

RESEARCH ARTICLE

Platelet mitochondrial respiration and coenzyme Q₁₀ could be used as new diagnostic strategy for mitochondrial dysfunction in rheumatoid diseases

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Abstract

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder affecting both small and large synovial joints, leading to their destruction. Platelet biomarkers are involved in inflammation in RA patients. Increased circulating platelet counts in RA patients may contribute to platelet hyperactivity and thrombosis. In this pilot study we evaluated platelet mitochondrial bioenergy function, CoQ₁₀ levels and oxidative stress in RA patients.

Methods

Twenty-one RA patients and 19 healthy volunteers participated in the study. High resolution respirometry (HRR) was used for analysis of platelet mitochondrial bioenergetics. CoQ₁₀ was determined by HPLC method; TBARS were detected spectrophotometrically.

Results

Slight dysfunction in platelet mitochondrial respiration and reduced platelet CoQ₁₀ levels were observed in RA patients compared with normal controls.

Conclusions

The observed decrease in platelet CoQ₁₀ levels may lead to platelet mitochondrial dysfunction in RA diseases. Determination of platelet mitochondrial function and platelet CoQ₁₀ levels could be used as new diagnostic strategies for mitochondrial bioenergetics in rheumatoid diseases.

roles of these authors are articulated in the 'author contributions' section.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder affecting both small and large joints leading to their destruction. RA is frequently a progressive disease, with inflammation and tissue degradation in joints leading to painful deformity and immobility.

RA can also include effects on the cardiovascular system, cancer, and a wide spectrum of conditions such as depression, mental difficulties, fatigue and physical disabilities. Clinical symptoms in early stage RA are characterized by swollen tender joints and morning stiffness. Indications of disease activity and progression are elevated levels of C-reactive protein (CRP) and an increased erythrocyte sedimentation rate [1]. Serum CRP levels in RA patients of around 3 mg/L are associated with an elevated risk of cardiovascular disease (CVD) and levels up to 10 mg/L are associated with a very high risk of CVD [2].

The chronic inflammatory state in insufficiently treated RA patients leads to a complex clinical picture with systemic manifestations noticed in lungs, musculoskeletal system (cartilage destruction, bone erosion and loss range of motion) and hematologic abnormalities, such as anemia, leukopenia, and thrombocytopenia [3–5]. Tissue destruction erupts as synovitis, an inflammation of the joint capsule consisting of the synovial membrane and synovial fluid. Pro-inflammatory molecules such as cytokines contribute to the inflammation and platelet dysregulation in RA [6].

Genetic and environmental factors contribute to the clinical picture of RA. Genetic factors include the generation of autoreactive T and B cells with secondary viral or bacterial infections. Other factors, such as smoking, obesity, age, drugs, infections, lung disease and periodontal disease participate in RA [7, 8]. In early stage RA the production of reactive oxygen species (ROS) is elevated [9–13]. One of the main sources of ROS and adenosine triphosphate (ATP) production are the mitochondria. Several studies have reported mitochondrial dysfunction (not in platelets) and enhanced ROS production in rheumatoid arthritis [14–16].

In the pathophysiology of RA crucial role is played by the platelets, which contain only 5–8 mitochondria per platelet [17]. In the resting state platelets obtain ATP predominantly from glycolysis rather than oxidative phosphorylation (OXPHOS) [18]. Glycolysis produces lactate and generates two ATP molecules per one glucose molecule. OXPHOS produces 36 ATP molecules per one glucose molecule. Glycolysis produces ATP faster than OXPHOS [19].

An essential component of the mitochondrial respiratory chain for energy production is coenzyme Q₁₀ (CoQ₁₀) which also has antioxidant properties. Our previous study on the rat model of rheumatoid arthritis (adjuvant-induced arthritis) showed reduced mitochondrial oxidative phosphorylation in myocardial and skeletal muscle [20], increased markers of inflammation and decreased concentrations of coenzyme Q_{9-OX} in skeletal muscle tissue and mitochondria [21].

Platelet mitochondrial dysfunction has been documented in patients with several human diseases [18] including Alzheimer's disease [22], depression [23], chronic kidney disease [24–26], patients after kidney transplantation [27]. Platelet mitochondrial dysfunction has also been observed in human ageing [28] and in winter time [29]. Data on platelet mitochondrial function in patients with RA are not available.

We performed studies to test the hypothesis that a deficit of CoQ₁₀ and disturbance of platelet mitochondrial function occurs in RA patients. A deeper knowledge of RA effects on platelet mitochondrial activities and CoQ₁₀ levels may contribute to the understanding of the pathobiochemical mechanisms of RA.

2. Materials and methods

2.1. Subjects

Twenty-one patients with confirmed RA participated in the study: 20 women and one man, aged from 38 to 79 years with a mean age of 61.2 ± 28.6 years and a mean body mass index (BMI) of $25.8 \pm 1.08 \text{ kg.m}^{-2}$. All chronic RA patients (*RA_ALL*) were treated with relevant conventional therapy (aceclofenac, vitamin D, methotrexate, plaquenil). From these patients, 5 of them were included in a subgroup *RA_CRP* (with high CRP from 9.50 mg/L to 11.99 mg/L) and 4 patients in a subgroup *RA_CVD* treated also with conventional therapy for cardiovascular diseases. The control group consisted of 19 human subjects (7 men and 12 women), aged from 56 to 81 years, with a mean age of 68.4 ± 13.3 years, with a mean of BMI of $25.0 \pm 1.16 \text{ kg.m}^{-2}$. RA patients on statins therapy were not included in the study.

The study was carried out according to the principles expressed in the Declaration of Helsinki, and the study protocol was approved by the Ethical Committee of the Academic Ladislav Dérer's Hospital, Bratislava, Slovakia (1/0245/19, 27 June 2018). Written informed consent form was obtained from each subject before enrollment in the study.

2.2. Observed parameters

The following parameters were measured in a clinical biochemical laboratory using standard methods: blood hemoglobin, leucocytes and platelets count, serum c-reactive protein, serum creatinine, uric acid, AST (aspartate aminotransferase), ALT (alanine aminotransferase), GMT (gama-glutamyltransferase), total proteins (TP).

2.3. Coenzyme Q₁₀ and oxidative stress

Coenzyme Q_{10-TOTAL} (ubiquinol+ubiquinone) in whole blood, plasma and isolated platelets were estimated using HPLC method with UV detection [30, 31]. For the oxidation of ubiquinol to ubiquinone, 100 μl of 1,4-benzoquinone (2 mg/1ml double distilled water—daily fresh) was added to 500 μl of blood or plasma and vortexed for 10 seconds [32]. After 10 minutes incubation at room temperature 2 ml of the mixture hexane/ethanol (5/2 v/v) was added, shaken for 5 minutes and centrifuged at 1 000 g for 5 minutes. The hexane layer was separated and extraction procedure was repeated with 1 ml of the extraction mixture. Collected organic layers were evaporated under nitrogen at 50°C. The residues were taken up in 99.9% ethanol and injected into a reverse phase HPLC column (SGX C18, 7 μm , Tessek Ltd). Elution was performed with methanol/acetonitrile/ethanol (6/2/2 v/v/v) at a flow rate 0.9 ml/min. The concentrations of CoQ_{10-TOTAL} were detected with an UV-detector at 275 nm, using an external standard (Sigma). Data were collected and processed with a CSW32 chromatographic station (DataApex Ltd). Concentrations of CoQ_{10-TOTAL} were calculated in $\mu\text{mol.l}^{-1}$.

Coenzyme Q_{10-TOTAL} determination in platelets. Isolated human platelets (approx 150–250 millions) were disintegrated with 500 μl of cold methanol [33]. Oxidation of ubiquinol to ubiquinone was performed with 1,4-benzoquinone as described for plasma extraction. The cell suspension was extracted with 2 ml hexane by shaking for 5 minutes. After centrifugation, organic layer was separated, evaporated and measured as described above. Concentrations of CoQ_{10-TOTAL} were calculated in pmol.10^{-9} cells.

A parameter of oxidative stress—thiobarbituric acid reactive substances (TBARS) was estimated by spectrophotometric method [34].

2.4. Platelet preparation

Blood samples were collected by venipuncture in two 9 mL K3EDTA (tripotassium ethylenediaminetetraacetic acid) tubes each day between 7:00 and 8:00 a.m. and transported at 25°C room temperature to the laboratory. For platelet (PLT) isolation, the tubes with blood were centrifuged at room temperature at 200×g for 10 min using swing-out rotor without breaking. Platelet-rich plasma (PRP) was transferred into a new plastic tube and mixed with 100 mM EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) to a final concentration of 10 mmol·L⁻¹. The pellet after centrifugation at 1200×g, was washed with 4 mL of DPBS (Dulbecco's Phosphate-Buffered saline) plus 10 mM EGTA and finally resuspended in 0.4 mL of the same solution. The PLT suspension was counted (10 times diluted) on hematology analyzer Mindray BC-2800 (Mindray, China) [35].

2.5. Platelet mitochondrial respiration and oxidative phosphorylation

Oxygen consumption in the intact and permeabilized platelets and the capacity of oxidative phosphorylation (OXPHOS) at Complex I were determined with the high-resolution respirometry (HRR) method. HRR is a sensitive technique to determine mitochondrial bioenergetic function in human platelets isolated from peripheral blood [36, 37]. For mitochondrial respirometric analysis, 200×10⁶ platelets were used in a 2 mL chamber of an O2k-Respirometer (Oroboros Instruments, Innsbruck, Austria). The respiration was measured at 37°C in a mitochondrial respiration medium, MiR05 and 20 mM creatine following SUIT protocol RP1 (Substrate-Uncoupler-Inhibitor-Titration). The data were collected with the DatLab software (Oroboros Instruments, Innsbruck, Austria) using a data recording interval of 2 s [36, 38]. A representative trace from the respirometric measurement is shown in Fig 1.

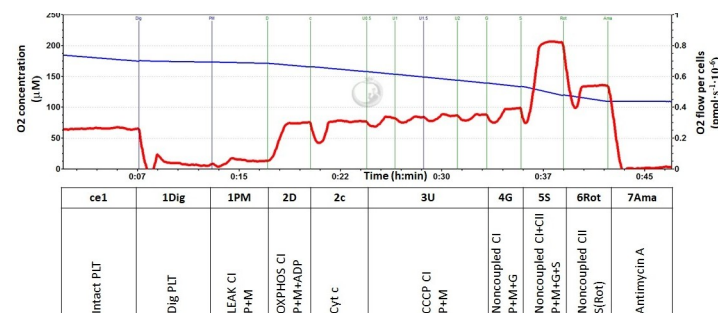


Fig 1. Respirometric analysis of mitochondrial function in human platelets. The trace from the measurement of platelet (PLT) respiration at 37°C in a respiration medium MiR05 and 20 mM creatine. The blue line represents oxygen concentration (µM), and the red trace represents oxygen consumption as flow per cells (pmol O₂·s⁻¹·10⁻⁶ cells). The modified substrate-uncoupler-inhibitor-titration (SUIT) reference protocol 1 (RP1) [38], includes following steps: **ce1**: Oxygen consumption rate of intact PLT (routine respiration); **1Dig**: Respiration rate of mitochondria in PLT permeabilized with digitonin; **1PM**: Complex I-linked LEAK (State 4) respiration reflects the rate of mitochondrial respiration with exogenous substrates (pyruvate and malate); **2D**: Complex I-linked OXPHOS (State 3) after ADP addition reflects CI-linked ATP production; **2c**: Cytochrome c addition—a test for the integrity of outer mitochondrial membrane; **3U**: The rate after addition of an uncoupler CCCP represents maximal CI-linked oxidative capacity with substrates pyruvate and malate (uncoupled from OXPHOS). **4G**: Noncoupled Complex I-linked oxygen consumption after the addition of substrate glutamate; **5S**: Noncoupled Complex I- and Complex II-linked oxygen consumption after the addition of CII substrate succinate; **6Rot**: Noncoupled Complex II-linked oxygen consumption after the addition of rotenone—an inhibitor of Complex I; **7Ama**: Residual oxygen consumption (ROX) after the addition of antimycin A—an inhibitor of CIII represents respiration that is not associated with electron transfer pathways. This respiration is subtracted from all values for the determination of mitochondrial electron transfer pathways-related oxygen consumption.

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2.6. Data analysis

The results are reported as the mean \pm standard error (sem). Statistical analyses were performed in Graph-Pad Prism 6 for Windows. One-way ANOVA with pairwise tests and Holm-Sidak's correction for multiple comparisons were used for comparisons of groups with the control group. Differences are marked statistically significant where the respective adjusted p-values are less than 0.1. The levels of adjusted p-values vs control group are marked as follows: ****p<0.0001, ***p<0.001; **p<0.01 *p<0.05, †p<0.1.

3. Results

3.1. Metabolic parameters of human volunteers and of RA patients

The concentration of *hemoglobin* in all groups of RA patients was similar to the control group. *The leucocyte count* was not significantly increased in RA_ALL patients (110.98%), in RA_CRP group (120.45%), in RA_CVD patients (101.20%) vs control group.

The platelet count in group of RA_ALL patients was significantly increased to 122.8% (p<0.05) vs control group; count of platelets in group RA_CRP was increased to 124.9% (p<0.1), the highest count of platelets was in RA_CVD subgroup to 140.8% (p<0.05) vs control data (Table 1 and Fig 3). *CRP*, the marker of inflammation, in RA_ALL patients was increased to 219.3% vs control data. The highest CRP was in group RA_CRP, increased to 613.3%, (p<0.0001) vs control data, in group RA_CVD patients CRP was similar to control group. *Creatinine* concentration in plasma was without significant differences between control and RA groups. Only in RA_CVD group was this parameter slightly decreased to in comparison with the control group. *Uric acid* concentration was significantly decreased in RA_ALL patients, vs control data (p<0.05), representing 84.55%. Liver enzymes showed various results. *AST* was in reference values, slightly influenced by RA. In RA_ALL group of patients *AST* was not significantly increased to 113.48%, in RA_CRP decreased to 86.25%, in RA_CVD increased to 124.80% in comparison with control data. *ALT* activity in RA patients was not significantly increased from 120.07% (RA_ALL) to 168.46% (RA_CRP group). On the contrary, *GMT* activity was significantly

Table 1. Metabolic parameters of human volunteers and of RA patients.

Groups	Control	RA_ALL	RA_CRP	RA_CVD
Parameter	(N = 12)	(N = 21)	(N = 5)	(N = 3)
Hgb (g/L)	140.5 \pm 2.3	136.2 \pm 2.1	136.0 \pm 4.3	140.0 \pm 4.0
Le (10x9/L)	6.65 \pm 0.44	7.38 \pm 0.64	8.01 \pm 1.05	6.73 \pm 1.22
PLT (10x9/L)	219.2 \pm 9.4	269.3 \pm 13.1*	273.8 \pm 23.6†	308.7 \pm 65.9*
Creatinine (μ mol/L)	73.3 \pm 5.0	70.2 \pm 3.2	76.6 \pm 8.5	72.7 \pm 9.8
Uric acid (μ mol/L)	308.7 \pm 17.0	261.1 \pm 11.0*	273.8 \pm 16.4	260.0 \pm 17.6
AST (μ kat/L)	0.371 \pm 0.030	0.421 \pm 0.042	0.320 \pm 0.016	0.463 \pm 0.080
ALT (μ kat/L)	0.297 \pm 0.018	0.335 \pm 0.043	0.470 \pm 0.142	0.250 \pm 0.020
GMT (μ kat/L)	0.751 \pm 0.139	0.345 \pm 0.036***	0.392 \pm 0.121*	0.457 \pm 0.019†
CRP (mg/L)	1.50 \pm 0.20	3.29 \pm 0.79	9.20 \pm 1.12****	1.66 \pm 0.66
Total proteins (g/L)	28.7 \pm 4.7	69.6 \pm 0.8****	68.2 \pm 0.8****	71.6 \pm 0.8****

Hgb–hemoglobin; Le–leucocytes; PLT–platelets; AST–aspartate aminotransferase; ALT–alanine aminotransferase; GMT–gamma-glutamyltransferase; CRP- c-reactive protein; Data of all groups are presented as mean \pm sem and statistically evaluated in comparison with control group.

****p<0.0001,

***p<0.001;

**p<0.01

*p<0.05, †p<0.1 vs control group.

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reduced in RA_ALL patients ($p < 0.001$) to 45.94% vs control data. In RA_CRP group of patients it was decreased to 52.20% ($p < 0.05$), in RA_CVD patients to 60.85% ($p < 0.1$). Total proteins were significantly increased in all RA groups in comparison with healthy human volunteers. In RA_ALL group ($p < 0.0001$), in RA_CRP ($p < 0.0001$), in RA_CVD group ($p < 0.0001$), (Table 1).

3.2. Platelet mitochondrial respiration and oxidative phosphorylation of control subjects and RA patients

In our trial we found slight differences in platelet mitochondrial oxygen consumption between groups of RA patients and control subjects. Differences between RA groups and control group statistically are evaluated by ANOVA test and in %.

Oxygen consumption in intact platelets (*ce1*) in RA_CRP group was decreased to 86.58% vs control group, in group RA_ALL and RA_CVD changed slightly vs control data. The rate of mitochondrial respiration with CI-linked substrates (*1PM-state 4*) in group of RA_ALL decreased to 87.90%; in group RA_CRP decreased to 81.45% vs control data and in group RA-CVD increased to 123.39% vs control data. CI-linked respiration coupled with ATP production (*2D* -CI-linked oxidative phosphorylation) was increased in group in RA_ALL to 132.21% ($p < 0.1$); in RA_CRP to 152.00% ($p < 0.1$); in RA_CVD to 135.16% vs control group. The respiration after addition of cytochrome c (*2c*) was increased in groups RA_ALL to 127.72%; in RA_CRP to 146.34%; in RA_CVD to 130.30%. Maximal oxidative capacity after uncoupler titration (*3U*) in group of RA_ALL was increased to 121.07%; in RA_CRP to 135.62%; in RA_CVD to 128.26% vs control group. After addition of exogenous substrate glutamate (*4G*) mitochondrial respiration was slightly increased in all RA groups, in RA_ALL to 118.89%; in RA_CRP to 133.72%; in RA_CVD to 126.60% vs control data. Noncoupled CI+CII mitochondrial respiration (*5S*) in all RA groups were similar to control group. Mitochondrial respiration of CII after inhibition of CI with rotenone (*6Rot*) in RA groups was similar to control group (Fig 2 and Table 2).

3.3. Coenzyme Q₁₀-TOTAL and TBARS in control subjects and in RA patients

Endogenous concentration of CoQ₁₀-TOTAL (ubiquinone + ubiquinol) in platelets of RA_ALL patients significantly decreased to 57.79% ($p < 0.0001$), in group RA_CRP

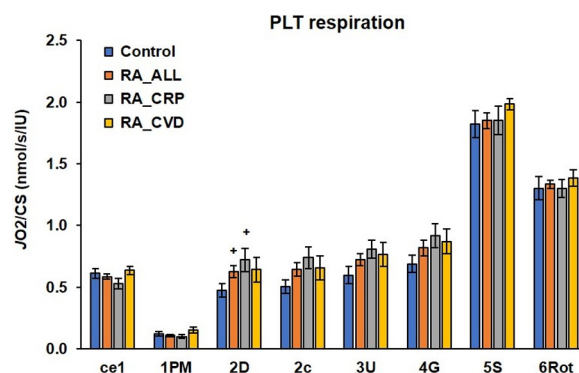


Fig 2. Platelet mitochondrial function in control subjects and groups of RA patients. The parameters of mitochondrial respiration in human platelets expressed as oxygen (O₂) flux per mitochondrial marker—the activity of citrate synthase (CS). The bars show mean ± sem. The names on x-axis represent steps in the SUIT protocol RP1 (see the legend for Fig 1). Control = control subjects; RA_ALL = all patients with rheumatoid arthritis; RA_CRP = rheumatic patients with high c-reactive protein concentration; RA_CVD = rheumatic patients with cardiovascular diseases; ⁺ $p < 0.1$.

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Table 2. Platelet mitochondrial function in control subjects and groups of RA patients.

Groups	JO2/CS [nmol/s/IU]			
	Control (N = 15)	RA_ALL (N = 21)	RA_CRP (N = 5)	RA_CVD (N = 3)
<i>ce1</i>	0.611±0.042	0.585±0.021	0.529±0.044	0.637±0.035
1Dig	0.045±0.013	0.038±0.006	0.041±0.015	0.053±0.018
1PM	0.124±0.017	0.109±0.008	0.101±0.015	0.153±0.027
2D	0.475±0.054	0.628±0.050 ⁺	0.722±0.094 ⁺	0.642±0.102
2c	0.505±0.054	0.645±0.052	0.739±0.089	0.658±0.096
3U	0.598±0.070	0.724±0.050	0.811±0.073	0.767±0.098
4G	0.688±0.070	0.818±0.063	0.920±0.098	0.871±0.099
5S	1.821±0.110	1.851±0.065	1.852±0.115	1.984±0.044
6Rot	1.303±0.096	1.336±0.034	1.301±0.074	1.385±0.069

Names of the parameters are in Fig 1. Data of all groups are presented as mean±sem and statistically evaluated in comparison with control group; ⁺p<0.1 vs control group.

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decreased to 59.42% (p<0.01) and in RA_CVD group to 87.02% (not significant) vs control group. Concentration of CoQ_{10-TOTAL} in whole blood and in plasma slightly decreased in RA_ALL and in RA_CRP groups, in RA_CVD decreased to 89.0% and 86.5% respectively vs control group. TBARS concentration was slightly increased in RA_ALL and in RA_CRP vs control data (Table 3).

3.4. Platelet count and platelet coenzyme Q_{10-TOTAL} in control subjects and in groups of RA patients

Mean of platelet count and platelet CoQ_{10-TOTAL} concentration in control subjects and in RA patients shown in Table 1 and 3 and Fig 3.

4. Correlations between platelet CoQ_{10-TOTAL} and platelet mitochondrial respiration in control subjects and RA_ALL patients (Fig 4A–4D)

Oxygen consumption by intact platelets (*ce1*) in RA_ALL patients positively correlated with CoQ_{10-TOTAL} concentration in platelets (p = 0.069), This correlation was not found in control subjects (Fig 4A). Oxygen consumption by mitochondria in permeabilized platelets (1PM) positively correlated with CoQ_{10-TOTAL} concentration in platelets of RA_ALL

Table 3. Endogenous coenzyme Q_{10-TOTAL} and TBARS in control subjects and in RA patients.

Groups	Control (N = 18)	RA_ALL (N = 21)	RA_CRP (N = 5)	RA_CVD (N = 3)
<i>Parameter</i>				
CoQ_{10-TOTAL}				
Platelets (pmol.10⁻⁹cells)	165.6.0±12.8	95.7±5.0****	98.4±4.9**	144.1±30.9
Blood (μmol.L⁻¹)	0.271±0.031	0.289±0.028	0.254±0.063	0.241±0.047
Plasma (μmol.L⁻¹)	0.409±0.039	0.408±0.029	0.383±0.049	0.354±0.064
TBARS (μmol.L⁻¹)	4.71±0.14	4.90±0.15	5.21±0.28	4.58±0.23

**** p<0.0001,

** p<0.01- statistically significant difference vs control group.

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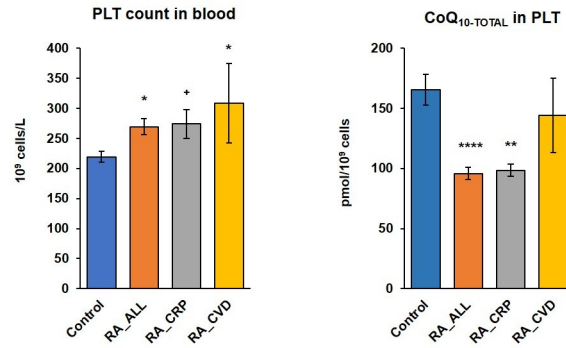


Fig 3. Platelet count and platelets coenzyme Q₁₀-TOTAL in control subjects and in groups of RA patients. CoQ₁₀-TOTAL (ubiquinol + ubiquinone); RA_ALL = all patients with rheumatoid arthritis; RA_CRP = rheumatic patients with high c-reactive protein concentration; RA_CVD = rheumatic patients with cardiovascular diseases; *p<0.1, **p<0.05, ***p<0.01, ****p<0.0001.

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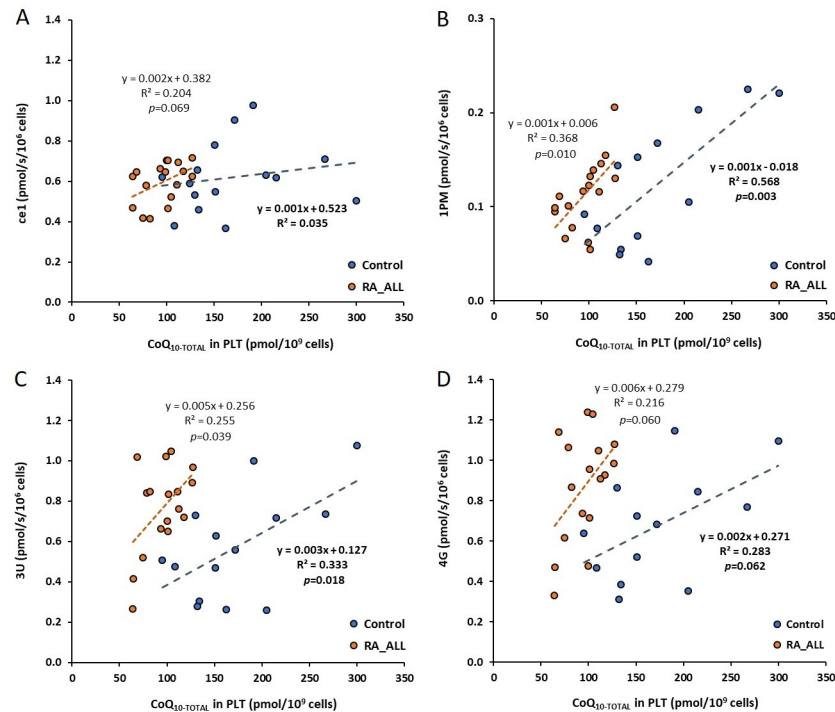


Fig 4. 4A: Correlation between CoQ₁₀-TOTAL in Platelets and Respiration of Intact Platelets in Control Subjects and RA_ALL Patients. ce1 = the rate of oxygen consumption in intact platelet; RA_ALL = all patients with RA; p = 0.069; CoQ₁₀-TOTAL = ubiquinol + ubiquinone; PLT = platelet. 4B: Correlation between CoQ₁₀-TOTAL in Platelets and Mitochondrial Respiration in Permeabilized Platelets in Control Subjects and RA_ALL Patients. 1PM = the rate of oxygen consumption in mitochondrial of permeabilized platelets (State 4); RA_ALL = all patients with RA; p = 0.010, p = 0.003 = statistically significant; CoQ₁₀-TOTAL = ubiquinol + ubiquinone; PLT = platelet. 4C: Correlation between CoQ₁₀-TOTAL in Platelets and 3U Platelets Mitochondrial Respiration in Control Subjects and RA_ALL Patients. 3U = the rate of oxygen consumption in platelet mitochondrial noncoupled respiration at CI; RA_ALL = all patients with RA; p = 0.039, in control group: p = 0.018; CoQ₁₀-TOTAL = ubiquinol + ubiquinone; PLT = platelet. 4D: Correlation between CoQ₁₀-TOTAL in Platelets and 4G Platelets Mitochondrial Respiration in Control Subjects and RA_ALL Patients. 4G = the rate of oxygen consumption in platelets mitochondrial noncoupled CI after addition of substrate glutamate CI; RA_ALL = all patients with rheumatoid arthritis; p = 0.060; CoQ₁₀-TOTAL = ubiquinol + ubiquinone; PLT = platelet.

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patients ($p = 0.010$) and control subjects ($p = 0.003$), (Fig 4B). We did not find significant correlation between CI-linked oxidative phosphorylation—ATP production (State 3) after ADP addition (2D) and CoQ_{10-TOTAL} concentration in platelets of control subjects or RA patients. The correlations between CoQ_{10-TOTAL} in platelets and 3U –platelet mitochondrial noncoupled respiration at CI in control subjects and RA_ALL patients were significant ($p = 0.018$, respectively $p = 0.039$), (Fig 4C). The correlation between platelets CoQ_{10-TOTAL} and noncoupled Complex I-linked oxygen consumption after addition of substrate glutamate (4G) was marginally significant in both control group ($p = 0.062$) and RA_ALL patients ($p = 0.060$), (Fig 4D). Noncoupled CI+CII-linked oxygen consumption after the addition of CII substrate succinate (5S), non-coupled CII-linked oxygen consumption after addition an inhibitor of Complex I (6Rot) did not correlate with CoQ_{10-TOTAL} concentration in platelets of control subjects and RA_ALL patients.

5. Discussion

This is the first study to examine platelet mitochondrial respiratory function and the relationship between platelet CoQ₁₀ level and mitochondrial respiration in patients with rheumatoid arthritis (RA).

Platelets play a crucial role in pathophysiology of RA, they are small (2–4 μm) anucleate circulating fragments generated from the megakaryocytes in the bone marrow [39]. They are released from bone marrow into circulation, where they live for 7–10 days. Platelets are involved in wound healing, angiogenesis, immunoregulation, and inflammatory processes and they play an integral role in intracellular communication [40]. Their main function in the blood stream is a rapid binding to damaged blood vessels. In patients with RA activated PLTs release pro-inflammatory microparticles, which interact with white blood cells causing joint and systemic inflammation [41].

Platelet mitochondrial function is believed to reflect the mitochondrial health of the organism. A deeper knowledge of mitochondrial energy function in isolated platelets may contribute to a better understanding of the pathogenesis of RA and similar musculoskeletal diseases in humans [42, 43]. Individuals with mitochondrial disorders have comorbid conditions that may increase their risk for poor bone health [43].

In this trial several metabolic parameters were affected by RA: the count of leucocytes and platelets, CRP levels and total proteins were increased and GMT activity was reduced in RA patients (Table 1). Very high levels of CRP can be associated with very high risk of CVD [2]. Platelet count in RA patients was increased from 269.3 to 308.7x10⁶ platelets per mL of blood compared to control group at 219.2 x10⁶ platelets per mL of blood. Platelet counts in healthy adults are between 150 x10⁶ and 450 x10⁶ per mL of blood, and these counts are changed with diseases [39]. Increased platelet count in RA patients can contribute to platelet hyperactivity and to thrombosis [44].

Increased platelet mitochondrial mass was observed in patients with myeloproliferative neoplasms and increased tumor necrosis factor- α (TNF- α). Similar effects of TNF-alpha on platelets in ageing was shown recently. The authors suggest altered bioenergetics in platelets and higher levels of baseline ADP and ATP could be key players in platelet hyperactivity in old mice [45].

Platelets are metabolically active cell fragments with high energy consumption. At rest, platelets supply 60% of energy from glycolysis and 30–40% from mitochondrial oxidative phosphorylation [15]. ATP is essential for platelet function. Mitochondria are key to platelet function and survival [37]. Mitochondria are important for energy supply in all organisms and platelet mitochondria act as the main energy suppliers during thrombus formation. Platelets contain a small count of mitochondria [14], they are metabolically flexible, they are capable to

adapt to different metabolic changes and can utilize glycolysis or fatty acid metabolism instead of mitochondrial ATP production via oxidative phosphorylation.

The present study found slight differences in mitochondrial respiration of permeabilized platelets of RA patients in comparison with control human volunteers (Fig 2 and Table 2), the differences in platelet mitochondrial function between control and RA data we expressed in %, when control data were considered 100%. The lower oxygen consumption of intact platelets (ce1) in RA_CRP patients can indicate damaged platelet membrane permeability. In permeabilized platelets (1PM) in RA_CRP group, platelet mitochondrial respiration linked to Complex I (State 4) was decreased, which reflects decreased platelet mitochondrial oxygen consumption at Complex I. Mitochondrial oxygen uptake is slightly reduced and could be caused by increased anaerobic metabolism, reprogrammed platelet mitochondrial OXPHOS metabolism to glycolysis in rheumatoid arthritis. The increase of ADP-stimulated respiration at Complex I (State 3) in RA_ALL patients to 132.21%, in RA_CRP group of patients with higher CRP to 152.0% vs control data and the increased of OXPHOS-coupling efficiency could be platelet mitochondria adaptation to inflammation. OXPHOS-coupling efficiency parameter (1-L/P) [46] as respiratory control index, was higher in RA_ALL and RA_CRP groups in comparison with control group (Fig 5). Our results showed higher platelet counts and ATP production by OXPHOS in patients with rheumatic arthritis which may correspond with reported hyperreactivity of platelets in RA patients [41]. Upon platelet activation both glycolysis and OXPHOS are upregulated and platelets could receive relatively more energy from glycolysis or fatty acids metabolism than from OXPHOS in comparison with resting platelets [47]. In our study stimulated ATP production at CI, stimulated mitochondrial oxygen consumption in noncoupled state at complex I, in non-coupled states at CI+CII and at Complex II (after rotenone CI inhibition), support the hypothesis of platelet activation accompanied by mitochondrial energy reprogramming in RA patients.

Mitochondria are important not only for energy production, but also responsible for regulation of intracellular signalling through ROS. Mitochondrial function is impaired during uncontrolled ROS production, which has a critical role in the development and progression of diseases. Mitochondria initiate apoptosis through the release of cytochrome *c* into the cytoplasm. Released cytochrome *c* from mitochondria by inducing mitochondrial cristae remodeling was supported by inflammation as well as by increased oxidative stress [48]. An increase in the permeability of the membrane, a release of cytochrome *c* and a loss of mitochondrial membrane potential are signs of cell death [49]. Decreased level of CoQ₁₀ in platelets and increased TBARS in RA patients with very high CRP concentration indicate increased oxidative stress that can contribute to the inflammatory processes (Table 3).

In the most common form of arthritis, osteoarthritis, not only mitochondrial dysfunction but also mitochondrial DNA damage is involved [50]. Mitochondrial dysregulation and oxidative stress in patients with chronic kidney disease was reported [51] and in the last years disturbances in platelet mitochondrial function were documented in several human diseases [15].

Oxidative stress supported glycolytic metabolism may contribute to the acceleration of inflammatory mechanisms in RA [52]. Elevated levels of TBARS were found in several studies in the inflamed joints of RA patients [9–13].

CoQ₁₀ plays a key role in mitochondrial energy production via OXPHOS, by way transferring electrons from Complex I and Complex II to Complex III along the respiratory chain in the inner mitochondrial membrane. Significantly reduced CoQ_{10-TOTAL} level in platelets together with oxidative stress in chronic inflammation in RA can lead to a deficit in platelet mitochondrial energy production, as well as to oxidative imbalance, which can enhance inflammation and tissue damage (Table 3 and Fig 3). Lipid peroxidation (TBARS) as an oxidative stress marker was increased in RA_ALL patients, in group of RA_CRP the values were highest. Decreased antioxidants level (ubiquinone + ubiquinol) and increased oxidative stress

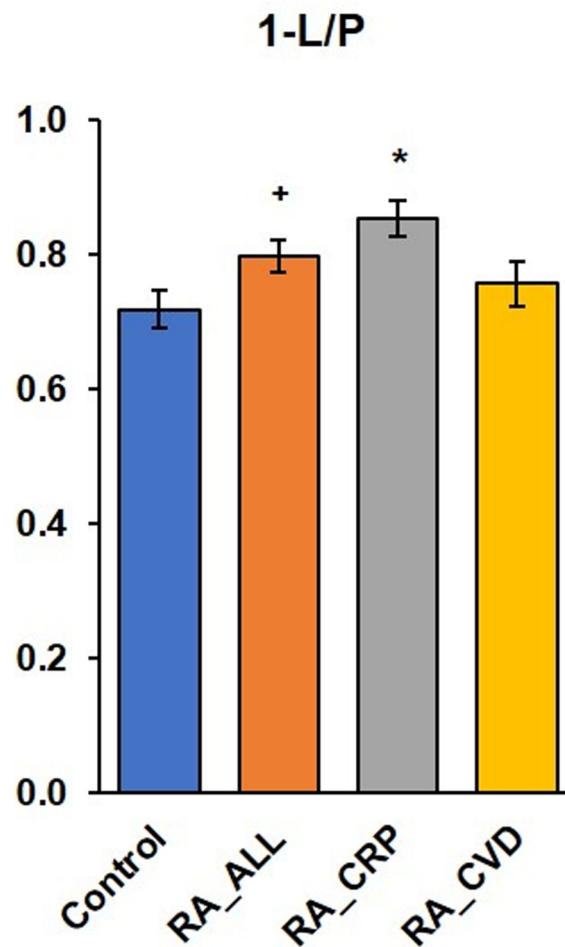


Fig 5. The OXPHOS-coupling efficiency in healthy control volunteers and groups of RA patients. RA_ALL = all patients with rheumatoid arthritis; RA_CRP = rheumatic patients with high c-reactive protein concentration; RA_CVD = rheumatic patients with cardiovascular diseases; + $p < 0.5$; * $p < 0.05$ statistical significance vs control group.

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participate in dysbalance of oxido-redox potential of RA patients [11–13, 16, 52]. In other chronic inflammatory diseases, ankylosing spondylitis (AS), leading to joint disability, increased oxidative stress and decreased levels of total antioxidant status were observed in meta-analysis of 22 studies, including 931 AS patients [53]. Increased lipid peroxidation in RA patients may be a reason for platelet membrane damage (ce1) in RA patients. In a previous study we confirmed that high lipid peroxidation is a very important parameter in metastatic urothelial carcinoma patients, which was associated with poor survival [54].

Mitochondrial respiration significantly correlated with platelet CoQ_{10-TOTAL} concentration in RA patients as well as in control group (Fig 4B and 4C). Marginally significant correlations were found between platelet CoQ_{10-TOTAL} concentration and ce1 (Fig 4A) for RA_ALL patients and between CoQ_{10-TOTAL} concentration and 4G (Fig 4D) for both RA_ALL patients and control group. These results support mitochondrial energy reprogramming and flexibility of platelet mitochondrial function in RA patients.

CoQ_{10-TOTAL} level, measured in blood plasma may reflect mitochondrial bioenergy damage of various tissues. Based on our data in patients with RA the level of platelet CoQ₁₀ seems to be

crucial for oxygen consumption by intact platelets or by mitochondria in platelets. Platelet CoQ_{10-TOTAL} level was positively correlated with platelet mitochondrial respiratory function in RA patients and it seems to be the most sensitive marker of platelet mitochondrial function in RA.

Decreased platelet CoQ_{10-TOTAL} level in chronic inflammatory RA diseases may lead to platelet mitochondrial dysfunction. Mitochondria are an important therapeutic target in osteoarthritis. Supplementary CoQ₁₀ therapy can suppress the glycolytic pathway, decrease lactate production and upon glycolytic suppression the intracellular energy metabolism is reprogrammed toward mitochondrial OXPHOS [55]. CoQ₁₀ supplementation in RA patients has beneficial effects on inflammatory cytokines (suppressed overproduction of TNF- α) and oxidative stress [56]. According to FL Crane, supplemental CoQ₁₀ regulates outer mitochondrial membrane permeability, which depends on the VDAC (Voltage dependent Anion Channels) [57]. CoQ₁₀ supplementation in RA patients can improve mitochondrial health and prevent or slow the progress of rheumatoid arthritis in humans.

6. Conclusion

This is the first study showed slight dysfunction of platelet mitochondrial respiration and reduced platelet CoQ₁₀ level in patients with rheumatoid arthritis (RA). The most sensitive marker of platelet mitochondrial dysfunction in RA patients seems to be platelet CoQ_{10-TOTAL} levels which positively correlated with platelet mitochondrial respiratory function in RA patients. High resolution respirometry method for measurement of platelet mitochondrial function, and platelet coenzyme Q₁₀ level determination could be used as a new diagnostic bioenergetic strategy in patients with rheumatoid arthritis. We conclude that decreased platelet CoQ_{10-TOTAL} level in chronic inflammatory RA diseases leads to platelet mitochondrial dysfunction. Reprogramming of platelet mitochondrial energy function could be possible by targeting supplementary therapy of RA patients with CoQ₁₀. The results contribute to the understanding the pathobiochemical mechanisms of rheumatic diseases.

Supporting information

S1 Data.
(XLSX)

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