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White blood cell mitochondrial DNA copy number is decreased in rheumatoid arthritis and linked with risk factors. A twin study

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ABSTRACT

Low mitochondrial DNA copy number (mtDNA CN) has been associated with e.g. cancer, cardiovascular and autoimmune diseases. We aimed to study a potential association between mtDNA CN and rheumatoid arthritis (RA).

The relative quantity of mitochondrial DNA compared to nuclear DNA was measured in peripheral white blood cells from 149 RA affected twin pairs and 1321 non-affected twin pairs. Multiple regression analysis including RA discordant twin pairs was performed in order to separate specific effects of RA and familial RA predisposition using non-RA affected twin pairs as reference group. In addition, we performed a twin pair level analysis including only RA discordant twin pairs evaluating the effect of cell type, auto antibodies and RA genetic risk factors.

Both the RA twins and their non-affected co-twins had significantly lower mtDNA CN than non-affected twins (−28.7 and −23.1 mtDNA CN, respectively). Adjusting for cell count attenuated these differences (−23.1 mtDNA CN and −20.1 mtDNA CN respectively). Within RA discordant twin pairs *PTPN22*(T) positive RA twins had a significantly lower amount than their co-twins (−16.3 mtDNA CN). *PTPN22*(T) had no effect among twins from non-affected twin pairs.

MtDNA CN is significantly lower in persons with established RA and in predisposed non-affected RA co-twins suggesting that mitochondrial variation may be involved in the RA disease pathways. Our results also suggest that the RA associated genetic risk factor, PTPN22(T), further decreases the mtDNA CN, but only in carriers with established RA.

1. Introduction

The mitochondrion is implicated in many important physiological processes, including metabolism, signaling, apoptosis, cell cycle control, and cell-differentiation [1]. In particular, the mitochondrion is responsible for the production of cellular energy by generating ATP through the electron transport chain (ETC) located on the inner mitochondrial membrane [2]. Mitochondrial biogenesis is therefore essential for proper cellular functioning. Human mitochondrial DNA (mtDNA) is a circular double-stranded molecule, 16,569 base pairs (bp) in length, that encodes 13 subunits of the oxidative phosphorylation system, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) [3]. Each mitochondrion contains 2–10 mtDNA copies and is present in hundreds to thousands of copies in each cell [4].

Altered mtDNA copy number (CN) that is commonly expressed as the mitochondrial to nuclear genome content using real-time quantitative polymerase chain reaction (PCR), is a proxy marker of mitochondrial function [5] and has been associated with many diseases including multiple sclerosis, type 2 diabetes, liver disease, cardiomyopathy as well as cancer [6].

Among the rheumatic diseases decreased leucocyte mtDNA CN has been associated with the development and progression of systemic lupus erythematosus (SLE), and production of proinflammatory cytokines and may contribute to persistent low-grade inflammation [7,8]. Also oxidative DNA and mtDNA alterations are implicated in progression of SLE and correlate with mtDNA CN in SLE patients [8]. In

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addition, mtDNA from rheumatoid arthritis (RA) synoviocytes has demonstrated twice the number of mutations compared with mtDNA from osteoarthritis synoviocytes [9], and higher levels of extracellular mtDNA has been detected in both RA plasma and synovial fluid compared to healthy controls [10] probably reflecting cellular injury [11]. However, mtDNA CN in peripheral blood cells from RA patients has not been investigated previously.

The increased *relative risk* of contracting *RA* conditioned on having an affected *co-twin* compared to the background population risk is somewhere between 17.3 and 35.4 [12] indicating a high degree of RA predisposition in these co-twins The aim of our study was therefore to examine whether mtDNA CN was associated with both RA and RA predisposition by measuring mtDNA CN in both RA affected twins, their healthy co-twins and in twins from twin pairs where neither twin were affected by RA. The predisposition in the RA co-twins may be genetic and we have therefore explored the potential effect of the two major RA genetic risk factors *PTPN22*(T) [13] and the "shared epitope" [14] on mtDNA CN.

2. Material and methods

2.1. Subjects

Cases: We included 81 RA twins and 68 of their non-affected co-twins from the Danish RA twin cohort [12]. The RA diagnosis was validated by clinical examination, medical records and presence of rheumatoid factor according to the modified ARA 87 criteria [15,16] Among the RA patients, 78%, 14% and 8% were currently, previously or never treated with disease modifying anti-rheumatic drugs (DMARDs) respectively. Zygosity among same-sexed twins was determined by genetic markers.

Controls: 1321 non-RA affected twin pairs from the study of Middle Aged Danish Twins (MADT) [17]. These twins were randomly selected to investigate how health, lifestyle and functioning at midlife contribute to differences in late life and mortality. Zygosity among same-sexed twins was based on questions about the degree of similarity between co-twins. The questionnaire method has been proved to assign correct zygosity in more than 95% of all twin pairs, compared to zygosity determined by genetic markers [18].

2.2. DNA extraction

For twin pairs with at least one twin with RA, DNA was extracted from peripheral blood that was centrifuged at 1,000*g* for 15 min followed by removal of plasma (buffy coat), using a standard manual DNA extraction kit (Gentrapure, Qiagen-LGC, Hertfordshire, UK). For 26 pairs (36% of the total sample of RA and co-twins) DNA was extracted by a semi-automatic method (Autopure, Qiagen-LGC, Gentrapure, Hertfordshire UK), but using the same chemistry as for the manual extraction. Twin pairs from whom DNA was extracted by automated extraction had mtDNA CN that on average was 10.2 mtDNA CN lower than for twins pairs subjected to manual DNA extraction, equivalent to 3.6 lower mtDNA CN for the complete RA twin population. This did not affect the pairwise analysis as extraction method was similar within twin pairs.

For controls buffy coat and DNA was prepared in the same manner as for twins with RA, using a standard manual DNA extraction kit (Gentrapure, Qiagen-LGC, Hertfordshire, UK).

2.2.1. HLA typing

HLA-DRB1 genotyping was performed using LABType SSO DRB1 Typing Test kit (One Lambda), coupled with the Luminex xMAP technology (Luminex) according to the manufacturer's instructions.

The PTPN22 C1858T single nucleotide polymorphism (R620W) was detected as described in the online Supplementary file 1. Subjects were categorized as PTPN22 1858 T-allele positive if they were either heterozygous or homozygous for the allele.

2.2.2. Autoantibodies

Anti-cyclic citrullinated peptide antibodies (ACPA) were determined by ELISA (Euro-Diagnostica, Malmö, Sweden) as described by the manufacturer.

2.3. Quantification of mitochondrial DNA copy number

An assay based on real-time quantitative PCR and SYBR Green technology was adapted to measure the amount of mitochondrial DNA compared to nuclear DNA. Each DNA sample was assayed in triplicate in a procedure identical to that described by Mengel-From et al. [19,20] (online supplementary file 1).

2.4. Statistical analysis

Descriptive statistics of RA twins and controls were performed by chi-square tests for categorical variables and t-tests for continuous variables using stata 14.2 (StataCorp).

Because our samples consist of twin pairs, we introduced two analytical models for performing individual level and twin-pair level analyses.

Individual level analysis: Individual twins were treated as unrelated subjects with correction for intra twin-pair correlation by introducing a mixed effects model defining RA and covariates as fixed effects and twin pairing as a random effect. The mixed effect model allows inclusion of RA discordant and concordant twin pairs as well as singletons and healthy controls. The mixed effects model was fitted by the free R package lmerTest [21].

Twin-pair level analysis: The twin-pair level analysis regressed intra-pair difference in mtDNA CN on intra-pair differences in the covariates of the RA discordant twin pairs. The analysis controls for the overall genetic effect on mtDNA CN partly in dizygotic (DZ) twin pairs and completely in monozygotic (MZ) twin pairs. Thus, only DZ pairs will contribute to genetic variation. In this model, the intercept is the mean of intra-pair differences in mtDNA CN across all RA discordant twin pairs after adjusting for covariates, while the slope parameters measure if a covariate increases or decreases the difference in mtDNA CN from RA to controls. We introduced a stepwise regression with both backward and forward variable selection to construct the best performing model. Linear regression was done using the lm() function in R and the stepwise regression was performed using the MASS package in R [22].

3. Results

Table 1 depicts the characteristics of the study population consisting of 81 RA twins, 68 of their co-twins and 1321 controls. There were more DZ twins, females and a lower mean age among the RA affected twin pairs compared to the controls and they had a relatively higher proportion of neutrophils than of lymphocytes and monocytes.

Individual level analysis: The healthy control twins had a significantly higher unadjusted mtDNA CN (91.7) than the twin pairs (69.6) in which at least one twin was affected by RA ($p < 0.001$, see Table 1).

In the multiple regression analysis, treating the RA affected twins and their non-affected co-twins as categorical predictors and using the twins from the healthy control twin pairs as the reference group, adjusting for age, sex and the twin correlation structure (model 1 in Table 2) both the RA twins (Δ: −28.7 mtDNA CN) and their healthy co-twins (Δ: −23.1 mtDNA CN) had a significantly lower mtDNA CN than the controls ($p < 0.001$). When stratifying the analysis according to zygosity the same pattern was observed for both MZ and DZ twins (model 1 in Table 2). As the RA affected twins had a lower mtDNA content than their co-twins, irrespective of zygosity, a new regression

Table 1
Demographic and para-clinical data of participating twins.

	Twins with RA and co-twins	Twin pair controls	p-values
N	149	1321	
MZ, (%)	42 (28)	553 (42)	
DZ SS, (%)	61 (41)	363 (27)	
DZ OS, (%)	46 (31)	404 (31)	
Complete twin pairs, N	73	513	
Complete MZ pairs, N	21	255	
Complete DZ SS pairs, N	30	123	
Complete DZ OS pairs, N	22	135	
Age,y (SD) [range]	61.0 (10.5) [33–78]	66.8 (6.2) [55–80]	< 0.001
Female, (%)	98 (66)	633 (48)	< 0.001
Discordance RA time, y	20.4 (10.4)	–	–
Neutrophils (%)	65.7	61.8	< 0.001
Lymphocytes (%)	26.6	29.3	< 0.001
Monocytes (%)	5.8	6.5	0.002
Eosinophils (%)	2.0	2.2	0.24
Basophils (%)	0.3	0.3	0.21
Leukocytes (10^9/L)	755.1	701.3	0.10
CRP "mg/L"	4.9 (10.9)	–	–
CCP "1",(%)	69 (46)	–	–
SE carriers, (%)	105, (70)	–	–
PTPN22(T) carriers, (%) [$N_{heterozygotes}/N_{homozygotes}$]	33, (22) [31/2][b]	241, (21) [227/14]	0.78 0.78[a]
mtDNA CN, (SD)	69.6 (21.1)	91.7 (31.3)	< 0.001

ss: same sex, os:opposite sex.
[a] Genetic additive model.
[b] RA-twins [N heterozygotes/Nhomozygotes]: [17/1], co-twins [N heterozygotes/Nhomozygotes]: [14/1].

Table 2
Multiple linear regression analysis of mtDNA CN difference between RA and controls and RA co-twins and controls respectively.

mtDNA CN	Model 1 age and sex adjusted reg. coef.,p-values	Model 2 age, sex and cell comp. adjusted reg. coef.,p-values
All twins: (N = 1470)		
Control twins	Ref	Ref
Co-twins - controls	−23.1, < 0.001	−20.1, < 0.001
RA twins - controls	−28.7, < 0.001	−23.1, < 0.001
MZ twins: (N = 595)		
Control twins	Ref	Ref
Co-twins - controls	−22.7, < 0.001	−22.8, < 0.001
RA twins - controls	−27.3, < 0.001	−23.0, < 0.001
DZ twins: (N = 875)		
Control twins	Ref	Ref
Co-twins - controls	−23.8, < 0.001	−19.1, < 0.001
RA twins - controls	−30.0, < 0.001	−24.2, < 0.001

analysis was performed adjusting for differential cell counts as the inflammatory state in the RA twins was slightly higher than in the co-twins (mean CRP 7.6 versus 1.4) and with relatively more neutrophils in the RA twins (67.0% versus 64.1%). This adjustment attenuated the difference between the twins from RA pairs and the healthy reference group. Also, the difference between the RA twins and their co-twins almost disappeared in MZ twin pairs (Δ 0.2 mtDNA CN) but less so in the DZ pairs (Δ 5.1 mtDNA CN) (Table 2).

Twin pair level analysis: We explored the effect of RA status itself, anti-CCP antibodies (ACPA), and the two major genetic risk factors associated with RA, i.e. the shared epitope (SE) and the *PTPN22*(T) polymorphism on mtDNA CN, adjusting for cell composition. In addition, in this twin pair analysis the within-pair differences in mtDNA CN among RA discordant twin pairs makes adjustment for the confounding effect of age, sex, and shared environment early in life. Thus, Table 3 depicts the results from this analysis in which the outcome is the difference between mtDNA CN in the RA twin and the non-RA co-twin.

Table 3
RA discordant twin paired analysis ($N_{twin\ pairs}$ = 48).

mtDNA CN	Full model Twins with RA Reg. coef, p-value	Optimized model Twins with RA Reg. coef, p-value
RA/intercept	−0.52, 0.92	–
Median age[1]	8.21, 0.57	–
Sex	−0.21, 0.97	–
Discordance RA time, y[1]	0.02, 0.95	–
Neutrophilocytes	6.13, 0.13	2.40, 0.01
Lymphocytes	7.02, 0.09	3.30, 0.003
Monocytes	3.43, 0.38	–
Eosinosphilocytes	6.60, 0.10	3.21, 0.04
Basophilocytes	4.82, 0.49	–
se	1.96, 0.77	–
ptnp22	−15.60, 0.03	−16.28, 0.01
CCP	−0.06, 0.99	–

First we ran a full model including all the selected variables presented in Table 3 (full model) to reach the most parsimonious model (optimized model). This model included neutrophils, lymphocytes, eosinophils and *PTPN22*(T) as predictors whereas RA status, ACPA or SE had no significant effect (Table 3, column 1). This model, adjusted for cell composition, shows that PTPNN22(T) carriers had a significantly lower mtDNA CN than their non-affected co-twins but only when the carrier had established RA (Δ - 16.3 mtDNA CN) (Table 3, column 2).

We also performed an unadjusted intra pairwise analysis comparing both DZ and MZ RA discordant twin pairs (Supplementary Table 1). Here we found that in seven DZ RA discordant twin pairs, the unadjusted mean mtDNA CN in the *PTPN22* (T) positive RA twin was much lower compared to their *PTPN22*(T) negative healthy co-twins (−29.4 mtDNA CN) ($p < 0.008$), but only a small (0.7 mtDNA CN) and non-significant difference between four *PTPN22*(T) negative RA DZ twins compared to their *PTPN22*(T) positive healthy co-twins. In five RA discordant *PTPN22*(T) positive MZ twin pairs the difference was small (0.9) as was also the case in 13 RA discordant *PTPN22* (T) negative MZ twin pairs (−0.4). *PTPN22*(T) positive MZ twin pairs had an average mtDNA CN of 64.3, whereas the *PTPN22*(T) negative MZ twin pairs had a mtDNA CN of 73.8, thus lowest for the *PTPN22*(T) positive MZ twin pairs.

To examine a potential effect of *PTPN22*(T) on mtDNA CN in non-RA disposed individuals we ran a regression analysis in the healthy non-affected twin population (N = 1321 twins), adjusting for age, sex and cell composition, but did not find any effect of *PTPN22*(T) (N = 241) on the mtDNA CN (−1.65, $p > 0.80$).

4. Discussion

To our knowledge this is the first study to investigate mtDNA CN in leukocytes from patients with RA. Our study revealed that mtDNA CN was reduced in the RA affected twins and their healthy co-twins compared to a control group of healthy twin pairs, thus indicating that both RA and RA predisposition are associated with a lower mtDNA content. A pairwise difference in mtDNA CN in RA discordant twin pairs was observed for DZ but not MZ twin pairs, suggesting that genetic variation influences mtDNA CN variation. This is also inferred from the observation that the presence of the genetic variant, *PTPN22*(T), reduced mtDNA CN further in the RA twins but not in the RA unaffected co-twins. The *PTPN22* (T) variant had no effect on mtDNA CN in twins from non-RA affected twin pairs. This indicates an interaction between the *PTPN22* (T) variant, the RA phenotype and the level of mtDNA CN. Neither the other major genetic risk factor for RA, the shared epitope, nor the presence of anti-CCP antibodies were associated with mtDNA CN.

It has recently been reported by our group that the relative mtDNA CN in peripheral blood cells declines with age for those older than 50

years of age and longitudinal data from twins above the age of 70 demonstrated an individual decline of 12.7 mtDNA CN in 10 years independent of sex and zygosity [19]. Thus, our finding of a decline of more than 20 mtDNA CN in RA twin pairs compared to healthy twin pairs when adjusting for age, sex and blood cell composition corresponds to an almost 20 year age-related decline, which may be one of the largest differences in mtDNA CN found for a disease to date. Patients with RA have increased mortality compared to the background population, mainly due to cardiovascular disease [23–25]. As mtDNA CN depletion is an independent risk factor for heart failure and predicts higher cardiovascular mortality in patients with heart failure [26,27], the lower mtDNA content, and perhaps even the related mitochondrial metabolism in leukocytes, may be key factors in the increased risk of cardiovascular disease in RA patients.

RA patients also have a marked increase of reactive oxygen species (ROS) in the blood and a decrease in the activity of antioxidant defense system leading to oxidative stress [28,29]. It has been demonstrated that oxidative stress increases mtDNA CN and that antioxidants may suppress mtDNA replication by an alleviation of oxidative stress in leukocytes [30]. These mechanisms would tend toward a higher mtDNA CN in RA twins compared to the healthy co-twins. However, the majority of our RA twins had been exposed to treatments with NSAIDs, corticosteroids and non-biologic DMARDs, which have been shown to decrease ROS production in neutrophils [31] and might have attenuated the oxidative stress in the RA twins. Furthermore, a Finnish study found a significantly lower anti-oxidant index in preclinical serum samples from subject who subsequently developed RA [32]. This would tend to further attenuate the difference in mtDNA CN between the RA twins and their predisposed healthy co-twins.

A low mtDNA CN has been associated with low grade inflammation indicated by elevated high sensitivity-CRP, IL-6, fibrinogen, and white blood cell count in a healthy population of age 65 [33]. Thus, inflammation may contribute to the lower mtDNA CN in the RA twins, but may also add to the lower mtDNA CN observed in the predisposed healthy co-twins, as biomarkers of inflammation have been observed in preclinical RA several years before RA onset as well as in unaffected first-degree relatives of probands with RA [34–36].

We observed a negative effect of the nuclear *PTPN22(T)* variant on mtDNA CN in RA twins when compared to their respective healthy co-twins but no effect of *PTPN22*(T) was observed among the healthy twin pairs. This indicates the existence of an interaction between the *PTPN22*(T) variant and the specific genetic and/or environmental effects that are needed to elicit the development of RA beyond the predisposing factors carried by the healthy co-twins from RA discordant twin pairs. Besides, almost no difference in mtDNA CN was observed within MZ twin pairs discordant for RA. Yet, MZ *PTPN22*(T) positive RA discordant twin pairs had reduced mtDNA CN compared to MZ *PTPN22*(T) negative twin pairs, which supports the existence of an interaction between *PTPN22*(T) and mtDNA CN in RA twins, as observed within the DZ RA discordant twin pairs. Moreover, a recent in vitro study demonstrated that the *PTPN22* (T) variant enhanced the neutrophil ROS production fourfold in healthy individuals but only 1.75 fold in RA patients in response to TNF-α priming and stimulation with the chemotactic factor Fmlp (N-formylmethionyl-leucocyl-phenylalanine) [37]. This support the notion that the *PTPN22*(T) variant has an interactive role in mitochondrial regulation. Since oxidative stress is positively associated with mtDNA CN in leukocytes, and the main contribution of mtDNA CN come from neutrophil leukocytes [38], this observation indirectly corroborate our finding of a negative effect on mtDNA CN in *PTPN22*(T) positive RA twins compared with their *PTPN22*(T) negative co-twins, and that *PTPN22*(T) has no significant effect on mtDNA CN in healthy twins.

Mitochondrial dysfunction and mtDNA CN have been implicated in a range of diseases such as diabetes, cancer, neurodegeneration, and muscle atrophy [6], but nevertheless little is known about the exact mechanisms behind these variations in each of the conditions. It is known, though, that mtDNA biogenesis and maintenance is influenced by a coordinated expression of both nuclear and mitochondrial genes [39]. According to twin and family studies, genetic variation accounts for 33–65% of the variation of mtDNA CN in DNA extracted from whole blood [40,41]. However, we do not know whether the lower number of mtDNA CN observed in blood leukocytes from RA patients is a result of decreased synthesis or increased degradation, but most mitochondrial proteins are nuclear encoded and nuclear DNA methylation is linked to both mtDNA CN and mtDNA haplotypes [42]. Of note, several of the key nuclear encoded mtDNA replication factors, e.g. the mitochondrial transcription A (*TFAM*), the mitochondrial DNA single stranded binding protein (*SSBP1*), and the twinkle mtDNA helicase (*C10orf2*) [43] were found to be differentially methylated in peripheral blood from patients with ACPA positive RA [44], which may indicate that nuclear DNA methylation is involved in the mtDNA CN set point in RA.

Although our study is cross-sectional, the fact that we observe a significantly reduced mtDNA CN in RA co-twins predisposed to RA implies that genetic and also likely environmental effects involved in RA pathogenesis may influence mtDNA CN biogenesis or degradation. Our results stem from a mixture of cells from peripheral blood and as mtDNA CN is regulated in a cell specific manner [43] we have adjusted for cell type composition to reduce a possible bias, but we have not been able to estimate the contribution from each specific cell type in peripheral blood.

In conclusion, our study has demonstrated that mtDNA content is decreased in peripheral blood cells, not only from RA patients but also from persons predisposed to RA, suggesting that mitochondrial biogenesis and/or degradation may be involved in the RA disease process. Furthermore, we find that the RA genetic risk factor *PTPN22*(T) is associated with a lower content of mtDNA in leukocytes from RA patients but not in leukocytes from non-RA affected persons irrespective of RA predisposition.

Declarations of interest

None.

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Author's contributions

AJS and JMF conceived the study. JMF, QT, LC, BT, MN, AJS analysis and interpretation of the data. MAJ, JMF contributed reagents/materials/analysis tools. AJS, QT, JMF and BT prepared the manuscript. All authors read and approved the manuscript.

Ethics

The study was approved by all the regional scientific ethics committees in Denmark (Projekt ID: S- 20070088) and the Danish Data Protection board (J.nr. 2007-41-0747). We obtained informed written consent from all participants in the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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