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A Pilot Study on Mitochondrial Dysfunction, Autophagy, and Inflammation in Rheumatoid Arthritis Patients on Methotrexate Treatment

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ABSTRACT

Background and Aims: Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease characterized by persistent synovial inflammation, cartilage degradation, and joint destruction. Several studies have reported mitochondrial dysfunction, dysregulated autophagy, and inflammation in RA, but few have tracked their dynamics following therapy. The aim of our study was to evaluate the direct effect of methotrexate (MTX) on metabolic parameters and markers of autophagy and inflammation in patients with RA.

Materials and Methods: Ten newly diagnosed RA patients were enrolled in the study. They were treated with MTX in optimally approved daily doses for six months. Conventional biochemical and immunological indicators, metabolic parameters, and biomarkers for inflammation and autophagy were examined at the start of treatment and after the follow-up period.

Results: All examined clinical and laboratory parameters were improved after treatment. We observed decreased basal respiration (p=0.018) and adenosine triphosphate (ATP) production (p=0.03), along with a significant reduction in plasma levels of chitinase-3-like protein 1 (YKL-40) (p=0.007) and lysosomal-associated membrane protein 1 (LAMP1) (p=0.03) following MTX therapy. Strong correlations were found between LAMP1 and basal respiration (p=0.022, r=0.928); LAMP2 and ATP production (p=0.033, r=-0.886); and Disease Activity Score in 28 joints (DAS28) and proton leak (p=0.017, r=0.941) before therapy. After therapy, associations were observed between LAMP1 and spare respiratory capacity (p=0.003, r=0.919), and between the 7-joint ultrasound score (GUS7) and LAMP2 (p=0.027, r=0.692).

Conclusion: In conclusion, we present novel data on correlations between mitochondrial functional parameters and autophagy- and inflammation-related proteins in RA patients after treatment with MTX. These pilot findings may pave the way for future clinical applications related to monitoring disease severity and treatment response.

Keywords: Autophagy, inflammation, mitochondrial dysfunction, rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease marked by persistent synovial inflammation, cartilage degradation, and joint destruction. While its etiopathogenesis remains multifactorial, increasing evidence implicates mitochondrial dysfunction as a central contributor to immune dysregulation and chronic inflammation in RA.[1]

Mitochondria are essential organelles not only for cellular energy metabolism but also for the regulation of redox signaling, apoptosis, and innate immune responses. In RA, mitochondrial impairment has been observed in both synovial fibroblasts and immune cells, resulting in excessive production of reactive oxygen species (ROS), altered bioenergetics, and the cytosolic release of mitochondrial DNA (mtDNA) and other mitochondrial components.[2-4] In RA, synovial fibroblasts, macrophages, and infiltrating immune cells exhibit disrupted mitochondrial bioenergetics, often characterized by a metabolic shift from oxidative phosphorylation to glycolysis. This reprogramming is accompanied by excessive production of mitochondrial reactive oxygen species (mtROS), which further damages mitochondrial DNA, lipids, and proteins, perpetuating a cycle of oxidative stress.[5]

Along with mitochondria, lysosomes are another type of multifunctional organelles implicated in a variety of diseases. Lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2) are integral glycoproteins located primarily on the lysosomal membrane, playing essential roles in maintaining lysosomal integrity, autophagy, and immune regulation.[6] Recent research has highlighted their involvement in the pathogenesis of autoimmune diseases, including RA, where synovial fibroblasts and macrophages are known to exhibit enhanced autophagy. This process is regulated in part by LAMP1 and LAMP2. These proteins facilitate the fusion of autophagosomes with lysosomes and support the degradation of cellular debris and damaged organelles, including dysfunctional mitochondria. Dysregulated autophagy can lead to persistent synovial inflammation and resistance to apoptosis in RA synoviocytes.[7,8] Both LAMP1 and LAMP2 have been shown to translocate to the cell surface under inflammatory and neoplastic conditions, where they may interact with immune cells or contribute to the release of lysosomal contents.[9] This surface expression is associated with increased cytokine production and joint tissue damage in RA. Moreover, LAMP proteins may influence the formation of extracellular vesicles, which transport inflammatory mediators between synovial cells.[10]

As immune mediated inflammation is a key pathogenetic driver in RA, it is essential to examine the involvement of proinflammatory cytokines such as interleukins 1 and 6 (IL1 and IL-6). Both cytokines contribute to synovial inflammation-induced cartilage degradation and to the systemic manifestations of the disease. IL-1, particularly IL-1β, is produced by activated macrophages, synovial fibroblasts, and infiltrating immune cells in the RA joint.[11] IL-6 is abundantly expressed in the RA synovium and contributes to both local joint inflammation and systemic symptoms such as fatigue, anemia, and osteoporosis. IL-6 signals through the gp130 receptor complex and activates the JAK/STAT3 (Janus kinase/signal transducer and activator of transcription 3) pathway, promoting Th17 differentiation, B cell activation, and acute-phase protein synthesis.[12] IL-6 is also involved in enhancing angiogenesis and synovial pannus formation.[13]

Another molecule related to inflammation is the chitinase-3-like protein 1 (YKL-40) glycoprotein, which plays a role in tissue remodeling and the immune response. Elevated levels of YKL-40 are associated with various inflammatory conditions, making it a useful biomarker for assessing inflammation.[14] Our previous studies have shown an increase of over 50% in YKL-40 levels across different pathologies, while in patients with clinically active RA, the glycoprotein is elevated in 95% of cases.[15]

Our working hypothesis is that the interplay between mitochondrial dysfunction, interleukins, and lysosome-associated membrane proteins constitutes a self-amplifying loop of oxidative stress and inflammation. This loop contributes to the persistence of synovitis and the progression of joint damage in RA. The elucidation of these mechanisms will not only enhance our understanding of RA pathogenesis but may also open new therapeutic avenues targeting mitochondrial integrity and inflammatory signaling pathways. Therefore, we aimed to examine molecules related to mitochondrial function, autophagy, and inflammation in patients with RA, and to follow their dynamics after treatment with one of the most commonly used therapeutics—methotrexate (MTX).

We present novel data on the correlations between key markers of these fundamental pathogenetic processes and the effects of treatment in RA patients.

METHODS

Patients

The study involved 10 treatment-naive RA patients diagnosed according to the 2010 criteria of the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR). They were treated with MTX at optimally approved daily doses (mean of 15 mg/week orally) for six months. Conventional biochemical and immunological markers, such as rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) antibodies, and acute phase reactants including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), were measured at the start of treatment and after the follow-up period. Venous blood samples were collected before and after therapy. The study was approved by the Medical University-Plovdiv Scientific Ethics Committee (Approval Number: 4/08.06.2022, Date: 20.06.2022). Informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

The activity of RA in all patients was assessed using the Disease Activity Score 28 (DAS28), which evaluates 28 joints. This score is based on counts of tender and swollen joints, the patient's global assessment of disease activity, and either ESR or CRP levels. The thresholds for classifying disease activity are as follows: remission ≤2.6, low activity ≤3.2, moderate activity ≤5.1, and high activity >5.1.[16]

Isolation of Plasma and Peripheral Blood Mononuclear Cells (PBMCs)

Ethylenediaminetetraacetic acid (EDTA) venous blood was obtained from each patient, following standard procedures for venipuncture. Samples were centrifuged at 1800 rpm for 10 minutes. Plasma was collected, aliquoted, and stored at -80^{0}C until analysis. The buffy coat layer was mixed with 2 mL of phosphate-buffered saline (PBS) (pH=7.4) and layered onto Histopaque (Sigma-Aldrich, d=1.077 g/mL) at a ratio of 1:1, followed by density gradient centrifugation at 1800 rpm for 30 minutes. The PBMC layer was aspirated, washed twice with 10 mL PBS, and centrifuged at 1800 rpm for 10 minutes. PBMCs were cultured in RPMI-1640 medium (Gibco CAT#P04-18000) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown overnight in 24-well plates and incubated at 37°C with 5% CO2 and high humidity. Cell viability and counts were assessed using the "LUNA" automated cytometer (Logos Biosystems, Anyang, Korea). For metabolic assays, cells were adjusted to a final concentration of 2×105 cells per well in 8-well Seahorse microplates using RPMI-1640. Viability and cell number were confirmed immediately prior to metabolic analysis.

Ultrasound Examination

Ultrasound assessment of the wrists, hands, and forefeet was conducted using a GE Logic E9 machine with an ML6-15-D Matrix Array linear probe for joint and tendon evaluation. Two-dimensional ultrasound (US) (B-mode/gray-scale US) and power Doppler US were performed. Gray-scale ultrasound (GSUS) frequency was set between 11-15 MHz, depending on the joint examined, and GSUS gain was adjusted based on joint region and patient characteristics, with an average value of 50%. The following settings were used for power Doppler ultrasound (PDUS): frequency of 8.3 MHz, pulse repetition frequency of 600-800 Hz, PDUS gain adjusted according to joint regions and patients with an average value of approximately 50%, as well as a low wall filter. The 7-joint ultrasound (GUS7) scoring system of Backhaus et al.[17] was applied.

We assessed GSUS and PDUS in seven joints of the clinically dominant hand or foot, selecting those most affected by swelling or tenderness, according to the GUS7 scoring system: the wrist, second and third metacarpophalangeal (MCP) joints, second and third proximal interphalangeal (PIP) joints, and second and fifth metatarsophalangeal (MTP) joints. Several parameters were evaluated following the definitions and standardized protocols of Outcome Measures in Rheumatology (OMERACT), including the presence of synovitis and tenosynovitis.[18] The scoring ranges were as follows: 0-27 for GSUS synovitis, 0-7 for GSUS tenosynovitis/paratenonitis, 0-39 for PDUS synovitis, and 0-21 for PDUS tenosynovitis/paratenonitis. The GUS7 score was calculated as the sum of synovitis and tenosynovitis/paratenonitis scores on GSUS, and the synovitis and tenosynovitis scores on PDUS. The DAS28 reference values for disease activity are as follows: remission ≤2.6; low disease activity >2.6-3.2; moderate disease activity >3.1-5.1; and high disease activity >5.1.

ELISA for Detection of Plasma Levels of YKL-40, IL-1β, IL-6, and LAMPs

Plasma levels of YKL-40 (Quidel, CAT#8020), IL-1β (Rand D, CAT#DLB50), IL-6 (Elabscience, CAT#E-EL-H6156), and LAMPs (SunRed, CAT#DZE201124185/6D) were measured in all patients using ELISA (Enzyme-Linked Immunosorbent Assay). The assays were performed in duplicate according to the manufacturer's instructions. The intra-assay coefficients of variation (CV) was 10%, and the inter-assay CV was <12 %. The assay employed a sandwich-based ELISA method, and optical density was measured at 450 nm using a Tecan Sunrise ELISA reader.

Assessment of Mitochondrial Function by Mito Stress Test

The Mito Stress Test measures oxygen consumption rate (OCR), a real-time indicator of mitochondrial respiration in living cells. The bioenergetic profile is determined by five key parameters: adenosine triphosphate (ATP) production, proton leak, maximal respiratory capacity (MRC), spare respiratory capacity (SRC), and non-mitochondrial oxygen consumption (NMOC). The plates were hydrated with double-distilled water, and PBMCs were cultured on the first day after venipuncture. On the following day, after assessing the viability and number of cultured cells, they were seeded onto hydrated plates at a concentration of 2×105 cells/mL in Seahorse XFp Base Medium, pH 7.4 (Agilent CAT#103576-100). PBMCs were visualized using an inverted microscope prior to analysis to confirm even distribution in the wells. The PBMCs were analyzed in triplicate in a series of consecutive runs, following strict adherence to the same experimental procedure. All PBMCs were isolated and processed in the same manner. Cells were counted immediately after isolation, followed directly by bioenergetic quantitation. Each bioenergetic experiment was performed in triplicate. Patient data were statistically processed in triplicate both before and after therapy.

Mitochondrial respiration was examined in real time following the application of inhibitors. Basal OCR was measured using oligomycin to assess mitochondrial ATP production. After treatment of the cells with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), maximum mitochondrial respiration and spare respiratory capacity were determined. Finally, the addition of rotenone and antimycin A allowed for the evaluation of non-mitochondrial respiration rates.

Statistical Analysis

The data were statistically processed using Prism software, version 10. Descriptive statistics were applied to determine group means. The normality of data distribution was assessed, and all variables were found to follow a normal distribution, justifying the use of a paired t-test to determine statistical significance in both groups. P-values less than 0.05 were considered statistically significant. Data from the metabolic tests were analyzed using Wave software.

Software

Clinical and laboratory data were analyzed statistically using Prism software, version 10. Metabolic test data were processed using Wave software.

RESULTS

We investigated disease activity in 10 RA patients, evaluating clinical and laboratory parameters before and after therapy. Table 1 presents laboratory values (CRP, RF, ESR, and anti-CCP) and ultrasound scores (DAS28, GUS7) used to assess disease activity in RA patients. All examined parameters showed improvement following treatment.

Table 1. Clinical laboratory parameters and ultrasound scores in RA patients before and after treatment with MTX

Parameters	Before therapy (Mean±SD)	After therapy (Mean±SD)
CRP (mg/l), < 5	51.62±12.9	32.7±4.9
RF (IU/ml), < 10	36.06±8.2	10.17±3.35
ESR (mm/h), < 15	129.4±25.77	87.18±29.09
Anti-CCP-Ab (IU/ml), < 17	739.9±157	471.8±430.2
DAS28, remission ≤ 2.6	5.63±0.16	3.59±0.23
GUS7 score, (0-108)	26.06±1.65	11.29±1.08

Data are presented as the mean±standard deviation by Graph Pad Prism 10. CRP: C - reactive protein; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; Anti-CCP-Ab: anti-cyclic citrullinated peptide-antibody; DAS28: diseases activity score; GUS7: Ultrasound score.

The metabolic Mito Stress test was used to evaluate mitochondrial function before and after therapy. The analysis revealed a decrease in two key parameters (Figs. 1A-B) after treatment—basal respiration (p=0.018) and ATP production (p=0.03). Data are presented as mean±standard deviation (SD).

To assess inflammation in both cohorts, we measured plasma levels of the cytokines IL-1β, IL-6, and YKL-40 before and after therapy.

We found a significant decrease in YKL-40 levels (p=0.0078) (Fig. 2), along with reductions in standard inflammatory markers (CRP and ESR) after six months of therapy. The decrease in both interleukin levels was evident but not statistically significant.

Assessment of plasma levels of LAMP molecules revealed a reduction in LAMP1 levels (p=0.03) following therapy (Fig. 3). While not statistically significant, an increase in LAMP2 levels was observed in treated RA patients.

To further elucidate the interplay between cellular mechanisms and clinical outcomes in RA, we analyzed correlations between clinical scores, inflammatory cytokines, autophagy-related proteins, and mitochondrial function before and after MTX therapy (Fig. 4). Before treatment, significant associations were observed between LAMP1 and basal respiration (p=0.022, r=0.928); LAMP2 and ATP production (p=0.033, r=-0.886); DAS28 and proton leak (p=0.017, r=0.941); as well as YKL-40 and IL-6 (p=0.035, r=0.759) (Fig. 4A).

After therapy, strong correlations were found between LAMP1 and spare respiratory capacity (p=0.003, r=0.919), and between GUS7 and LAMP2 (p=0.027, r=0.692) (Fig. 4B).

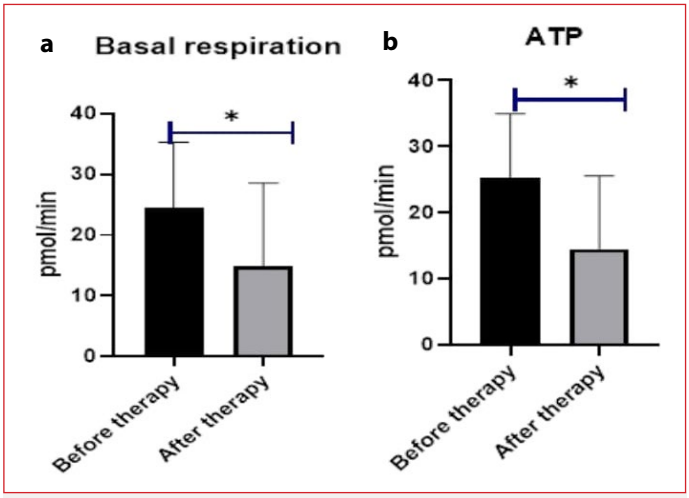

Figure 1. Basal respiration **(a)** and ATP **(b)** in PMBCs of RA-patients before and after theraphy with MTX.

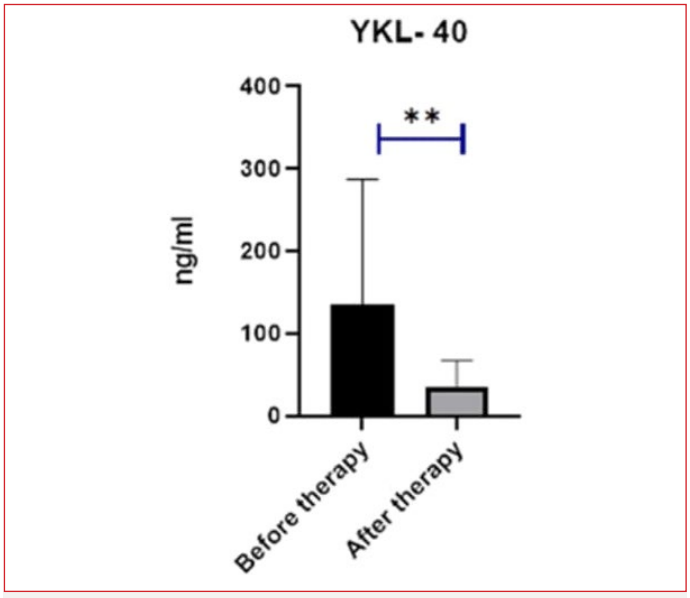

Figure 2. Plasma levels of YKL-40 of RA-patients before and after therapy with MTX. Presented as Mean±SD.

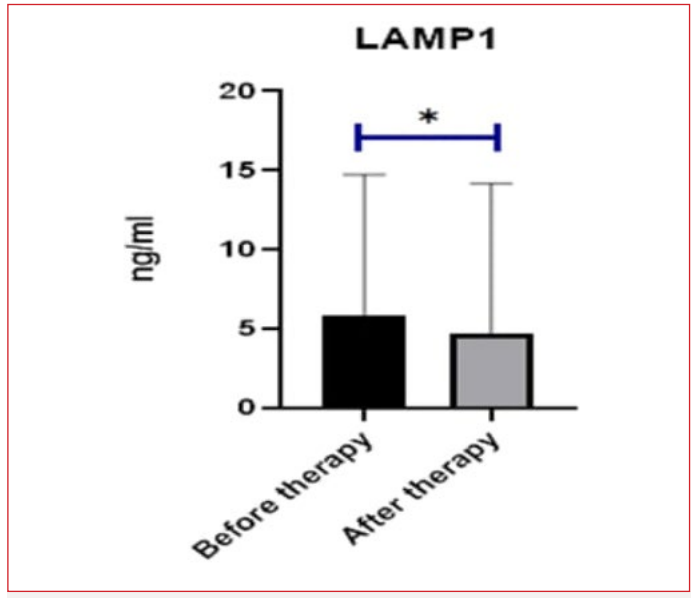

Figure 3. Plasma levels of LAMP1 of RA-patients before and after therapy with MTX. Presented as Mean±SD.

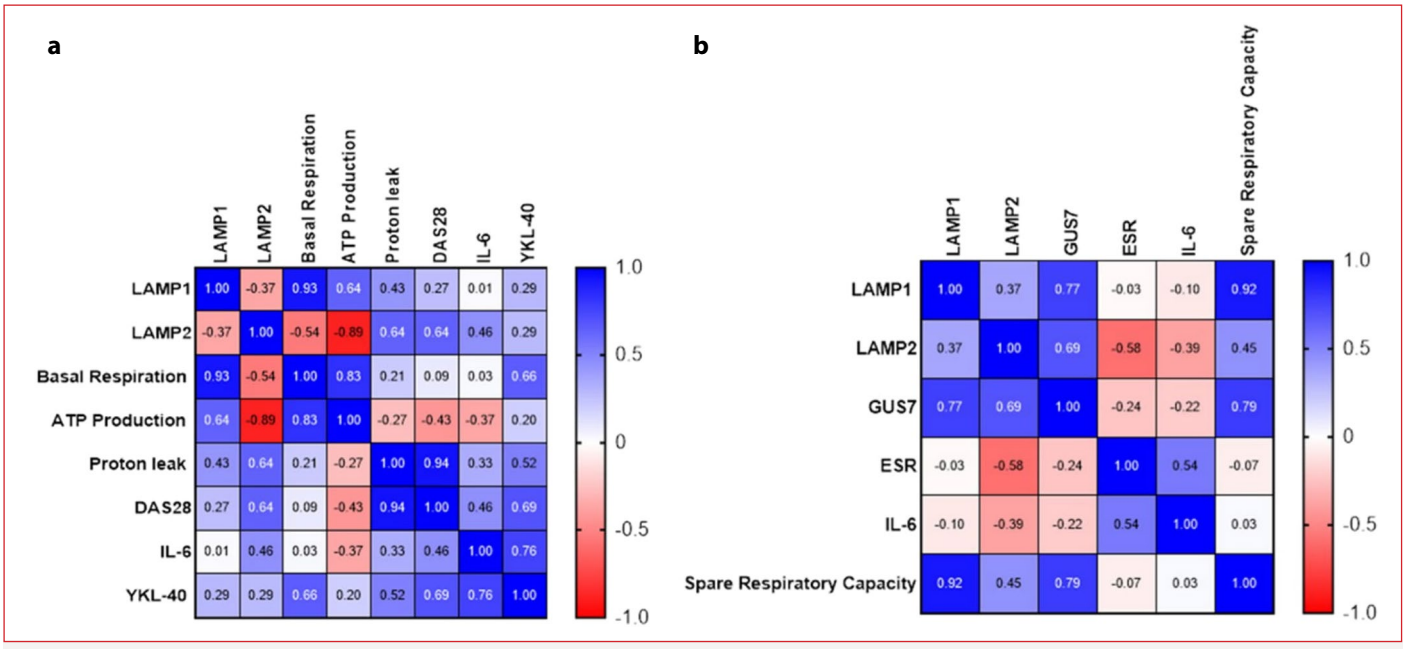

Figure 4. (a) Correlation matrix reflecting associations between laboratory indicators of inflammation, mitochondrial function and ultrasonographic findings before therapy. The numerical values reflect the correlation r coefficient. **(b)** Correlation matrix reflecting associations between laboratory indicators of inflammation, mitochondrial function and ultrasonographic findings after therapy with MTX. The numerical values reflect the correlation r coefficient.

DISCUSSION

This pilot study reinforces the pivotal role of mitochondrial dysfunction, inflammatory cytokines, and autophagy in the pathogenesis of RA, and demonstrates the beneficial modulatory effects of MTX on these processes. After six months of MTX therapy, we observed substantial clinical

improvement, evidenced by reductions in DAS28 and GUS7 scores, along with a marked decline in key inflammatory markers, including CRP, ESR, RF, and anti-CCP.

One of the interesting findings of our study was the significant decrease in basal oxygen consumption (p=0.018) and ATP production (p=0.03) following MTX therapy. This may reflect an overall improvement in mitochondrial efficiency or a reduction in the chronic hyperactivation of mitochondrial bioenergetics commonly associated with RA pathology. These observations are consistent with previous reports by Biniecka et al.,[5] who described a shift from oxidative phosphorylation to glycolysis in synovial cells, leading to elevated levels of mitochondrial reactive oxygen species (mtROS). Additionally, Zhou et al.[4] demonstrated that mtROS are potent activators of the NLRP3 (NOD-like receptor family pyrin domain-containing 3) inflammasome, contributing to sustained inflammation in RA. Therefore, the normalization of mitochondrial respiration observed in our patients may indicate a disruption of this self-perpetuating cycle of oxidative stress and inflammation.

Our data also revealed a significant reduction in plasma levels of LAMP1 (p=0.03) after treatment, while LAMP2 levels showed a non-significant upward trend. LAMP1 and LAMP2 are essential for the fusion of autophagosomes with lysosomes and are crucial for the degradation of damaged organelles, including dysfunctional mitochondria. Altered levels of LAMP1 and LAMP2 have been reported in the synovium of RA patients, facilitating pathological autophagy that enables synoviocytes to resist apoptosis and sustain inflammation.[19] The post-treatment decrease in LAMP1 observed in our cohort may reflect a shift toward normalization of autophagic flux and reduced turnover of damaged mitochondria, potentially diminishing the source of endogenous danger signals.

These findings align with the observations of Kato et al.,[20] who highlighted the dual role of LAMP proteins in maintaining lysosomal integrity and modulating antigen presentation. Furthermore, the altered surface expression of LAMPs under inflammatory conditions, as described by Kany et al.,[21] may amplify immune cell activation and cytokine production. Our findings suggest that MTX therapy may partially restore lysosomal homeostasis and reduce autophagy-associated inflammation in RA.

The significant reduction in YKL-40 (p=0.0078), along with decreased plasma levels of IL-1β and IL-6 after MTX therapy, underscores the anti-inflammatory effects of the treatment at the molecular level. IL-1β and IL-6 are key cytokines in RA pathogenesis, promoting synovial inflammation, cartilage degradation, and systemic symptoms.[11] The decline in these cytokines likely reflects reduced immune cell activation and an improved synovial microenvironment following therapy.

YKL-40, a glycoprotein implicated in tissue remodeling and chronic inflammation, has been proposed as a biomarker for disease activity in RA. Its reduction in our cohort after treatment supports previous findings linking YKL-40 to inflammatory burden in RA.[22] Furthermore, considering the role of IL-6 in driving Th17 differentiation and promoting chronic inflammation through the JAK/STAT3 pathway, the observed post-treatment decrease may have broader implications for immune rebalancing.[23]

Notably, we observed a significant association between LAMP1 and basal respiration (p=0.022), as well as between LAMP2 and ATP production (p=0.033), suggesting a direct link between lysosomal-autophagic activity and mitochondrial bioenergetics. These findings support the hypothesis that autophagy regulates mitochondrial turnover and function, particularly under inflammatory stress, as proposed by Ryter et al.[24] Furthermore, DAS28 scores correlated with proton leak (p=0.017), indicating that mitochondrial membrane integrity may reflect clinical disease activity. Similarly, YKL-40 and IL-6 levels were significantly correlated (p=0.035), supporting their roles as co-regulated inflammatory markers and reinforcing previous findings on their involvement in RA pathology.[12]

After MTX therapy, we found strong correlations between LAMP1 and spare respiratory capacity (p=0.003, r=0.919), suggesting that improvements in mitochondrial reserve capacity may be associated with normalized lysosomal function. Additionally, the GUS7 score correlated with LAMP2 (p=0.036, r=0.677), and ESR with IL-6 (p=0.05, r=0.640), reflecting the close relationship between clinical inflammation and molecular immune activity.

These correlations not only validate the biological relevance of the measured biomarkers but also support the concept that MTX modulates RA activity through interconnected pathways involving mitochondrial regulation, autophagy, and cytokine signaling.

The interplay between mitochondrial dysfunction, elevated proinflammatory proteins, and dysregulated autophagy creates a self-amplifying cycle of inflammation and tissue damage in RA. Our study contributes to a better understanding of these complex processes in the context of RA.

One of the main limitations of this study is the small sample size (n=10), which reduces statistical power and increases the risk of both false-positive (Type I) and false-negative (Type II) results. Nevertheless, this investigation was designed as a pilot study, aiming to identify potential trends and biomarkers related

to mitochondrial dysfunction, autophagy, and inflammation in patients with RA treated with MTX. Therefore, the findings should be interpreted as preliminary and hypothesis-generating, requiring confirmation in future studies with larger and more representative cohorts. Additionally, the absence of a control group (either untreated RA patients or healthy individuals) makes it difficult to determine whether the observed changes are specific to MTX treatment or part of the natural disease course. However, the combined approach (clinical assessment, molecular markers, and mitochondrial function) provides a comprehensive view of the pathogenetic processes in RA. Future studies with larger cohorts and long-term follow-up will allow for better stratification and personalization of the therapeutic approaches.

CONCLUSION

In conclusion, we present novel data on correlations between mitochondrial function parameters and autophagy- and inflammation-related proteins in RA patients after treatment with MTX. The pilot findings may pave the way for future clinical applications related to monitoring disease severity and evaluating treatment response.

Ethics Committee Approval: Ethics committee approval was obtained from Medical University-Plovdiv Scientific Ethics Committee (Approval Number: 4/08.06.2022, Date: 20.06.2022)

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest: The authors have no conflicts of interest to declare.

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