Mitochondrial DNA Copy Number in Peripheral Blood Cells and Risk of Developing Breast Cancer

Alina Lemnrau, Mark N. Brook, Olivia Fletcher, Penny Coulson, Katarzyna Tomczyk, Michael Jones, Alan Ashworth, Anthony Swerdlow, Nick Orr, and Montserrat Garcia-Closas

Abstract

Increased mitochondrial DNA (mtDNA) copy number in peripheral blood cells (PBC) has been associated with the risk of developing several tumor types. Here we evaluate sources of variation of this biomarker and its association with breast cancer risk in a prospective cohort study. mtDNA copy number was measured using quantitative real-time PCR on PBC DNA samples from participants in the UK-based Breakthrough Generations Study. Temporal and assay variation was evaluated in a serial study of 91 women, with two blood samples collected approximately 6-years apart. Then, associations with breast cancer risk factors and risk were evaluated in 1,108 cases and 1,099 controls using a nested case–control design. In the serial study, mtDNA copy number showed low assay variation but large temporal variation (assay intraclass correlation coefficient (ICC), 79.3%–87.9%; temporal ICC, 38.3%). Higher mtDNA copy number was significantly associated with younger age at blood collection, being premenopausal, having an older age at menopause, and never taking HRT, both in cases and controls. Based on measurements in a single blood sample taken on average 6 years before diagnosis, higher mtDNA copy number was associated with increased breast cancer risk (OR (95% CI) for highest versus lowest quartile, 1.37 (1.02–1.83); P = 0.007). In conclusion, mtDNA copy number is associated with breast cancer risk and represents a promising biomarker for risk assessment. The relatively large temporal variation should be taken into account in future analyses. Cancer Res; 75(14); 1–7. ©2015 AACR.

Introduction

Mitochondria are responsible for multiple cellular functions, including regulation of energy production, modulation of oxidation–reduction status, generation of reactive oxygen species, and apoptosis (1). Each mitochondrion possesses multiple copies of a mitochondrial genome comprised of independently replicating double-stranded DNA (mtDNA; ref. 1). mtDNA lacks protective histones and has limited capacity for repairing damage. This, in conjunction with a high level of exposure of the mitochondrial genome to oxidative damage, results in a comparatively higher mutation rate than typically observed in nuclear genomic DNA (gDNA). In contrast with gDNA, the integrity of which is maintained by sophisticated repair mechanisms, mitochondria compensate for mtDNA damage by increasing mtDNA copy number (2). Therefore, mtDNA copy number has been proposed as a marker of DNA damage and oxidative stress (3, 4).

Oxidative stress is thought to play an important role in breast carcinogenesis, with breast tumors showing frequent alterations in mtDNA (5–7). However, the role of these alterations in breast cancer initiation and development is not well understood (5). The evidence regarding the association between mtDNA copy numbers in peripheral blood cells (PBC) and risk of breast cancer is also limited. In a retrospective study with postdiagnostic blood samples including 103 case–control pairs, Shen and colleagues (8) found that high mtDNA copy number in PBCs was associated with increased risk of breast cancer, and several endogenous oxidants and antioxidants. A small prospective study of 183 breast cancer cases and 529 controls in China also found a similar association with breast cancer risk; however, data suggested that the association was present only in samples collected close to diagnosis (9). In contrast, reduced mtDNA copy number was shown to be correlated with tumor progression in Chinese patients with breast cancer (10).

Higher mtDNA copy number in PBCs has also been associated with the risk of developing other tumor types in prospective cohort studies, including non-Hodgkin lymphoma (11), lung cancer (12), pancreatic cancer (13), and colorectal cancer (14). Conversely, a retrospective case–control study showed an increased risk of renal cancer associated with decreased mtDNA copy number (15). All of these studies measured mtDNA copy number at a single point in time, and little is known about the sources of variation of this biomarker in the population, which may be important to interpret findings from epidemiologic studies (16).

Here, we evaluate sources of variation of mtDNA copy number and its association with breast cancer risk in the Breakthrough Generations Study (BGS), a prospective cohort study of more than 113,000 women in the United Kingdom.
Patients and Methods

Study population

The study design is depicted in the flow chart in Fig. 1. BGS is a prospective cohort study of breast cancer that has recruited 113,073 women over the period 2003 to 2013 (17). All participants were asked to complete a risk factor questionnaire and provide a blood sample at recruitment (T0). All women are followed up via repeat questionnaires; for women recruited during 2004 and 2005 a second blood sample was also requested at follow up, 5 to 6 years after recruitment (T1). Repeat blood samples were available for 8,738 women.

Analyses in this report are based on two study populations nested within the BGS. The first population (referred to hereafter as the “serial study”) included a random sample of 91 participants who were recruited in 2004 and provided a blood sample at baseline (T0) and follow-up (T1), and met the following selection criteria: free of breast cancer at T1, no family relatives in the serial study, and less than 2 days between venipuncture and blood sample processing.

The second study population (referred to as the “nested case-control study”) included incident invasive or in situ breast cancer cases and controls individually matched to cases by recruitment source (i.e., Breakthrough Breast Cancer charity supporters, self-referral, or referral by existing study members), year of enrollment (i.e., year of baseline questionnaire/blood sample), ethnicity, birth date (within 12 months), availability of blood sample, and interval between venipuncture and blood arrival at the processing laboratory.

We originally identified 1,110 eligible case-control pairs. Thirteen samples were excluded from the analysis due to low amount of DNA available or assay failure, leaving 2,207 subjects available for analyses; these included 1,108 cases (953 invasive, 142 in situ, and 13 unknown invasiveness) and 1,099 controls.

Women were classified as postmenopausal if they had a natural menopause or a bilateral oophorectomy at least 2 years before blood draw at T0. Where the women’s menopausal status was unknown, they were classified as postmenopausal if they were ≥55 (current smokers) or ≥56 (never/former smokers) years old at enrollment. Otherwise, women were classified as premenopausal. Oral contraceptive (OC) and hormone replacement therapy (HRT) status was classified as never, former (last use ≥1 year before enrollment), and current (last use <1 year before enrollment) use. Smoking status was classified as never, former (last smoked ≥1 year before enrollment), and current (last smoked <1 year before enrollment) smoker.

The local Institutional Review Board and the South East Multi-Centre Research Ethics Committee approved the study protocol. All study participants provided signed consent to participate in the study.

Blood processing and DNA extraction

Blood samples were collected into EDTA coated Vacutainers (BD Biosciences) and returned to our laboratories by post. Samples were separated into buffy coat and plasma and stored in liquid nitrogen tanks. Buffy coat samples were sent for DNA extraction in one batch (2011) for the serial study, and in three batches (2009, 2011, and 2013) for the case–control study. Qiagen DNA Blood Mini Kits were used for DNA extraction, with the exception of samples from 29 cases and 15 controls extracted by Tepnel Life Sciences. DNA was quantified using Quant-iT PicoGreen dsDNA kits (Invitrogen) according to the manufacturer’s recommendations.

mtDNA copy number

mtDNA copy number was measured using a quantitative real-time PCR-based protocol based upon previously published methods (13, 15). All quantitative real-time PCR reactions were performed using an Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems).

Briefly, the amount of genomic and mitochondrial DNA in each sample was estimated from standard curves prepared by serial dilution of reference DNA. PCR primers were designed to amplify the mitochondrial gene MT-ND1 and the single-copy constitutioal gene ALB (18) in separate 14-µL reactions containing 7 µL of SYBR Green Master Mix (Applied Biosystems), 200 nmol/L of forward and reverse primers, and 5 ng gDNA. Primer sequences and thermal cycling conditions are shown in Supplementary Table S1. For ALB, a seven-point standard curve (range, 0.3125–20 ng) was constructed using gDNA of known concentration. To estimate total mtDNA copy number, a 69-bp fragment of the mitochondrial gene MT-ND1 was cloned into a pCR 2.1-TOPO vector, which was subsequently linearized and used to construct a seven-point standard curve (range, 3.125 × 10⁻³ – 2.0 ng). Both standard curves were optimized to give an R² of 0.99 or greater and PCR efficiency (slope) of between −3.1 and −3.5.

The number of copies of MT-ND1 and ALB per sample were then calculated using the following formula:

\[
\text{copy number per sample} = \left( \frac{6.02 \times 10^{23}}{\text{DNA length (bp)}} \right) \times \frac{\text{DNA amount (ng)}}{660 \times 1 \times 10^9}
\]

For each sample, average mtDNA copy number per cell was expressed as a ratio of MT-ND1 copy number to ALB copy number.
Estimates of sample MT-NT1 and ALB copy number were based on three replicates for samples from the serial study and two replicates for samples from the case–control study. Replicated samples were included in the same real-time experiment.

mtDNA copy number assays were performed in two phases. Phase I (April 2012) included samples from the 91 serial samples and 230 case–control pairs. Phase II (August 2013) included samples from an additional 880 case–control pairs. Duplicate samples from 97 subjects (47 subjects in phase I and 50 subjects in phase II) were included for quality control (QC) purposes. The same 47 phase I QC samples were also processed in phase II to enable evaluation of variation between phases.

The pairs of DNA samples for T0–T1 pairs and case–control pairs were randomly sorted within the same DNA plate. All samples were processed and analyzed in the same manner, and laboratory personnel were blinded to T0–T1 or case–control status, and QC status.

The intraassay coefficient of variability (CV) for the mtDNA copy number in the serial study was 7.3% (95% confidence intervals [95% CI], 5.7%–8.8%) in T0 and 7.0% (95% CI, 5.9%–8.2%) in T1. For the samples in the case–control study, the CV was 10.5% for phase I (95% CI, 8.1%–12.8%) based on blinded duplicated aliquots from 47 women and 3.7% (95% CI, 3.0–4.6%) for phase II (based on blinded duplicated aliquots from 50 women). The interphase CV was 7.6% (95% CI, 5.6%–9.5%) based on 47 women with blinded aliquots included in both phases).

Statistical analysis

Z-score for mtDNA copy number. Based on 47 samples with duplicated aliquots included in phase I and II, the mtDNA copy number was slightly lower in phase I than phase II: median (25th, 75th percentiles) of 160.8 (121.0, 269.0) in phase I and 172.2 (128.2, 278.6) in phase II. This difference was larger when comparing samples in phase I with samples in phase II (paired Wilcoxon rank sum P = 0.10).

Serial study. Between-subject and within-subject variation was assessed using serial samples from 91 participants collected at two time points, baseline (T0) and approximately 6 years later (T1), measured in triplicates. The three measures in each time point allowed separating the assay and temporal components of the within-subject variation. Nine measurements were removed from the analysis; four had undetermined measurements of mtDNA (three at T0, and one at T1), four had undetermined measurements of gDNA (all at T0), and one had an out-of-range ratio of mtDNA to gDNA (at T0). This resulted in a total of 537 measurements on samples from 91 subjects included in the analyses.

The intraclass correlation coefficient (ICC) of the Z-score of mtDNA copy number was used to quantify the between-subject variation in the study population relative to the total variation (i.e., sum of the between-subject variance and within-subject variation). Within-subject variation reflects the variation due to changes in a subject’s measurement over time, as well as assay variation. The temporal ICC was estimated by removing the assay variation from the total within-subject variation to estimate the temporal variance, and was defined as the ratio of between-subject variance to the sum of between-subject variance plus temporal variance. The assay ICC at each time point, T0 and T1, was also calculated based on three measurements within each time point, and was defined as the ratio of between-subject variance to the sum of between-subject variance plus assay variance at a given time point.

Nested case–control study. The Z-score of mtDNA copy number was analyzed as both categorical (quartiles or deciles defined according to the distribution in the control population) and continuous variables. Distributions of individual characteristics across quartiles of mtDNA Z-score were assessed separately for cases and controls. Median and 25th, 75th percentiles were used describe the central tendency and spread of the distribution of continuous characteristics as the distribution of many of these variables was skewed. Distributions of continuous characteristics were compared for top and bottom quartiles using the Wilcoxon–Mann–Whitney rank sum test. For categorical characteristics, we compared frequencies across quartiles of mtDNA using a χ2 test. We also performed tests for trend using linear regression models for continuous characteristics, with the Z-score of mtDNA copy number as the outcome variable.

Conditional logistic regression for matched pairs was used to estimate odds ratios (OR) and 95% CI for breast cancer risk in relation to mtDNA copy number. Models included indicator variables for 19 shipping dates when buffy coats were pulled from LN2 freezers and sent to the DNA extraction laboratory (extractions were done in three batches, including 10, 6, and 3 different buffy coat shipping dates each) in the conditional logistic analyses. In addition, we ran restricted analyses including only case–control pairs with samples extracted within the same batch. Interactions between mtDNA copy number and covariates were evaluated by including main effect and interaction terms in the conditional logistic models. A likelihood ratio test (LRT) comparing models with main effects only to models with main effects and interaction terms was used to test for interaction. Conditional analyses resulted in the loss of 55 cases and 46 controls due to missing data in one of the case–control pairs, resulting in a total of 1,053 matched pairs available for analyses.

All statistical tests were two-sided with P < 0.05 used as the threshold for statistical significance. All analyses were performed in STATA (Release 12; StataCorp).

Results

Serial study

Participants in the serial study (N = 91) had a median (25th, 75th percentile) age of 53 (47, 60) years at enrollment, and had an average of 6 years (range, 5–7 years) between the two blood collections at baseline (T0) and follow-up (T1). The median time that buffy coats were frozen in LN2 before being pulled for DNA extraction (LN2 freezing time) was 7.0 years (25th, 75th percentile: 6.9, 7.3) for T0 samples and 1.2 years (1.1, 1.2) for T1.

There was a relatively low correlation (Spearman ρ = 0.35; P = 0.0006) between the mtDNA copy number for each subject (average of the triplicate measurements) in T0 and T1 (Supplementary Fig. S1). The Z-scores for mtDNA copy number within a subject tended to be lower in T1 than T0 [mean (SD), –0.151 (0.94) and
Table 1. Sources of variation and components of the ICC for mtDNA copy number Z-score, based on serial samples from 91 women measured in triplicate at two time points approximately 6-years apart

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Variance</th>
<th>ICC components</th>
<th>ICC (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total variance</td>
<td>0.98</td>
<td>Total ICC</td>
<td>32.7 (14.3–51.1)</td>
</tr>
<tr>
<td>Between subject variance</td>
<td>0.31</td>
<td>Assay ICC</td>
<td>7.2 (7.2–85.8)</td>
</tr>
<tr>
<td>Within subject variance</td>
<td>0.66</td>
<td>T0 (baseline)</td>
<td>87.9 (84.0–91.9)</td>
</tr>
<tr>
<td>Assay variation</td>
<td>0.16</td>
<td>T1 (follow-up)</td>
<td>87.9 (84.0–91.9)</td>
</tr>
<tr>
<td>Temporal variance</td>
<td>0.50</td>
<td>Temporal ICC</td>
<td>38.3 (18.0–55.5)</td>
</tr>
</tbody>
</table>

0.163 (0.94), respectively; paired t test *P = 0.006*. The total ICC for the Z-score of mtDNA copy number was estimated to be 32.7% [95% CI [14.3%–51.1%; Table 1]]. Separation of the assay and temporal components of the within-subject variation indicated low assay variation, whereas changes in measurements between the T0 and T1 explained most of the within-subject variation. The assay ICC was 79.3% [72.9%–87.9%] at T0 and 87.9% [84.0%–91.9%] at T1. In contrast, the temporal ICC after accounting for assay variation was 38.3% [18.0%–55.5%]. Changes in age between T0 and T1 had little impact on estimates of the temporal variation (data not shown).

Nested case–control study

The median (25th, 75th percentile) age at enrollment was 55 (47, 61) for both controls and cases. The median (25th, 75th percentile) time between blood collection and breast cancer diagnosis in cases was 2.3 (1.3, 4.0) years. Approximately 60% of cases and controls were postmenopausal at baseline. Selected characteristics among cases and controls are summarized by quartiles of mtDNA copy number in Table 2 and Table 3. Compared with women with lower levels of mtDNA copy number, those with higher levels were younger, more often premenopausal, and when postmenopausal, they had later ages at menopause and had more often never taken HRT. A higher percentage of cases in the higher quartiles were former users of OCs (*P < 0.001*). However, this association was not seen in controls (*P = 0.928*). Similarly, women in the highest quartile had shorter duration of alcohol use in controls (*P = 0.007*) but not in cases (*P = 0.535*).

Cases had a higher average Z-score for mtDNA copy number than controls [mean (SD), 0.145 (1.03) and −0.005 (0.99), respectively; paired t test *P = 0.0004*]. Conditional logistic analyses including 1,053 matched pairs, adjusting for buffy coat shipping date, showed strong evidence for an association between increasing mtDNA copy number and breast cancer risk when mtDNA was considered as a continuous variable (*P trend = 0.007*). Women in the highest quartile of mtDNA copy number had a higher risk of developing breast cancer compared with women in the lowest quartile (OR, 1.37; 95% CI, 1.02–1.83; *P = 0.037*; Table 4). Decile analyses confirmed a dose–response relationship with increasing levels of mtDNA copy number and suggested a plateau effect (Supplementary Fig. S2). Analyses restricted to the 713 case–control pairs with DNA samples extracted with the same method (Qiagen Mini Kits) and in the same batch (68% of the total) showed similar risk associations to those in analyses based on the remainder of the samples (*N = 340 pairs*) adjusted by buffy coat shipping date (Supplementary Table S2; *P interaction = 0.657*).

The mtDNA copy number association with breast cancer risk was not significantly modified by age at blood collection (*P* [df = 1 df] = 0.091), parity [*P* [df = 3 df] = 0.572], menopausal status [*P* [df = 1 df] = 0.182], age at menopause [*P* [df = 1 df] = 0.915], HRT use [*P* [df = 2 df] = 0.961], OC use [*P* [df = 2 df] = 0.593], alcohol consumption [*P* [df = 1 df] = 0.290], family history [*P* [df = 1 df] = 0.473], smoking status [*P* [df = 2 df] = 0.568], or BMI [*P* [df = 1 df] = 0.045]. A stronger association of mtDNA copy number and breast cancer risk was observed for women with DNA samples taken at longer time intervals before diagnosis [Supplementary Table S3: OR (95% CI) for top to bottom quartiles were 0.83 (0.43–1.61) for <1 year between blood collection and diagnosis/selection; 0.79 (0.43–1.16) for 1–<2 years; 1.29 (0.78–2.14) for 2–4 years; and 3.29 (1.83–5.90) for 4 years or more; *P interaction = 2.8 × 10⁻⁴*]. We saw no significant differences in the risk association by invasiveness (in situ vs. in situ, case-only *P* heterogeneity = 0.781) or by estrogen receptor (ER) status of invasive tumors (case-only *P* heterogeneity = 0.524, based on 609 ER⁺ and 106 ER⁻ tumors).

Discussion

Our data provide evidence for an association between mtDNA copy number measured in PBC samples and risk of subsequently developing breast cancer, based on analyses of more than 1,000 case–control pairs from a prospective cohort study. Two previous reports, a nested case–control study of 183 breast cancers and 529 controls in China using prediagnostic samples (9) and a case–control study of 103 case–control pairs in the United States using postdiagnostic samples (8), reported an association between increasing mtDNA copy number and breast cancer risk. Our data provide strong confirmation of these results and more precise measures of association based on a study with more than five times the number of cases in previous reports.

Multiple factors are likely to determine mtDNA copy number in a particular cell type. Evidence in the literature suggests that there is an increase in mtDNA copy number per cell due to some forms of oxidative stress, age, T-cell activation, or exposure to benzene compounds (2, 3, 19). We observed an inverse association between mtDNA copy number in PBCs and age at blood collection: women in the highest quartile were on average 2 to 3 years younger than women in the lowest quartile. This unexpected result has not been reported in previous studies of smaller size (12–14, 20) and therefore requires confirmation. Most individual characteristics evaluated in this report were not associated with mtDNA copy number, which is consistent with previous reports (9, 13, 14, 20). mtDNA copy number has been associated with smoking in a previous study (13). We did not confirm this association; however, this could be due to low levels of smoking in our study population. Reproductive history was not associated with mtDNA copy number, with the possible exception of an association between later age at menopause and HRT never usage and increased mtDNA copy number. The potential associations with OC and alcohol use require further confirmation. Other factors related to the methods used, such as DNA extraction and white blood cell type composition could also influence the measures of mtDNA. We accounted for DNA extraction batch in our analyses. However, we did not have a measure of blood cell composition and if it varied between cases and controls, it could have mediated the observed associations.
Table 2. Distribution of subject characteristics (continuous) by quartiles of mtDNA copy number in 1,099 controls and 1,108 cases

<table>
<thead>
<tr>
<th>Quartiles of mtDNA copy number (Z-score)</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Controls</td>
<td>266</td>
<td>265</td>
<td>265</td>
<td>266</td>
<td></td>
</tr>
<tr>
<td>Median (25th, 75th)</td>
<td>56.0 (49.0, 61.0)</td>
<td>56.0 (49.0, 61.0)</td>
<td>55.0 (46.0, 61.0)</td>
<td>52.0 (44.0, 60.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Time between venipuncture and selection, y</td>
<td>2.5 (1.3, 4.2)</td>
<td>2.4 (1.4, 4.0)</td>
<td>2.3 (1.1, 4.0)</td>
<td>2.3 (1.1, 4.0)</td>
<td>0.388</td>
</tr>
<tr>
<td>Age at menarche, y</td>
<td>13.0 (12.0, 14.0)</td>
<td>13.0 (12.0, 14.0)</td>
<td>13.0 (12.0, 14.0)</td>
<td>13.0 (12.0, 14.0)</td>
<td>0.796</td>
</tr>
<tr>
<td>Age at first birth (parous women), y</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
<td>0.712</td>
</tr>
<tr>
<td>Number of full-term pregnancies</td>
<td>30.0 (27.0, 33.0)</td>
<td>29.0 (27.0, 33.0)</td>
<td>29.0 (27.0, 33.0)</td>
<td>29.0 (27.0, 33.0)</td>
<td>0.573</td>
</tr>
<tr>
<td>Age at last birth (parous women), y</td>
<td>50.0 (43.0, 52.0)</td>
<td>50.0 (43.0, 52.0)</td>
<td>50.0 (43.0, 52.0)</td>
<td>50.0 (43.0, 52.0)</td>
<td>0.005</td>
</tr>
<tr>
<td>Alcohol intake (ever drinkers), y</td>
<td>31.0 (22.0, 39.0)</td>
<td>33.0 (25.0, 39.0)</td>
<td>33.0 (25.0, 39.0)</td>
<td>33.0 (25.0, 39.0)</td>
<td>0.007</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.6 (22.5, 27.5)</td>
<td>24.7 (22.3, 27.4)</td>
<td>24.9 (22.6, 28.5)</td>
<td>24.8 (22.5, 27.9)</td>
<td>0.663</td>
</tr>
<tr>
<td>Height, cm</td>
<td>162.6 (160.0, 167.6)</td>
<td>162.6 (158.3, 167.6)</td>
<td>162.6 (159.0, 167.6)</td>
<td>162.6 (160.0, 170.2)</td>
<td>0.483</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>65.8 (59.0, 74.4)</td>
<td>66.2 (59.9, 76.0)</td>
<td>67.1 (59.0, 76.0)</td>
<td>66.4 (60.8, 75.3)</td>
<td>0.258</td>
</tr>
<tr>
<td>Duration of smoking (ever smokers), y</td>
<td>15.0 (8.0, 26.0)</td>
<td>14.0 (7.0, 26.0)</td>
<td>17.0 (9.0, 28.0)</td>
<td>15.0 (9.0, 28.0)</td>
<td>0.670</td>
</tr>
<tr>
<td>Number of full-term pregnancies</td>
<td>201 26.0 (23.0, 29.0)</td>
<td>213 26.0 (24.0, 29.0)</td>
<td>252 26.0 (23.0, 29.0)</td>
<td>277 26.0 (24.0, 29.0)</td>
<td>0.152</td>
</tr>
<tr>
<td>Age at last birth (parous women), y</td>
<td>26.0 (23.0, 29.0)</td>
<td>26.0 (24.0, 29.0)</td>
<td>26.0 (23.0, 29.0)</td>
<td>26.0 (24.0, 29.0)</td>
<td>0.152</td>
</tr>
<tr>
<td>Number of full-term pregnancies</td>
<td>21.0 (2.0, 3.0)</td>
<td>21.0 (2.0, 3.0)</td>
<td>21.0 (2.0, 3.0)</td>
<td>21.0 (2.0, 3.0)</td>
<td>0.756</td>
</tr>
<tr>
<td>Age at first birth (parous women), y</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
<td>2.0 (2.0, 3.0)</td>
<td>0.573</td>
</tr>
<tr>
<td>Age at last birth (parous women), y</td>
<td>50.0 (46.0, 53.0)</td>
<td>50.0 (47.0, 53.0)</td>
<td>50.0 (49.0, 53.0)</td>
<td>50.0 (49.0, 53.0)</td>
<td>0.027</td>
</tr>
<tr>
<td>Alcohol intake (ever drinkers), y</td>
<td>30.0 (20.0, 38.0)</td>
<td>32.0 (23.0, 40.0)</td>
<td>29.5 (20.5, 37.0)</td>
<td>29.0 (21.0, 37.0)</td>
<td>0.081</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.6 (22.6, 27.8)</td>
<td>25.4 (22.2, 29.0)</td>
<td>25.0 (22.8, 28.8)</td>
<td>24.5 (22.4, 27.4)</td>
<td>0.359</td>
</tr>
<tr>
<td>Height, cm</td>
<td>162.6 (160.0, 167.6)</td>
<td>165.1 (160.0, 168.0)</td>
<td>163.0 (160.0, 168.0)</td>
<td>165.1 (160.0, 170.2)</td>
<td>0.056</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>66.7 (60.3, 74.4)</td>
<td>68.5 (61.7, 77.6)</td>
<td>69.0 (60.3, 76.0)</td>
<td>66.2 (59.9, 73.7)</td>
<td>0.651</td>
</tr>
<tr>
<td>Duration of smoking (ever smokers), y</td>
<td>16.0 (7.0, 26.0)</td>
<td>17.0 (11.0, 28.0)</td>
<td>15.0 (10.0, 25.0)</td>
<td>15.0 (9.0, 25.0)</td>
<td>0.821</td>
</tr>
<tr>
<td>Height, cm</td>
<td>10.2 (2.4, 18.0)</td>
<td>7.0 (3.8, 17.1)</td>
<td>9.8 (3.2, 18.0)</td>
<td>9.3 (2.7, 20.0)</td>
<td>0.651</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>20.0 (12.0, 30.0)</td>
<td>19.0 (15.0, 28.0)</td>
<td>21.0 (11.0, 28.0)</td>
<td>20.0 (12.0, 28.0)</td>
<td>0.584</td>
</tr>
<tr>
<td>Duration of smoking (ever smokers), y</td>
<td>22.5 (10.0, 32.0)</td>
<td>25.0 (14.0, 32.0)</td>
<td>22.5 (14.0, 32.0)</td>
<td>22.5 (14.0, 32.0)</td>
<td>0.023</td>
</tr>
<tr>
<td>Pack-years (ever smokers)</td>
<td>9.3 (3.5, 19.0)</td>
<td>7.4 (3.8, 20.0)</td>
<td>8.6 (3.3, 20.1)</td>
<td>9.3 (5.5, 16.5)</td>
<td>0.759</td>
</tr>
<tr>
<td>Time since quitting (former smokers), y</td>
<td>22.5 (10.0, 32.0)</td>
<td>25.0 (14.0, 32.0)</td>
<td>22.5 (14.0, 32.0)</td>
<td>22.5 (14.0, 32.0)</td>
<td>0.349</td>
</tr>
</tbody>
</table>
Strengths of our study include having prospectively collected blood samples and the large sample size. Contrary to the only previous prospective study (9), our data showed a stronger association with risk when mtDNA copy number was measured in samples collected longer before the date of diagnosis or control selection, with the strongest association found for samples collected 4 years or longer before diagnosis/selection. This indicates that preclinical disease is unlikely to explain the observed association with increased breast cancer risk, and suggests early rather than late-acting biologic effects of oxidative stress or other correlates of mtDNA copy number on breast cancer risk. Other explanations for an apparent lag effect include preclinical effects of the tumor that result in a lower average mtDNA copy number in blood cells of samples taken close to diagnosis. However, this finding requires confirmation in further prospective studies, preferably with a larger range of follow up times or multiple samples collected at different points in time. Other measures of mitochondrial genome instability, such as length heteroplasmy in the hypervariable regions of mtDNA, have been suggested to influence breast cancer risk (21); however, they are not evaluated in this report.

The serial study with repeated measurements of mtDNA copy number in the same subject approximately 6-years apart indicated that although the assay variation was small, repeated measurements for the same individual at two points in time varied substantially, resulting in a low total ICC when both assay and

### Table 3. Distribution of subject characteristics (categorical) by quartiles of mtDNA copy number in 1,099 controls and 1,108 cases

<table>
<thead>
<tr>
<th>Quartiles of mtDNA copy number (Z-score)</th>
<th>Controls</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>Q2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Menopausal status
- Postmeno
  - Controls: 154 (66.4)
  - Cases: 122 (61.3)
- Premeno
  - Controls: 78 (33.6)
  - Cases: 77 (38.7)

#### HRT status
- Never
  - Controls: 168 (61.1)
  - Cases: 148 (61.4)
- Former
  - Controls: 67 (24.4)
  - Cases: 46 (19.1)
- Current
  - Controls: 40 (14.5)
  - Cases: 47 (19.5)

#### Use of oral contraceptives
- Never
  - Controls: 49 (18.0)
  - Cases: 56 (23.2)
- Former
  - Controls: 209 (76.8)
  - Cases: 173 (71.8)
- Current
  - Controls: 14 (5.1)
  - Cases: 12 (5.0)

#### Alcohol consumption
- Never
  - Controls: 46 (16.7)
  - Cases: 46 (16.7)
- Ever
  - Controls: 230 (83.3)
  - Cases: 234 (88.0)

#### Breast cancer family history in first-degree relatives
- No
  - Controls: 154 (66.4)
  - Cases: 122 (61.3)
- Yes
  - Controls: 78 (33.6)
  - Cases: 77 (38.7)

#### Smoking Status
- Never
  - Controls: 163 (59.5)
  - Cases: 212 (88.0)
- Former
  - Controls: 97 (35.4)
  - Cases: 81 (33.6)
- Current
  - Controls: 14 (5.1)
  - Cases: 12 (5.0)

### Table 4. OR and 95% CI for the association between breast cancer risk and quartiles of mtDNA copy number (Z-score), based on 1,053 matched case-control pairs in the BGS

<table>
<thead>
<tr>
<th>mtDNA copy number (Z-score)</th>
<th>Cases</th>
<th>Controls</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>P trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartile 1</td>
<td>230</td>
<td>264</td>
<td>Ref.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quartile 2</td>
<td>234</td>
<td>267</td>
<td>1.19 (0.90–1.57)</td>
<td>0.220</td>
<td></td>
</tr>
<tr>
<td>Quartile 3</td>
<td>283</td>
<td>263</td>
<td>1.39 (1.05–1.85)</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>Quartile 4</td>
<td>306</td>
<td>259</td>
<td>1.37 (1.02–1.83)</td>
<td>0.037</td>
<td>0.007</td>
</tr>
</tbody>
</table>

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temporal variation were taken into account (total ICC, 32.7%). Although DNA samples from T0 and T1 were processed and analyzed at the same time, samples in T0 had been stored approximately for 6 years longer than samples in T1, with very little variation in storage time within the two time points. Changes in mtDNA during storage could contribute to the observed differences between T0 and T1; however, this is unlikely given that storage time was unrelated to mtDNA copy number in the case–control study after adjusting for batch effects ($P = 0.813$).

Temporal variation in mtDNA copy number in prospective studies with a single DNA sample taken before diagnosis (11–14) is likely to introduce measurement error that is nondifferential to disease status, and thus would tend to underestimate of the underlying risk association. Accounting for temporal changes in disease status, and thus would tend to underestimate of the risk association. Accounting for temporal changes in disease status, and thus would tend to underestimate of the risk association.

In conclusion, our prospective study provides evidence for an association between higher mtDNA copy number in PBCs at a single sample per subject due to the challenges and expense of collecting multiple samples before diagnosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Authors’ Contributions

Conception and design: A. Lemnrau, A. Swedlow, N. Orr, M. Garcia-Closas
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Lemnrau, M.N. Brook, O. Fletcher, M. Garcia-Closas
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Lemnrau, P. Coulson, M. Jones

Study supervision: A. Lemnrau, N. Orr, M. Garcia-Closas

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