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LYMPHOID NEOPLASIA

# Mitochondrial DNA copy number and future risk of B-cell lymphoma in a nested case-control study in the prospective EPIC cohort

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## Key Points

- This study strengthens the previous observation of elevated mitochondrial DNA copy number and future risk of chronic lymphocytic leukemia.

**It has been suggested that mitochondrial dysfunction and DNA damage are involved in lymphomagenesis. Increased copy number of mitochondrial DNA (mtDNA) as a compensatory mechanism of mitochondrial dysfunction previously has been associated with B-cell lymphomas, in particular chronic lymphocytic leukemia (CLL). However, current evidence is limited and based on a relatively small number of cases. Using a nested case-control study, we extended these findings with a focus on subtype-specific analyses. Relative mtDNA copy number was measured in the buffy coat of prospectively collected blood of 469 lymphoma cases and 469 matched controls. The association between mtDNA copy number and the risk of developing lymphoma and histologic subtypes was examined using logistic regression models. We found no overall association between mtDNA and risk of lymphoma. Subtype analyses revealed significant increased risks of CLL (n = 102) with increasing mtDNA copy number (odds ratio = 1.34, 1.44, and 1.80 for quartiles 2-4, respectively; *P* trend = .001). mtDNA copy number was not associated with follow-up time, suggesting that this observation is not strongly influenced by indolent disease status. This study substantially strengthens the evidence that mtDNA copy number is related to risk of CLL and supports the importance of mitochondrial dysfunction as a possible mechanistic pathway in CLL ontogenesis. (*Blood*. 2014;124(4):530-535)**

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The online version of the article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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## Introduction

Mitochondria as "cellular power plants" generate cellular adenosine triphosphate through aerobic respiration. In addition to energy, however, reactive oxygen species (ROS) with the potential to cause DNA damage are produced.[1] Each mitochondrion has 2 to 10 copies of mitochondrial DNA (mtDNA), which is a prime target of ROS damage because of the lack of protective histones, limited DNA repair mechanisms, and its close proximity to the electron transport chain, which releases ROS.[2] Thus, the mutation rate of mtDNA has been reported to be up to 15-fold higher than for nuclear DNA in response to DNA-damaging agents[3] such as UV, cigarette smoke, benzene, and ionizing radiation.[4-6]

It has been hypothesized that mitochondria function and mtDNA damage are involved in carcinogenesis.[7] Studies have suggested that mitochondria possibly increase the number of mtDNA copies to compensate for mtDNA damage and mitochondrial dysfunction.[1,8,9] Many of the mtDNA mutations found in human cancer samples are located in the D-loop region, which is involved in the control of replication and transcription of mtDNA and might cause an alteration in the copy number and/or gene expression of the mitochondrial genome.[10] Recent prospective studies have shown an association between increasing mtDNA copy number in peripheral blood and increased risk of B-cell non-Hodgkin lymphoma (B-NHL),[11] lung cancer,[12] pancreatic cancer,[13] and colorectal cancer,[14] but not with gastric cancer.[15]

Lan and colleagues reported elevated mtDNA copy numbers of peripheral white blood cells in prediagnostic blood samples of healthy subjects that later in life developed B-NHL, with a suggestion that the effect was most pronounced for chronic lymphocytic leukemia (CLL).[11] However, the study was limited by its relatively small sample size (CLL cases, n = 34), hampering firm conclusions on the subtype-specific analyses. Replication of these results in other prospective cohort studies with a larger number of B-NHL and especially CLL cases would provide additional evidence on the possible role of mtDNA function/damage in (CLL) lymphomagenesis.

In this study, we investigated the association between mtDNA copy number and incidence of Hodgkin lymphoma (HL), T-cell NHL (T-NHL), B-NHL, and histologic subtypes among participants of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study using a nested case-control design.

## Materials and methods

### Study population

A detailed description of the EPIC cohort study can be found elsewhere.[16,17] In the period 1992 to 2000, recruitment of over half a million (520 000) people in 10 European countries (Denmark, France, Germany, Greece, Italy, The Netherlands, Norway, Spain, Sweden, and the United Kingdom) was completed. The cohort includes participants of both genders, mostly in the age range of 35 to 70 years at recruitment. After providing informed consent, diet, lifestyle, and personal medical history questionnaires were collected from most participants. Additionally, participants were invited to donate a blood sample. The EPIC study was approved by the review boards of the International Agency for Research on Cancer and by the local institutes in the participating countries. The study was conducted in accordance with the Declaration of Helsinki.

### Follow-up for cancer incidence and vital status

Data on vital status in EPIC are collected through record linkage with regional and/or national mortality registries, except in Germany and Greece, where data are collected through active follow-up and checks through municipal population registries. Cancer incidence is determined through record linkage with regional cancer registries or via a combination of methods, including the use of health insurance records, contacts with pathology registries, and active follow-up through participants and their next of kin. Cases with lymphoid cancers were originally classified according to the second revision of the International Classification of Diseases for Oncology (ICD-O-2) but were subsequently reclassified according to the third edition of the World Health Organization classification of hematopoietic and lymphoid tissue cancers,[18] using a program available on the United States National Cancer Institute Surveillance, Epidemiology and End Results Web site (http://seer.cancer.gov/) and the expertise of pathologists. Participants of the French cohort (n = 24 371 with available blood samples) were excluded from the study because of incomplete coding for lymphoid neoplasms when the present project was started.

For each incident lymphoma case, 1 random control was selected among all cohort members alive and free of cancer at the time of diagnosis of the index case matched by center, gender, date of recruitment (±1 year), and age at recruitment (±3 years). Through August 1, 2008, a total of 483 lymphoma cases were identified who were eligible to be included in the study. Subjects whose blood specimen were missing (1 case and 10 controls) and respective matched subjects were excluded, so a total of 472 pairs entered the study.

### mtDNA copy-number assay

The mitochondrial copy number (MCN) was calculated based on the copy-number measurement of mitochondrial gene (NADH dehydrogenase, subunit 1 [*ND1*]) and nuclear gene (hemoglobin subunit β [*β-globin*]) according to the protocol described by Liu et al[9] with some modifications. Briefly, fluorescence-based quantitative polymerase chain reaction (qPCR) was applied to determine the mtDNA copy number in human leukocytes. The LightCycler-FastStart DNA Master kit was applied for qPCR (Roche Molecular Biochemicals, Pleasanton, CA). Two primers (forward: 5′-GAAGAGCCAAGGACAGGTAC-3′; reverse: 5′-CAACTTCATCCACGTTCACC-3′) complementary to the sequences of the *β-globin* gene were used to amplify a 268-bp product. For studying the mitochondrial gene products, the forward primer 5′-AACATACCCATGGC CAACCT-3′ and the reverse primer 5′-AGCGAAGGGTTGTAGTAG CCC-3′, which are complementary to the sequence of the *ND1* gene, were used to amplify a 153-bp PCR product. qPCR was performed under the following conditions: initial denaturation at 95°C for 300 seconds followed by 40 cycles of 0.1 second at 95°C, 6 seconds at 58°C, and 18 second at 72°C. A total of 20 ng DNA was used in qPCR for determination of the threshold cycle number (Ct) of the nuclear and mitochondrial genes, respectively. The Ct values for *β-globin* gene and mitochondrial *ND1* gene were concurrently determined in each sample during the same qPCR run. The MCN in leukocytes of each subject was calculated by the equation $(2 \times 2^{(Ct(\beta\text{-globin}) - Ct(ND1))})$. Laboratory personnel were blinded with regard to case-control status. Cases and their matched controls were assayed on the same plate in the same batch. Each DNA sample was analyzed in duplicate in the same run. A third measurement was performed only when a discrepancy between the duplicate mtDNA copy-number values was observed. Quality control samples were run with the case-control sets in each batch. Mean within and between batch coefficients of variation based on the quality control samples were 7.7% and 3.6%, respectively. Mean within-batch coefficient of variation and the intraclass coefficient of variation based on the duplicate measurements (n = 130) was 11.4% and 0.84, respectively. One case and 2 controls along with the matched counterparts were excluded from the analysis due to missing measurements. The mtDNA copy number was determined successfully on 469 case-control pairs.

### Statistical analysis

Distributions of baseline characteristics for matched cases and controls were assessed by paired Student *t* test and $\chi^2$ test for continuous and categorical variables, respectively. Odds ratios (ORs) and 95% confidence intervals (CIs)

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**Table 1. General characteristics of lymphoma cases and matched controls**

| | Cases (n = 469) | Controls (n = 469) | P* |
|---|---|---|---|
| Sex | | | |
| Male, n (%) | 231 (49.3%) | 231 (49.3%) | |
| Female, n (%) | 238 (50.7%) | 238 (50.7%) | |
| Age at recruitment, y† | 56.6 (8.8) | 56.6 (8.8) | .55 |
| BMI† | 26.9 (4.4) | 26.6 (4.2) | .26 |
| Alcohol at recruitment (g/d)† | 13.7 (18.6) | 13.3 (19.5) | .78 |
| **Smoking status** | | | .31 |
| Never | 42.1% | 43.7% | |
| Former | 37.1% | 32.5% | |
| Current smoker | 20.8% | 23.8% | |
| **Physical activity** | | | .63 |
| Inactive | 26.4% | 28.6% | |
| Moderately inactive | 31.1% | 27.7% | |
| Moderately active | 19.0% | 21.1% | |
| Active | 18.3% | 18.8% | |
| Unknown | 5.1% | 3.8% | |
| **Education** | | | .40 |
| None | 8.1% | 6.9% | |
| Primary school | 35.9% | 34.3% | |
| Technical/professional school | 23.4% | 22.8% | |
| Secondary school | 9.9% | 14.5% | |
| Longer education (including university degree) | 19.5% | 18.9% | |
| Not specified | 3.3% | 2.6% | |
| **Lymphoma type, n (%)** | | | |
| HL | 31 (6.6%) | | |
| B-NHL | 418 (89.1%) | | |
| DLBCL | 60 (14.5%) | | |
| FL | 75 (18.1%) | | |
| CLL | 102 (24.6%) | | |
| MM | 109 (26.3%) | | |
| Other | 72 (17.2%) | | |
| T-NHL | 20 (4.3%) | | |
| mtDNA copy number‡ | 35.7 (23.9) | 30.6 (10.0) | .0001 |

*Paired *t* test for continues and $\chi^2$ test for categorical variables.
†Mean (standard deviation).
‡The copy number of mtDNA in 1 leukocyte (per diploid genome); mean (standard deviation).

were estimated using conditional logistic regression models. The mtDNA copy number was categorized into quartiles based on the distribution among controls and modeled as a categorical variable. Tests for trend were calculated using the quartile number as a continuous variable. The effect of smoking status (categorical; current, former, and never smokers), physical activity (categorical; inactive, moderately inactive, moderately active, active), educational level as indicator of socioeconomic status (categorical; none, primary school, technical/professional school, secondary school, longer education), body mass index (BMI) (continuous; kg/m$^2$), and alcohol intake at recruitment (continuous; grams/day) as potential confounding variables were examined for B-NHL and for the major subtypes separately. Most of the confounder variables did not change the mtDNA risk estimates in these analyses by more than 10% except for smoking status and educational level. Therefore, we used a unified model including these 2 variables for all analyses. Of note, results based on subtype-specific models, including additional subtype-specific confounders and the unified model, were essentially similar. In addition, the possible effect modification of smoking as a factor that induces oxidative stress was evaluated. We also investigated the possible associations by major histologic subtypes of B-NHL and stratified by median follow-up time. In order to retain as much power as possible, for subtype analyses, unconditional logistic regression was used, in which case in each subtype was compared with all controls additionally adjusted for matching variables (ie, country, center, gender, and age at recruitment) and plate number. We additionally investigated associations with mtDNA categories for B-NHL subtypes (ie, CLL, diffuse large B-cell lymphoma [DLBCL], follicular lymphoma [FL], multiple myeloma [MM], other B-NHL) using polytomous regression models adjusted for age at recruitment, year of recruitment, sex, EPIC country, smoking status, educational level, and plate number. Due to the slow-growing nature of indolent lymphomas such as CLL, we repeated the analyses using a 3-, 6-, and 9-year follow-up interval as sensitivity analyses.

Statistical analyses were performed using SAS version 9.2 (SAS Institute). All *P* values are 2 sided, with $P < .05$ considered as statistically significant.

## Results

### Description of the study population

The characteristics of the 469 lymphoma cases (231 men and 238 women) and an equal number of control subjects in this study are provided in Table 1. Median time between recruitment (ie, blood collection) in the study and diagnosis of lymphoma was 4.6 years (range, 0.02-14.3 years) (data not shown). Of all lymphoma cases, 418 were diagnosed with B-NHL, with the most common diagnosed subtypes being MM (n = 109), CLL (n = 102), FL (n = 75), and DLBCL (N = 60). There were 31 and 20 cases diagnosed with HL and T-NHL, respectively. Case and control subjects were comparable in terms of age, BMI, smoking status, physical activity, and educational level. The mtDNA copy number was significantly higher in cases compared with controls (Table 1; $P = .0001$). Means and standard deviations of the mtDNA copy number were 44.13 (36.23), 32.21 (16.13), 34.41 (20.17), and 32.54 (14.9) for major subtypes of CLL, DLBCL, FL, and MM cases, respectively (data not shown). No significant association was found between mtDNA copy number and age, sex, BMI, alcohol intake, and smoking status among all participants or controls only (data not shown).

### Risk analyses

Risk for developing B-NHL increased significantly with increasing mtDNA copy number (Table 2; *P* trend = .04). However, after adjustment for confounding variables, this result did not reach formal statistical significance. In subtype analyses of B-NHL including all control subjects (unconditional), the association was most pronounced for CLL (OR = 1.34, 1.44, and 1.80 for quartiles 2-4, respectively; *P* trend = .002; Table 2). Polytomous regression analyses were consistent with the logistic regression analyses, showing a significant effect only for CLL (OR = 1.56, 2.12, and 4.45 for quartiles 2-4, respectively; *P* trend = .001). Models excluding CLL cases showed no association between B-NHL and mtDNA copy number. Heterogeneity test by B-NHL subtype was significant ($P = .03$). Moreover, we found no association between mtDNA and HL and T-NHL (data not shown). Stratified analyses by gender showed similar results in logistic regression (online material) and polytomous models (data not shown).

To determine if the association is driven in part by elevated mtDNA copy number among cases with undiagnosed, subclinical CLL at the time of blood-sample collection, we additionally investigated the association stratified by median follow-up time. We found that mtDNA copy number significantly increased among cases diagnosed <4.6 years after blood collection, which remained significant and of equal magnitude among cases diagnosed >4.6 years after providing a blood sample (OR = 1.40, 1.43, and 2.39 for quartiles 2-4, respectively; *P* trend = .05) (Table 3). Heterogeneity test was not significant by median follow-up time for B-NHL

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**Table 2. OR and 95% CI for mtDNA copy number and B-NHL and major subtypes**

| | Logistic regression * | | | | |
|---|---|---|---|---|---|
| | Q1 (6.7-23.6) | Q2 (23.7-29.4) | Q3 (29.5-36.2) | Q4 (36.3-294.7) | *P* trend |
| **B-NHL** | | | | | |
| n (case/control) | 112/107 | 77/105 | 85/104 | 144/102 | |
| Unadjusted OR (95% CI) | Ref. | 0.66 (0.43-1.02) | 0.81 (0.53-1.24) | 1.36 (0.91-2.04) | .04 |
| Adjusted OR (95% CI) | Ref. | 0.63 (0.40-0.99) | 0.79 (0.50-1.24) | 1.24 (0.81-1.92) | .13 |
| **B-NHL without MM** | | | | | |
| n | 82/84 | 55/72 | 61/81 | 111/72 | |
| Unadjusted OR (95% CI) | Ref. | 0.76 (0.46-1.25) | 0.79 (0.49-1.29) | 1.59 (1.01-2.50) | .03 |
| Adjusted OR (95% CI) | Ref. | 0.73 (0.43-1.22) | 0.79 (0.47-1.35) | 1.53 (0.93-2.52) | .05 |
| **DLBCL** | | | | | |
| n (case/control) | 21/117 | 12/117 | 6/118 | 21/117 | |
| Unadjusted OR (95% CI) | Ref. | 0.61 (0.30-1.24) | 0.32 (0.13-0.79) | 1.00 (0.55-1.83) | .76 |
| Adjusted OR (95% CI) | Ref. | 0.68 (0.32-1.48) | 0.22 (0.07-0.66) | 1.08 (0.54-2.14) | .75 |
| **FL** | | | | | |
| n (case/control) | 23/117 | 13/117 | 15/118 | 24/117 | |
| Unadjusted OR (95% CI) | Ref. | 0.61 (0.31-1.20) | 0.69 (0.36-1.32) | 1.04 (0.59-1.84) | .83 |
| Adjusted OR (95% CI) | Ref. | 0.55 (0.27-1.12) | 0.63 (0.32-1.25) | 0.94 (0.50-1.78) | .94 |
| **CLL** | | | | | |
| n (case/control) | 14/117 | 20/117 | 21/118 | 47/117 | |
| Unadjusted OR (95% CI) | Ref. | 1.37 (0.69-2.70) | 1.41 (0.72-2.78) | 2.68 (1.48-4.87) | .0004 |
| Adjusted OR (95% CI) | Ref. | 1.34 (0.66-2.70) | 1.44 (0.72-2.89) | 1.80 (1.48-5.28) | .001 |
| **MM** | | | | | |
| n (case/control) | 23/117 | 22/117 | 24/118 | 33/117 | |
| Unadjusted OR (95% CI) | Ref. | 0.78 (0.45-1.34) | 0.83 (0.48-1.42) | 1.08 (0.66-1.77) | .71 |
| Adjusted OR (95% CI) | Ref. | 0.80 (0.44-1.44) | 0.92 (0.52-1.63) | 1.15 (0.66-2.02) | .55 |
| **Other B-NHL** | | | | | |
| n (case/control) | 24/117 | 10/117 | 19/118 | 19/117 | |
| Unadjusted OR (95% CI) | Ref. | 0.39 (0.17-0.87) | 0.84 (0.46-1.55) | 0.85 (0.46-1.56) | .96 |
| Adjusted OR (95% CI) | Ref. | 0.41 (0.18-0.95) | 0.92 (0.48-1.76) | 1.00 (0.51-1.95) | .68 |

The mtDNA copy number (per diploid genome) was categorized into quartiles (Q) based on the distribution among controls and modeled as categorical variable; Q number (minimum-maximum); *P* trend is the *P* value (2 sided) calculated by including the quartile number as a continuous variable.

Ref., reference.

*Conditional logistic regression model adjusted for smoking status and education level was used for B-NHL, and an unconditional logistic regression model adjusted for age at recruitment, sex, country, smoking status, educational level, and plate number was used for subtype analyses.

($P = .62$) and CLL ($P = .12$). Moreover, sensitivity analyses of CLL by 3-, 6-, and 9-year follow-up intervals showed significant increased risks of CLL in all these analyses (data not shown). No association was found between mtDNA copy number and follow-up time among B-NHL ($P = .21$) and CLL cases ($P = .78$). CLL subtype analyses stratified by median age at diagnosis (62 years) showed increased risks of CLL with elevated levels of mtDNA copy number among both groups, with the association reaching statistical significance among cases diagnosed at an older age (OR = 1.36, 1.40, and 3.21 for quartiles 2-4, respectively *P* trend = .002 compared with OR = 1.41, 1.42, and 2.00 for quartiles 2-4, respectively; *P* trend = .12).

## Discussion

In this prospective study, we found no significant association between mtDNA copy number and risk of developing B-cell lymphoma except for a strong significant positive association between higher mtDNA copy number and future CLL risk. The differential effect of mtDNA on B-cell lymphoma risk is in line with the current thinking that NHL consists of a heterogeneous group of tumors with distinct ontogenesis.

Lan et al[11] previously reported that elevated mtDNA copy number was associated with an increased risk of NHL and in particular CLL in a study of 104 incident male NHL cases and 104 matched controls within the prospective α-Tocopherol, β-Carotene Cancer Prevention cohort. Our study, with about 3 times the number of CLL cases and including both males and females, confirms this association between mtDNA copy number and subsequent CLL risk. Stratified analyses by median follow-up time suggest furthermore that the increased risk can be observed long before diagnosis (>4.6 years). Additional, analyses shifting the cut point progressively to 6 and 9 years showed still-increased risks for cases diagnosed after 9 years of blood draw. Together with the observation that we did not observe an association between follow-up time and mtDNA levels among CLL cases, it would suggest that reversed causality is not a likely cause of the found association. However, as CLL is a chronic disease that often progresses slowly, we cannot exclude fully that the observed effects are due to a precursor stage of CLL (ie, monoclonal B-cell lymphocytosis). Our analyses furthermore would suggest that the association between mtDNA and CLL is stronger for cases diagnosed at an older age.

The largest meta-analysis on CLL including 4 genome-wide association studies identified several single-nucleotide polymorphisms (SNPs) near genes involved in apoptosis, which suggests a possible underlying mechanism of biological relevance.[19] The 2 identified SNPs at 18q21.33 map to the 3′ untranslated region of *BCL2* (B-cell CLL/lymphoma 2), which encodes an essential outer mitochondrial membrane protein that blocks lymphocyte apoptosis. Both SNPs are located within a narrow region of *BCL2* in which the majority of t(14;18) translocation breakpoints occur. The 2q33.1

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**Table 3. OR and 95% CI for mtDNA copy number and B-NHL overall and subtype of CLL according to follow-up time**

| | Logistic regression | | | | |
|---|---|---|---|---|---|
| | Q1 (6.7-23.6) | Q2 (23.7-29.4) | Q3 (29.5-36.2) | Q4 (36.3-294.7) | *P* trend |
| **B-NHL*** | | | | | |
| ≤4.6 y follow-up | | | | | |
| n (case/control) | 48/50 | 43/56 | 38/47 | 77/53 | |
| Adjusted OR (95% CI) | Ref. | 0.81 (0.40-1.62) | 0.87 (0.42-1.80) | 1.34 (0.71-2.55) | .18 |
| >4.6 y follow-up | | | | | |
| n | 64/57 | 34/49 | 47/57 | 67/49 | |
| Adjusted OR (95% CI) | Ref. | 0.52 (0.28-0.96) | 0.75 (0.41-1.36) | 1.16 (0.62-2.16) | .53 |
| **B-NHL without MM*** | | | | | |
| ≤4.6 y follow-up | | | | | |
| n | 32/38 | 28/37 | 26/33 | 58/36 | |
| Adjusted OR (95% CI) | Ref. | 1.00 (0.44-2.27) | 1.25 (0.50-3.11) | 2.09 (0.97-4.51) | .02 |
| >4.6 y follow-up | | | | | |
| n | 50/46 | 27/35 | 35/48 | 53/36 | |
| Adjusted OR (95% CI) | Ref. | 0.63 (0.31-1.27) | 0.60 (0.30-1.18) | 1.31 (0.65-2.62) | .59 |
| **CLL†** | | | | | |
| ≤4.6 y follow-up | | | | | |
| n | 6/117 | 8/117 | 8/118 | 26/117 | |
| Adjusted OR (95% CI) | Ref. | 1.24 (0.41-3.68) | 1.46 (0.49-4.35) | 3.70 (1.44-9.52) | .001 |
| >4.6 y follow-up | | | | | |
| n | 8/117 | 12/117 | 13/118 | 21/117 | |
| Adjusted OR (95% CI) | Ref. | 1.40 (0.56-3.52) | 1.43 (0.58-3.54) | 2.39 (0.99-5.78) | .05 |

Quartiles (Q) number (minimum-maximum); *P* trend is the *P* value (2 sided) calculated by including the quartile number as a continuous variable.
Ref., reference.
*Conditional logistic regression model adjusted for smoking status and educational level.
†Unconditional logistic regression model adjusted for age at recruitment, sex, country, smoking status, educational level, and plate number.

SNP (rs3769825) resides in intron 2 of *CASP8* (*caspase-8*) and is in linkage disequilibrium with a missense SNP (rs13006529, $r^2 = 0.71$) in the nearby gene *CASP10* (*caspase-10*), both of which have a central role in cell apoptosis. Moreover, 2 smaller case-control studies previously reported SNPs in *CASP8/CASP10* associated with CLL risk, including one in moderate linkage disequilibrium with the SNP Berndt et al found (rs11674246, $r^2 = 0.66$).[20,21] Our findings of increased mtDNA copy number among CLL cases are consistent with impaired mitochondrial apoptosis, which would be consistent with the involvement of apoptotic genes in CLL development. However, the exact mechanism through which altered mtDNA may play a role in lymphomagenesis still remains unclear.

Alteration in intracellular levels of ROS is associated with changes in mitochondrial abundance, mtDNA copy number, and expression of respiratory genes.[1] However, the outcome of the events leading to the increase in mitochondrial abundance and mtDNA copy number is dependent on the level of oxidative stress, the capacity of the intracellular antioxidants system, and the quality of mitochondria and mtDNA.[1] Tumorigenesis requires active mitochondrial biogenesis to support high proliferation activity. It is noteworthy that increased mtDNA copy number has been detected in tumor cells from patients with CLL,[22] in Burkitt lymphoma cell lines, and in lymphoblastoid cell lines.[23] As such, the observation of preclinical elevations in mtDNA levels in peripheral blood mononuclear cells seems of clinical relevance. Furthermore, mtDNA copy number has been shown to be increased in workers exposed to benzene,[24] an established leukemogen and suspected lymphomagen. As such, it seems that mitochondrial biogenesis is a biologically plausible pathway in lymphomagenesis.

The major strength of our study is that the biological samples were collected prospectively, before lymphoma diagnosis, from a large population including both genders. Our study also has limitations. We did not have repeated measures of mtDNA copy number over time, and a single measurement may not reflect mtDNA copy-number status over a lifetime. In addition, it is not possible to distinguish whether the increased MCN in one cell is due to the increased mitochondria (constant MCN in each mitochondrion) or is due to the increased mtDNA replication without increased mitochondrial number. Indeed, the increase in MCN in a cell can indicate increased mitochondrial mass and/or mtDNA replication. Moreover, due to the slow-growing nature of indolent lymphomas such as CLL in which subclinical disease can be present for decades before diagnosis, we cannot exclude that we might have observed some epiphenomenon related to the presence of early disease stages even among subjects with >4.6 years between blood collection and diagnosis. Although we did not observe any trend among CLL cases between mtDNA copy number and follow-up time, we cannot dismiss that the observed association might be caused by indolent (preclinical) disease, especially as the relation between mtDNA and lymphoma was only seen for CLL, which of all lymphoma subtypes is the most indolent. Finally, it is possible that some controls had undiagnosed subclinical CLL at the time of blood collection. However, given that CLL is a rare disease, bias due to individuals with undiagnosed CLL would be unlikely.

In conclusion, we observed that mtDNA copy number was strongly associated with increased risk of CLL. Although the biological significance is yet to be clarified, this may lead to a better understanding of mitochondrial function and damage in lymphomagenesis, particularly in the development of CLL.

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## Authorship

Contribution: R.V. and P.V. designed the study; Q.L, N.R., C-S.L., and W-L.C. performed experiments; P.G., A.T., D.C., A.M., H.B., A.T., P.L., D.T., V.K., R.T., S.P., G.M., E.W., J.M.H.C., E.A., N.S., M.D., J.R.Q., M.J.S., B.M., A.S.J., J.M., S.B., P.H.P., H.B.B-d-M., N.W., K.T.K., R.C.T., P.B., A.S., E.R., and P.V. organized the data of the EPIC cohort study; A.N. provided intellectual input; F.S.H. analyzed the data with important intellectual input from R.V. and prepared and revised the manuscript; and all authors reviewed and approved the final manuscript.

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