reflects mitochondrial membrane potential, was significantly lower in JAK2 V617F-positive patients compared to healthy controls (p = 0.031) (Figure 3A). Conversely, the MFI of NAO, indicative of mitochondrial mass, as NAO binds to cardiolipin in the inner mitochondrial membrane, was significantly higher in the patient group (p = 0.032) (Figure 3B). The calculated MT/NAO ratio, representing overall mitochondrial functionality relative to mitochondrial mass, was

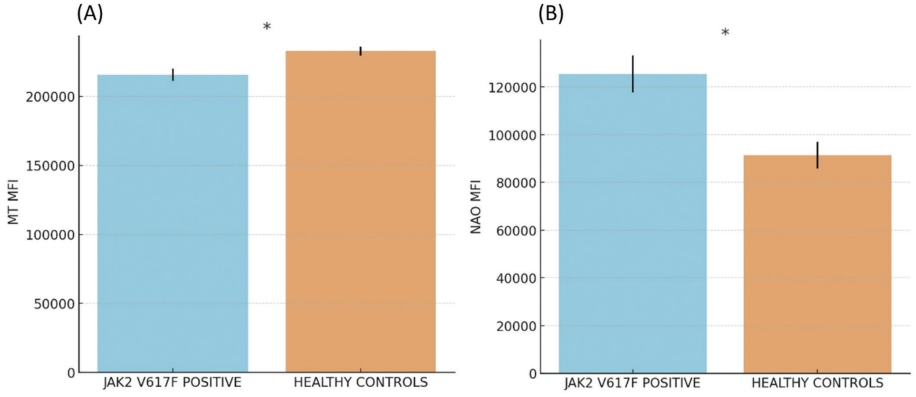

Figure 3. ***MFI of MT (A) and NAO (B) in cells from patients with confirmed JAK2 V617F mutation and healthy controls.*** *Data are presented as mean ± standard deviation. Statistically significant differences were observed between the groups: (A) p = 0.031 and (B) p = 0.032 (Mann-Whitney U test, * p < 0.05).*

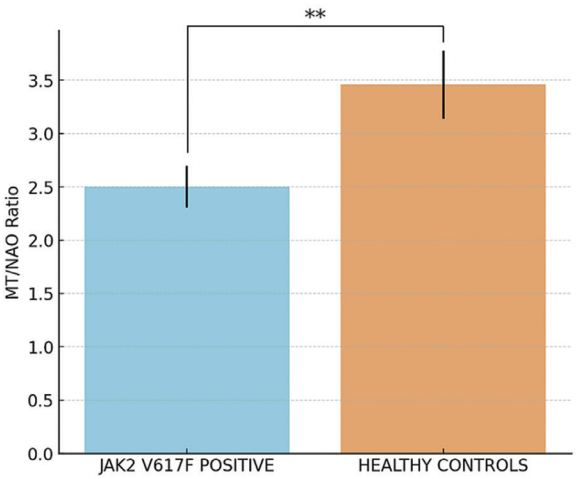

Figure 4. ***Ratio of MT to NAO fluorescence intensity (MT/NAO) in cells from JAK2 V617F-positive patients and healthy controls.*** *Data are presented as mean ± standard deviation. A statistically significant difference between the groups was observed (p = 0.003; Mann–Whitney U test, ** p < 0.01).*

significantly reduced in JAK2 V617F-positive patients (p = 0.003) (Figure 4). When analyzed by MPN subtype (PV, ET, MF), no statistically significant differences in MT MFI, NAO MFI, or MT/NAO ratio were observed (data not shown).

Gene Expression of Mitochondrial and Antioxidant Genes by qPCR

Compared to healthy controls, JAK2 V617F-positive patients showed significantly reduced relative expression of mitochondrial and antioxidant genes in granulocytes. Expression levels of *SIRT3* and *MT-ND1*, key regulators of mitochondrial function and oxidative phosphorylation, as well as *SOD2* and *CAT*, involved in antioxidant defense, were significantly lower in the patient group ($p < 0.001$) (Figure 5).

DISCUSSION

The aim of this study was to investigate symptom burden and mitochondrial alterations in patients with MPNs carrying the JAK2 V617F mutation, in order to better understand their potential contribution to disease pathophysiology. Fatigue was the most frequently reported

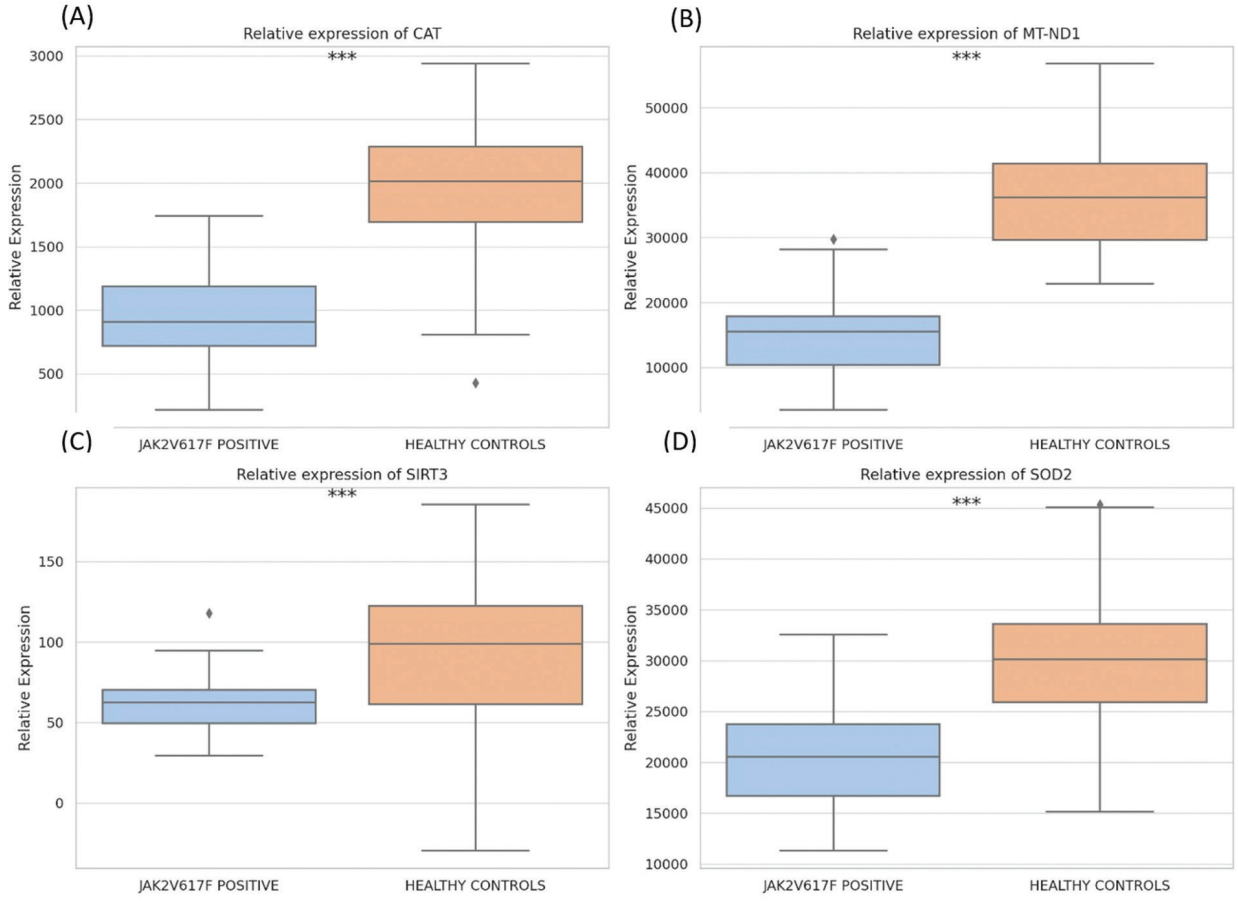

Figure 5. ***Relative expression of CAT, MT-ND1, SIRT3 and SOD2 in granulocytes from JAK2 V617F-positive patients and healthy controls.*** *Statistical significance was determined using the Mann–Whitney U test. Statistically significant differences were observed for all genes (**A–D**), ***p < 0.001.*

symptom among patients with MPN included in this study, which confirms its central role in the symptom profile of the disease. This finding is in concordance with previous studies that have identified fatigue as the most prevalent symptom in this patient population *(14)*. Although we observed a tendency for MF and ET patients to exhibit higher average TSS values per symptom compared to PV patients, this difference was not statistically significant. A similar finding was reported in a large German study involving 3,979 patients with PV, ET, and MF. Although MF patients reported slightly higher pain prevalence, overall TSS did not differ significantly across subtypes *(15)*. In contrast, a Canadian study including 784 patients reported significantly higher mean and maximum MPN-SAF TSS values in MF patients compared to those with PV and ET *(16)*. Variations in study findings may be attributed to differences in sample size, symptom assessment methods, and the clinical and demographic characteristics of the studied populations.

Mitochondria, as key regulators of cellular metabolism, oxidative stress, and apoptosis, are frequently disrupted in hematologic malignancies, including MPNs *(17, 18)*. In JAK2 V617F-positive patients, a significantly lower MFI of MT was observed compared to healthy controls, suggesting a reduction in mitochondrial membrane potential, which may reflect altered energetic status and mitochondrial dysfunction. This may result from metabolic alterations driven by chronic activation of the JAK-STAT pathway and subsequent energetic reprogramming. No significant differences in mitochondrial parameters were observed between MPN subtypes (PV, ET, MF), suggesting that these mitochondrial alterations are a common feature of JAK2 V617F-positive disease rather than being subtype-specific. Although our results indicate a decrease in mitochondrial membrane potential, this finding is not inconsistent with previous reports of mitochondrial hyperactivity. The term hyperactivity in that context refers to enhanced metabolic flux and excessive reactive oxygen species production, rather than an increase in membrane potential. Therefore, JAK2 V617F-driven mitochondrial dysfunction may involve elevated oxidative metabolism accompanied by a loss of membrane potential integrity, reflecting an energetically unstable mitochondrial state. Similar findings have shown that mitochondrial hyperactivity and membrane potential disturbances are linked to the myeloproliferative phenotype in hematopoietic cells *(19)*.

Conversely, the MFI of NAO was significantly higher in JAK2 V617F-positive patients compared to healthy individuals suggesting elevated mitochondrial mass. As NAO binds cardiolipin in the inner mitochondrial membrane, this signal likely reflects an accumulation of cardiolipin-rich membranes either due to enhanced biogenesis or impaired mitochondrial turnover *(20)*. The decreased MT/NAO ratio further supports the presence of dysfunctional mitochondria, where mass is preserved or elevated, but functional capacity is reduced. Such imbalance is characteristic of impaired mitochondrial homeostasis, whether due to defective mitophagy and oxidative stress, or insufficient mitochondrial quality control *(21)*. To our knowledge, this is the first study to simultaneously assess MFI of MitoTracker™, NAO, and their ratio as indicators of mitochondrial function in MPN patients. Although both dyes are established tools under oxidative stress conditions, their use in hematological malignancies has not previously been reported. In this context, increased mitochondrial mass may represent a compensatory response to oxidative stress or a metabolic shift toward OXPHOS dependence, providing survival benefits in the bone marrow niche. However, defective mitophagy may lead to the accumulation of non-functional mitochondria, contributing to sustained ROS production, genomic instability, and inflammatory signaling—features associated with clonal evolution and MPN progression *(22, 23)*. Our findings underscore the need for future mechanistic studies to determine whether mitochondrial expansion in MPNs results from increased biogenesis, impaired clearance, or both, and how these processes influence disease behavior.

The results of this study demonstrate significantly reduced expression of the *SIRT3, MT-ND1, SOD2* and *CAT* genes in JAK2 V617F-positive patients compared to healthy controls. *SIRT3*, a mitochondrial deacetylase, plays a key role in the regulation of oxidative phosphorylation and antioxidant defense mechanisms *(24)*. Its downregulation may impair mitochondrial function and promote increased production of ROS, a phenomenon previously associated with the pathogenesis of MPNs due to elevated ROS levels observed in affected individuals *(25)*. *MT-ND1* encodes a subunit of Complex I of the mitochondrial electron transport chain, and its decreased expression indicates Complex I dysfunction, which can impair ATP synthesis and, through inefficient electron transfer, increase ROS production *(5, 26)*. SOD2 and CAT, enzymes critical for the ROS detoxification *(27)*, were also downregulated, indicating compromised antioxidant capacity, which may contribute to oxidative damage and disease progression *(28)*. Elevated ROS levels have been shown to reduce the self-renewal capacity of HSCs in patients harboring the JAK2 V617F mutation, suggesting a mechanism involving mitochondrial dysfunction and increased oxidative stress. This mechanism has been proposed particularly in granulocytes and may play a central role in the pathophysiology of MPNs *(25)*. The observed downregulation of *SIRT3*, *MT-ND1*, *SOD2*, and *CAT* may result from transcriptional repression driven by persistent oxidative stress or metabolic alterations associated with JAK2 V617F signaling. Although global transcriptional silencing is not characteristic of MPNs, several studies suggest that oxidative stress can lead to a selective downregulation of genes involved in antioxidant defense via feedback mechanisms *(29)*. In this context,

elevated ROS levels may further suppress the transcription of ROS-detoxifying enzymes, creating a vicious cycle of mitochondrial dysfunction and oxidative damage. Epigenetic modifications, such as altered histone acetylation or DNA methylation, may also contribute to gene silencing, particularly for *SIRT3*, which is known to be sensitive to changes in cellular redox and NAD^+ status *(30)*. Further studies are needed to elucidate whether the suppression of these genes in MPNs is primarily driven by ROS-induced signaling, epigenetic remodeling, or transcription factor imbalance.

CONCLUSION

This study provides additional insights into mitochondrial alterations and impaired redox homeostasis in JAK2 V617F-positive MPN patients. The observed reduction in mitochondrial membrane potential (MT MFI) and MT/NAO ratio, along with elevated NAO signal, indicate mitochondrial dysfunction and altered structural integrity in patients' granulocytes. Detected downregulation of genes crucial for mitochondrial maintenance (*SIRT3),* cellular respiration *(MT-ND1*) and antioxidant defense (*SOD2, CAT*), indicate a shift towards oxidative stress and metabolic imbalance. Together, these results support the hypothesis that mitochondrial dysfunction and insufficient antioxidant response contribute to MPN pathogenesis in JAK2 V617F-positive patients. Targeting mitochondrial bioenergetics and ROS regulation may thus represent a promising therapeutic course. Further studies, including sequencing of the mitochondrial genome and nuclear genes involved in mitochondrial homeostatsis, assaying ATP and ROS production as well as mitochondrial membrane potential, will be necessary for defining potential therapeutic targets.

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