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Personalized Medicine and Imaging

Clinical Cancer Research

Low Tumor Mitochondrial DNA Content Is Associated with Better Outcome in Breast Cancer Patients Receiving Anthracycline-Based Chemotherapy

Marjolein J.A. Weerts, Antoinette Hollestelle, Anieta M. Sieuwerts, John A. Foekens, Stefan Sleijfer, and John W.M. Martens

Abstract

Purpose: In this study, we aimed to explore whether low levels of mitochondrial DNA (mtDNA) content in the primary tumor could predict better outcome for breast cancer patients receiving anthracycline-based therapies. We hypothesized that tumor cells with low mtDNA content are more susceptible to mitochondrial damage induced by anthracyclines, and thus are more susceptible to anthracycline treatment.

Experimental Design: We measured mtDNA content by a qPCR approach in 295 primary breast tumor specimens originating from two well-defined cohorts: 174 lymph node–positive patients who received adjuvant chemotherapy and 121 patients with advanced disease who received chemotherapy as first-line palliative treatment. The chemotherapy regimens given were either anthracycline-based (FAC/FEC) or methotrexate-based (CMF).

Results: In both the adjuvant and advanced settings, we observed increased benefit for patients with low mtDNA content in their primary tumor, but only when treated with FAC/FEC. In multivariable Cox regression analysis for respectively distant metastasis-free survival and progression-free survival, the HR for the FAC/FEC-treated mtDNA low group in the adjuvant setting was 0.46 [95% confidence interval (CI), 0.24–0.89; $P = 0.020$] and in the advanced setting 0.49 (95% CI, 0.27–0.90; $P = 0.022$) compared with the FAC/FEC-treated mtDNA high group. We did not observe these associations in the patients treated with CMF.

Conclusions: In our two study cohorts, breast cancer patients with low mtDNA content in their primary tumor had better outcome from anthracycline-containing chemotherapy. The frequently observed decrease in mtDNA content in primary breast tumors may be exploited by guiding chemotherapeutic regimen decision making. *Clin Cancer Res; 23(16); 4735–43. ©2017 AACR.*

Introduction

Mitochondria are cellular organelles involved in multiple cellular processes, but best known for efficient ATP generation through oxidative phosphorylation. Mitochondria contain their own genomic entity termed mitochondrial DNA (mtDNA) encoding proteins essential for the oxidative phosphorylation system, and rRNAs and tRNAs functioning in the mitochondrial translation apparatus. Multiple mtDNA molecules can reside within a single mitochondrion, and multiple mitochondria can reside within a single cell (1–3), making the total number of mtDNA molecules per cell (mtDNA content) variable. In general, the mtDNA content per cell is dependent on the tissue's energy demands (4).

In tumors, the mtDNA content is often changed compared with nonneoplastic adjacent tissue (5). For breast cancer specifically, there is a decline in mtDNA content: approximately three-quarter of primary breast tumor specimens have a decreased mtDNA content when compared with their nearby normal mammary epithelium (5–13). We recently reported an association of worse 10-year distant metastasis-free survival (DMFS) for node-negative primary breast cancer patients who did not receive any (neo-) adjuvant systemic treatment with low mtDNA content in their primary tumors, showing the impact of mtDNA content on tumor aggressiveness (14). However, how these findings influence response to systemic therapy in breast cancer patients is unknown.

The anthracyclines doxorubicin and epirubicin are currently the most frequently used agents in breast cancer treatment. However, despite multiple efforts to find predictors for anthracycline sensitivity, up to date no evidence-based biomarkers are applied clinically in neither early nor metastatic breast cancer. Several markers have been postulated to predict benefit from adjuvant anthracycline-based chemotherapy, including *TOP2A* gene amplification or protein expression, *ERBB2* (HER2) amplification, *TOP2A* and *ERBB2* coamplification, chromosome 17 polysomy (CEP17), TIMP1 protein expression, FOXP3 protein expression, or TP53 protein expression, but none of them have been recommended for clinical use (15). Anthracyclines induce severe oxidative stress (16) and are known to accumulate in mitochondria, where they can intercalate mtDNA (17) and damage mtDNA (18). In *in vitro* model systems, reduced mtDNA content increases

Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, the Netherlands.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Marjolein J.A. Weerts, Erasmus MC Cancer Institute, Erasmus University Medical Center, Wytemaweg 80, Rotterdam 3015 CN, the Netherlands. Phone: 311-0704-4375; Fax: 311-0704-4377; E-mail: m.weerts@erasmusmc.nl

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Translational Relevance

Anthracycline-based regimens are commonly used for breast cancer treatment. Despite multiple efforts to find predictors for anthracycline sensitivity, currently no biomarkers are applied clinically. Anthracyclines can induce severe oxidative stress in mitochondria and damage their DNA, as illustrated by off-target effects in terms of cardiotoxicity. Interestingly, multiple studies have indicated that tumor mitochondrial DNA (mtDNA) content is decreased in most breast cancer cases. We hypothesized that tumor cells with low mtDNA content are more susceptible to anthracycline-based treatment compared with tumor cells with high mtDNA content. Accordingly, our data demonstrate in two different clinical settings, adjuvant and palliative, that breast cancer patients with low mtDNA content in the primary tumor have better outcome after anthracycline-containing chemotherapy, whereas this is not the case after methotrexate-based chemotherapy. Our observations indicate that the frequently observed decrease in mtDNA content in primary breast tumors may be exploited by guiding chemotherapeutic regimen decision making.

sensitivity to doxorubicin (13, 19). We hypothesize that tumor cells with low mtDNA content are more susceptible to mitochondrial damage induced by anthracyclines than cells with high mtDNA content, and thus are more susceptible to anthracycline treatment. Before the introduction of anthracycline-based chemotherapy, methotrexate-based regimens were most often applied in breast cancer (20). The working mechanism of this compound is not directly at the DNA level, but it is an antimetabolite, ultimately leading to inhibition of DNA synthesis. Because methotrexate induces only low levels of oxidative stress (16), we do not expect differences in its efficacy in tumor cells with either low or high mtDNA content.

In this retrospective study, we aimed to explore if low levels of mtDNA content in the primary tumor can predict better outcome for patients receiving anthracycline-based therapies, but not for patients receiving methotrexate-based therapies.

Materials and Methods

Study cohort and sampling

DNA extracts from an earlier retrospective study (21) were selected from our bio-bank at the Erasmus MC (Supplementary Fig. S1). Tissue specimens from our selection were collected during 1979 to 1995. The study was approved by the medical ethics committee of the Erasmus MC (MEC 02.953) and conducted in accordance to the Code of Conduct of Federation of Medical Scientific Societies in the Netherlands, and thus provided patient records were anonymized and deidentified prior to analysis. We adhered to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK; ref. 22). Follow-up and tumor response were defined based on the criteria by the International Union Against Cancer (23) and the European Organization for Research and Treatment of Cancer (24) as described previously (21). Estrogen receptor (ER) status, progesterone receptor (PR) status, and *ERBB2* (HER2) amplification were determined as described before (25, 26, 27). The RT-qPCR Genomic Grade Index (GGI) was determined as described previously (28), which we used as an alternative grading system for histologic grading according to Bloom-Richardson since this grading was unavailable for nearly 30% of the samples.

For the adjuvant cohort (REMARK diagram in Supplementary Fig. S1A), a total of 528 DNA extracts were available from fresh-frozen primary breast tumor specimens originating from female patients with lymph node–positive disease (N1 or N2) without distant metastasis at primary diagnosis (M0). None of the patients received neoadjuvant therapy. We selected only samples from patients receiving as adjuvant systemic treatment either cyclophosphamide/methotrexate/5-fluorouracil (CMF) or 5-fluoracil/anthracycline/cyclophosphamide (FAC or FEC) and did not receive adjuvant hormonal treatment ($n = 307$; patient characteristics of complete set in Supplementary Table S1). Note that at the time of tumor collection, only patients with node-positive disease but not node-negative disease received adjuvant systemic chemotherapy in the Netherlands. Next, we selected based on uniformity in the DNA extraction method only the samples extracted with the DNeasy Tissue Kit (Qiagen, performed as described by supplier; $n = 289$). To minimize stromal cell contamination, we selected for an invasive tumor cell percentage of at least 50 in the specimen ($n = 176$). One patient ineligible in retrospect was excluded. Thus, mtDNA content was examined in a total of 175 patients. For 1 patient, a qPCR amplification signal was absent. In the final cohort of 174 patients, 56 received CMF and 118 received FAC/FEC adjuvant systemic therapy. At the end of follow-up, 111 events (distant metastasis) were observed within the group of these 174 patients.

For the advanced cohort (REMARK diagram in Supplementary Fig. S1B), a total of 231 DNA extracts from fresh-frozen primary disease resection specimen were available from female patients who received chemotherapy as first-line treatment for disease recurrence (loco-regional or distant metastasis). We selected only those samples originating from patients receiving either CMF or FAC/FEC as first-line chemotherapy regimen ($n = 206$; patient characteristics of complete set in Supplementary Table S2). We again only selected patients based on uniformity in the DNA extraction method [only the samples extracted with the DNeasy Tissue kit (Qiagen), performed as described by supplier; $n = 197$] and with an invasive tumor cell percentage of at least 50 in the specimen ($n = 126$). Five patients were excluded: no clinical data were available for four patient samples, and 1 patient who did not receive axillary lymph node dissection was ineligible in retrospect. Thus, mtDNA content was examined in a cohort of 121 patients, of which 44 received CMF and 77 received FAC/FEC first-line chemotherapy. A total of 29 (24%) patients received hormonal consolidation therapy during follow-up, and for 1 patient, it was unknown if she had been treated with consolidation therapy. Of the 121 patients, none (0%) received neoadjuvant therapy and 34 (28%) received prior adjuvant chemotherapy (7 CMF, 17 FAC/FEC, and 9 cyclophosphamide monotherapy). Overall response was defined as described previously (21), and a total of 69 patients responded to chemotherapy (5 with complete remission, 38 with partial remission, 26 with stable disease > 6 courses) and 51 patients showed no response to chemotherapy (36 with progression, 15 with stable disease $\leq$ 6 courses; Supplementary Table S3). For 1 patient, the type of response was ambiguous. In

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our analysis, we right-censored at 18-month follow-up because of low numbers of patients at risk. At the end of follow-up, 104 events (progression) were observed within the group of 121 patients.

mtDNA content

Mitochondrial DNA content was determined as described previously (14). Briefly, a multiplex real-time qPCR was used, which amplifies nuclear *HMBS* (chr11q23.2-qter) and mitochondrial *MT-TL1* (chrMT 3212–3319). ROX normalized quantification cycle values (Cq [dRn]) were obtained from duplicate runs on the MX3000 or MX3005P qPCR systems (Agilent Technologies) by the adaptive baseline approach (MxPro v4.10) up to cycle 35 with fixed fluorescence thresholds at 0.004 dRn. We quantified in 1 ng DNA the ratio of mitochondrial DNA opposed to nuclear DNA by the relative quantitation method ($2^{\Delta Cq}$ [54]) using the obtained Cq values. Multiplying this ratio by the copy number of *HMBS* (obtained as described below) resulted in the number of mtDNA molecules per cell as mtDNA content. A calibration curve containing a pool of DNA extracts from a different set of fresh-frozen tumors was taken along as internal control to monitor the performance of the PCR reactions (Supplementary Table S4). Also, no template reactions were taken along in each run and never resulted in an obtained Cq value.

Copy-number analysis

Copy-number variation of the nuclear-encoded *HMBS* gene—which served as a reference to obtain mtDNA content—was obtained using qBiomarker Copy Number assays (Qiagen) VPH000-0000000A (multi-copy reference) and VPH111-0594782A (*HMBS* target) as described by the supplier. Briefly, *HMBS* and the multi-copy reference were quantified using SYBR-green based real-time qPCR in 4 ng of DNA. The multi-copy reference is a stable sequence that is minimally affected by local genomic changes, because it appears in the human genome over 40 times. ROX-normalized Cq values were obtained as described above but with fixed fluorescence thresholds at 0.2 dRn. In 4 ng of DNA, using the $2^{\Delta\Delta Cq}$ calibrator genome method, the predicted copy number of *HMBS* was obtained for each individual tumor and corrected for tumor cell percentage. A calibration curve containing a pool of DNA extracts from a different set of fresh-frozen tumors was taken along as internal control to monitor the performance of the PCR reactions (Supplementary Table S4), of which the dilution containing 4 ng of DNA was used as the calibrator for copy-number calculations. Two cell line samples were taken along as high (MDA-MB-468) and low (MDA-MB-134VI) controls for copy-number variation, which gave results with respectively median 3.3 (min 2.5–max 4.5) and median 0.9 (min 0.7–max 1.0) *HMBS* copies/cell ($n = 12$ runs). Also, no template reactions were taken along in each run and never resulted in an obtained Cq value.

Statistical analysis

All analyses included the average mtDNA content obtained from the duplicate analysis for each individual sample. Data distribution was tested using the Shapiro–Wilk test for normality. Categorical comparisons of grouped clinical variables and the mtDNA content groups were employed using the Fisher exact test. The association with response rate to chemotherapy was analyzed with a logistic regression model to calculate ORs and 95% confidence intervals (95% CI). Kaplan–Meier survival plots were used to visualize the differences in time to distant metastasis (adjuvant setting) or time to progression (advanced setting) between mtDNA content groups (dichotomized). In the adjuvant cohort, the log-rank test was used to compare survival probability. In the advanced cohort, the Peto & Peto modification of the Gehan–Wilcoxon test was used to compare survival probability because most events occurred early on in this cohort. Proportional hazard analyses for DMFS (adjuvant setting) or progression-free survival (advanced setting) were performed using Cox regression methods to calculate HRs and their 95% CIs. Univariable analysis was done on the individual variables, and for the multivariable analysis, we used a base model (including traditional clinicopathologic variables; refs. 29, 30) and mtDNA content. In regression analyses, the Wald statistic was used to calculate corresponding *P* values. Proportionality over time was monitored for each Cox regression using the Schoenfeld residuals with the assumption tested using the χ^2 test, and was never violated ($P > 0.05$). All statistical tests were two-sided, and *P* values smaller than 0.05 were considered as statistically significant. Analyses were performed using R, version 3.2.3.

Results

mtDNA content in primary breast tumors

In total, we analyzed primary tumor DNA from 295 breast cancer patients derived from two cohorts: node-positive patients receiving either FAC/FEC or CMF adjuvant chemotherapy treatment (adjuvant cohort), and patients receiving either FAC/FEC or CMF first-line palliative chemotherapy treatment for their recurrent disease (advanced cohort). We obtained mtDNA content in these DNA extracts by multiplex real-time qPCR targeting a nuclear-encoded and a mitochondrial-encoded gene, and included a correction for sample-specific somatic copy-number variation of the nuclear-encoded gene as obtained by qPCR.

In the adjuvant cohort of 174 patients, the median mtDNA content was 758 mtDNA molecules per cell [interquartile range (IQR), 506], and in the advanced cohort of 121 patients, the median mtDNA content was 694 mtDNA molecules per cell (IQR, 402). Because mtDNA content was not normally distributed within both cohorts (both Shapiro–Wilk $P < 0.001$), we dichotomized each cohort based on the median mtDNA content, resulting in mtDNA low and mtDNA high groups. No differences were observed with respect to the chemotherapeutic regimens given between the mtDNA low and mtDNA high groups in both the adjuvant and the advanced cohorts (Fisher exact $P = 0.4$ and $P = 0.5$, respectively; Supplementary Table S5).

mtDNA content in association with clinicopathologic variables

Next, we evaluated the association between mtDNA content and clinicopathologic variables. In the adjuvant cohort, no statistically significant associations were observed between mtDNA content and age at diagnosis, menopausal status at diagnosis, nodal status (number of nodes positive), primary tumor size (T-stage), primary tumor GGI, ER, PR, and HER2 status (Fisher exact $P > 0.05$; Table 1). In the advanced cohort, no statistically significant associations were observed between mtDNA content and age at recurrence, menopausal status at recurrence, nodal status, primary tumor size, primary tumor GGI, PR status, HER2 status, dominant relapse site, disease-free interval, and treatment with consolidation therapy (Fisher exact $P > 0.05$; Table 2), but

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Table 1. Association between mtDNA content and clinicopathologic variables of node-positive patients receiving either FAC/FEC or CMF adjuvant chemotherapy treatment (adjuvant cohort)

Characteristic	mtDNA low *n* (%)	mtDNA high *n* (%)	*P*
Age (at diagnosis)			
≤40	27 (31.0%)	19 (21.8%)	0.3
>40–50	43 (49.4%)	53 (60.9%)	
>50	17 (19.5%)	15 (17.2%)	
Menopausal status (at diagnosis)			
Pre	80 (92.0%)	74 (85.1%)	0.2
Post	7 (8.0%)	13 (14.9%)	
Tumor size			
T1 (≤2 cm)	24 (29.3%)	13 (15.3%)	0.1
T2 (>2–5 cm)	43 (52.4%)	54 (63.5%)	
T3/4 (>5 cm)	15 (18.3%)	18 (21.2%)	
Unknown	5	2	
Nodal status			
1–3	53 (60.9%)	47 (54%)	0.4
>3	34 (39.1%)	40 (46%)	
Grade (GGI)			
1	15 (19.7%)	11 (15.7%)	0.5
2	27 (35.5%)	32 (45.7%)	
3	34 (44.7%)	27 (38.6%)	
Unknown	11	17	
ER status			
Negative	24 (27.6%)	24 (27.6%)	1
Positive	63 (72.4%)	63 (72.4%)	
PR status			
Negative	24 (29.6%)	27 (33.3%)	0.7
Positive	57 (70.4%)	54 (66.7%)	
Unknown	6	6	
HER2 status			
Balanced	64 (82.1%)	59 (83.1%)	1
Amplified	14 (17.9%)	12 (16.9%)	
Unknown	9	16	

ER-positive primary tumors were more prevalent in the mtDNA low group (Fisher exact $P = 0.018$).

mtDNA content in association with patient outcome

In the adjuvant cohort, patients treated with adjuvant FAC/FEC showed a significant longer DMFS when their primary tumor had low mtDNA content compared with patients with a high tumor mtDNA content (log-rank $P = 0.015$; Fig. 1A). The median lengths of DMFS in the mtDNA low and mtDNA high groups were respectively 85 months (95% CI, 40–NA) and 34 months (95% CI, 26–69). The 5-year DMFS in the mtDNA low and mtDNA high groups were respectively 59% (95% CI, 48–73) and 41% (95% CI, 30–57). In univariable Cox regression analysis for DMFS, the mtDNA low group compared with the mtDNA high group has an HR of 0.57 (95% CI, 0.36–0.9, $P = 0.017$; Table 3). Also in multivariable Cox regression analysis corrected for the base model including traditional prognostic factors, the patients in the low mtDNA content group had a longer DMFS compared with the mtDNA high group (HR, 0.46; 95% CI, 0.24–0.89, $P = 0.020$; Table 3). However, also in the adjuvant cohort, patients treated with adjuvant CMF showed an equal distant metastasis probability in the mtDNA low and mtDNA high groups (log-rank $P = 0.7$; Fig. 1B). The median lengths of DMFS in the mtDNA low and mtDNA high groups were respectively 49 months (95% CI, 41–NA) and 57 months (95% CI, 22–NA). The 5-year DMFS in the mtDNA low and mtDNA high groups were respectively 46% (95% CI, 30–71) and 44% (95% CI, 29–66). In univariable Cox regression analysis for DMFS, the mtDNA low group compared with the mtDNA high group has an HR of 0.88 (95% CI, 0.46–1.69, $P = 0.7$).

In the advanced cohort, in logistic regression analysis for overall response, patients treated with first-line FAC/FEC for their recurrent disease in the mtDNA low group had an OR of 2.48 (95% CI, 0.97–6.61, $P = 0.06$) compared with patients in the mtDNA high group (Table 4). These patients with a low primary tumor mtDNA content had a significant longer PFS compared with patients with high mtDNA content in their primary tumor (Peto $P = 0.022$; Fig. 1C). The median lengths of PFS in the mtDNA low and mtDNA high groups were respectively 9.20 months (95% CI, 6.44–16.39) and 5.91 months (95% CI, 2.99–9.13). In univariable Cox regression analysis for PFS, the mtDNA low group compared with the mtDNA high group has an HR of 0.6 (95% CI, 0.36–1.0, $P = 0.048$; Table 5). Also in multivariable Cox regression analysis corrected for the base model including also traditional predictive factors, the patients in the low mtDNA content group had a longer

Table 2. Association between mtDNA content and clinicopathologic variables of patients receiving either FAC/FEC or CMF first-line chemotherapy treatment for their recurrent disease (advanced cohort)

Characteristic	mtDNA low *n* (%)	mtDNA high *n* (%)	*P*
Age (at recurrence)			
≤45	14 (23.3%)	20 (32.8%)	0.1
>45–55	25 (41.7%)	15 (24.6%)	
>55	21 (35.0%)	26 (42.6%)	
Menopausal status (at recurrence)			
Pre	30 (50.0%)	31 (50.8%)	1
Post	30 (50.0%)	30 (49.2%)	
Tumor size			
T1 (≤2 cm)	17 (28.8%)	17 (28.3%)	0.8
T2 (>2–5 cm)	32 (54.2%)	30 (50.0%)	
T3/4 (>5 cm)	10 (16.9%)	13 (21.7%)	
Unknown	1	1	
Nodal status			
0	17 (28.3%)	25 (41.0%)	0.3
1–3	15 (25.0%)	13 (21.3%)	
>3	28 (46.7%)	23 (37.7%)	
Grade (GGI)			
1	8 (15.1%)	5 (8.5%)	0.3
2	14 (26.4%)	22 (37.3%)	
3	31 (58.5%)	32 (54.2%)	
Unknown	7	2	
ER status			
Negative	26 (43.3%)	40 (65.6%)	0.018
Positive	34 (56.7%)	21 (34.4%)	
PR status			
Negative	38 (63.3%)	41 (67.2%)	0.7
Positive	22 (36.7%)	20 (32.8%)	
HER2 status			
Balanced	38 (71.7%)	46 (76.7%)	0.7
Amplified	15 (28.3%)	14 (23.3%)	
Unknown	7	1	
Dominant relapse site			
Soft tissue	5 (8.3%)	6 (9.8%)	0.8
Bone	8 (13.3%)	11 (18.0%)	
Visceral	47 (78.3%)	44 (72.1%)	
Disease-free interval			
≤1 year	19 (31.7%)	26 (42.6%)	0.3
>1–3 years	29 (48.3%)	28 (45.9%)	
>3 years	12 (20.0%)	7 (11.5%)	
Hormonal consolidation therapy			
No	44 (74.6%)	47 (77.0%)	0.8
Yes	15 (25.4%)	14 (23.0%)	
Unknown	1		

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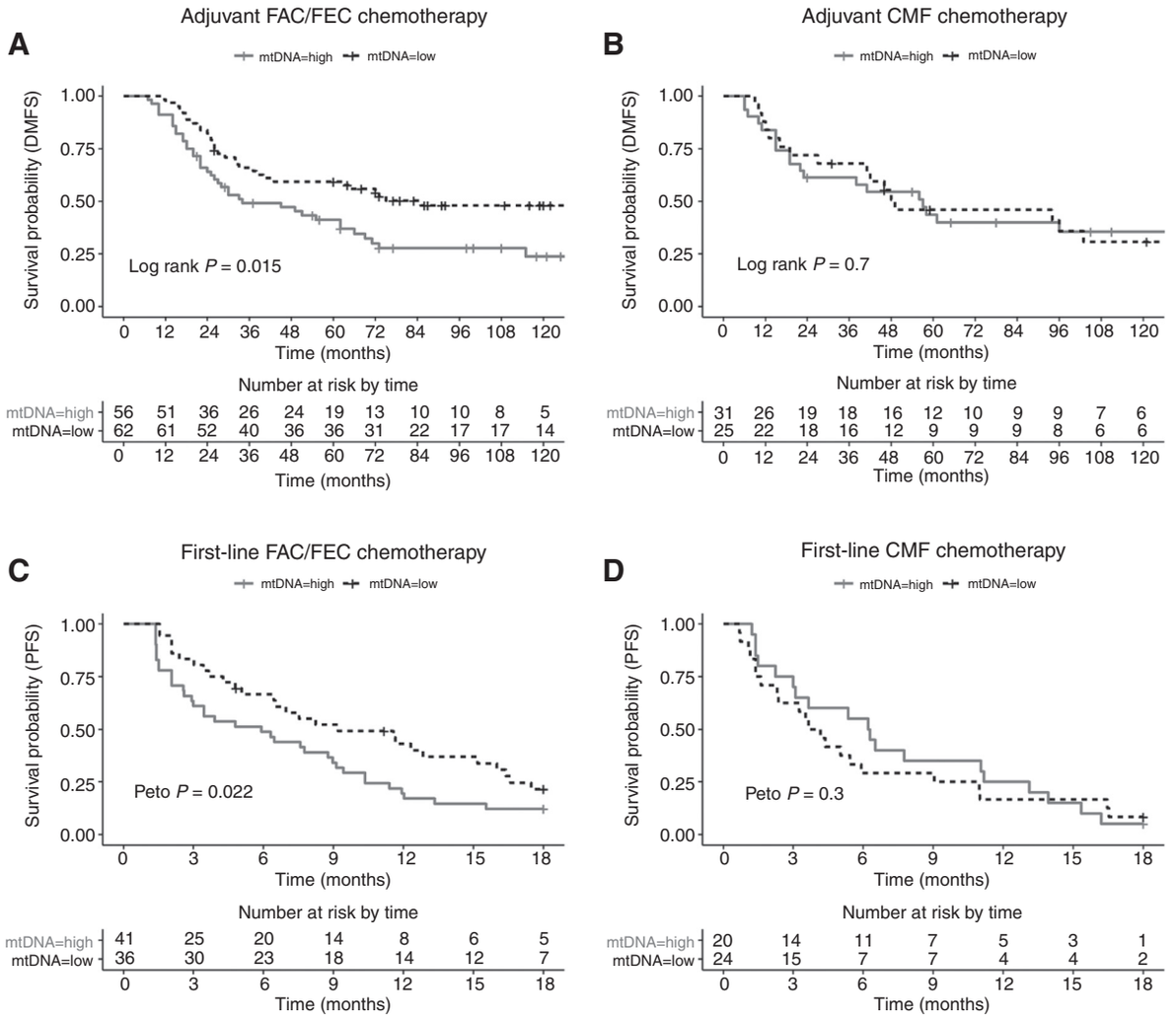

Figure 1.

Kaplan–Meier survival curves as a function of mtDNA content for DMFS in patients receiving FAC/FEC (**A**) or CMF (**B**) adjuvant chemotherapy, and for PFS in patients receiving FAC/FEC (**C**) or CMF (**D**) first-line chemotherapy for their recurrent disease.

PFS compared with the mtDNA high group with an HR of 0.49 (95% CI, 0.27–0.90, $P = 0.022$; Table 5). For the patients treated with first-line CMF for their recurrent disease, in logistic regression analysis for overall response the mtDNA low group had an OR of 0.48 (95% CI, 0.14–1.57, $P = 0.2$; Table 4). No significant differences were observed between these two groups in their probability to disease progression (Peto $P = 0.3$; Fig. 1D), with a median PFS of 3.91 months (95% CI, 2.37–9.03) and 6.24 months (95% CI, 3.09–13.11) in the mtDNA low and mtDNA high groups, respectively. In univariable Cox regression analysis for PFS, the HR of the mtDNA low group compared with the mtDNA high group was 1.14 (95% CI, 0.61–2.13, $P = 0.7$).

Discussion

In this retrospective study, we measured mtDNA content in primary breast tumors and demonstrate that patients with low mtDNA content in their tumors have increased benefit from anthracycline-based chemotherapies (FAC/FEC), in both the adjuvant and advanced disease settings. We hypothesized that tumor cells with low mtDNA content are more susceptible to mitochondrial damage induced by anthracyclines, and thus are more susceptible to anthracycline treatment. A previous study reported similar findings: for 27 patients receiving anthracycline-based adjuvant chemotherapy (13), the 5-year disease-free survival (tumor recurrence) was 84% in the mtDNA low group opposed to 50% in the mtDNA high group, and this difference was not observed in 24 patients who did not receive adjuvant chemotherapy. However, these cohorts had only small numbers of patients, were heterogeneous regarding prognosis (i.e., lymph node status), and it was not reported if these patients received adjuvant hormonal treatment to improve outcome. In this study, we measured mtDNA content in nearly 300 primary breast tumors of two well-defined cohorts: lymph node–positive patients receiving only adjuvant chemotherapy as systemic treatment for their disease, and patients with

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Table 3. Univariable and multivariable Cox regression analysis for DMFS of node-positive patients receiving FAC/FEC adjuvant chemotherapy (adjuvant cohort)

		Univariable			Multivariable	
Characteristic	**Events/*N***	**HR (95% CI)**	***P***	**Events/*N***	**HR (95% CI)**	***P***
Age (at diagnosis)						
≤40	24/33	1		19/25	1	
>40–50	39/66	0.67 (0.40–1.11)	0.1	23/44	0.34 (0.17–0.67)	0.002
>50	10/19	0.48 (0.23–1.01)	0.054	10/17	0.78 (0.33–1.85)	0.6
Menopausal status (at diagnosis)						
Pre	66/108	1		46/78	1	
Post	7/10	0.96 (0.44–2.10)	0.9	6/8	0.93 (0.33–2.60)	0.9
Tumor size						
T1 (≤2 cm)	14/28	1		12/21	1	
T2 (>2–5 cm)	43/68	1.70 (0.93–3.12)	0.09	31/53	1.15 (0.54–2.45)	0.7
T3/4 (>5 cm)	14/19	2.16 (1.02–4.55)	0.043	9/12	1.67 (0.62–4.49)	0.3
Nodal status						
1–3	41/73	1			1	
>3	32/45	1.48 (0.93–2.36)	0.1		1.39 (0.76–2.55)	0.3
Grade (GGI)						
1	7/16	1		6/13	1	
2	24/38	1.84 (0.79–4.28)	0.2	22/34	1.37 (0.50–3.74)	0.5
3	28/44	2.03 (0.88–4.64)	0.1	24/39	1.21 (0.44–3.34)	0.7
ER status						
Negative	22/34	1		17/26	1	
Positive	51/84	0.76 (0.46–1.26)	0.3	35/60	1.68 (0.55–5.13)	0.4
PR status						
Negative	22/32	1		20/30	1	
Positive	44/74	0.64 (0.38–1.07)	0.1	32/56	0.41 (0.13–1.25)	0.1
HER2 status						
Balanced	48/85	1		42/74	1	
Amplified	12/14	2.54 (1.33–4.85)	0.004	10/12	2.04 (0.92–4.54)	0.1
mtDNA						
High	39/56	1		26/38	1	
Low	34/62	0.57 (0.36–0.90)	0.017	26/48	0.46 (0.24–0.89)	0.020

NOTE: In the multivariable model, analysis was limited to 86 patients (52 events) with no missing values.

disease recurrence receiving chemotherapy as first-line palliative treatment.

We measured mtDNA content using a multiplex qPCR approach where the mitochondrial-encoded locus lies outside of the common deletion region (31). Also, we included a correction for copy-number variation of the nuclear-encoded reference locus to minimize a bias in mtDNA content determination due to tumor-related local genomic aberrations. By including a minimal tumor cell percentage of 50 (the fraction of tumor cell nuclei within the cryosection), we aimed to minimize the contribution of nontumor cells (i.e., stromal and immune cells) to the final mtDNA content. It is important to note that we evaluated the mtDNA content in the primary tumor specimens without comparing it with the tumor-adjacent normal mammary epithelium, and thus do not know whether the specimens with low mtDNA content have this due to variation of germline origin, or whether it is truly a somatic reduction in mtDNA copy number. In several other studies, mtDNA content in breast tumors has been compared with adjacent normal mammary tissue. In these studies, a somatic reduction of mtDNA was observed in approximately 70% of the cases (5–13), which makes it highly likely that those samples with a low mtDNA content in our dataset largely reflect the tumor-specific mtDNA content and is not due to germline origin.

Besides the stage in the disease trajectory, there are some differences between the two cohorts used in this study. The patient cohort receiving adjuvant chemotherapy was a younger cohort compared with the patients in the advanced cohort, with the majority of the patients being premenopausal. Also, the ER-positive cases were underrepresented in the advanced cohort compared with the general breast cancer population (approximately 75%), likely because ER-positive patients received (also) hormonal therapy (i.e., tamoxifen) and were thus excluded from our selection. In this advanced cohort, we observed an association between mtDNA content and ER status, with more ER-positive cases in the mtDNA content low group (Table 2). In both the adjuvant cohort of lymph node–positive patients, and in our previous study with lymph node–negative patients (14) or in other studies (7, 9, 12), no association between mtDNA content and ER status was observed. In addition, in the advanced cohort, there were some differences between patients treated with first-line CMF or with first-line FAC/FEC: patients receiving CMF were older (Fisher exact $P = 0.031$, Supplementary Table S7) and received less often consolidation therapy (Fisher exact $P = 0.048$, Supplementary Table S7). Also, similar to what we have done in the past for biomarker studies in the advanced setting, mtDNA content was measured in the primary tumor and not in the recurrence site. To our knowledge, it remains to be elucidated if the mtDNA content level in the recurrence site is similar to the primary tumor. Despite this, we did observe the hypothesized association between low mtDNA content and PFS in patients receiving first-line FAC/FEC chemotherapy, and this association was independent of established clinicopathologic variables affecting outcome. We did not observe a statistically significant association with overall response (complete remission, partial remission, or stable disease > 6 courses) within this group, but *post-hoc* power calculation with the current findings (72% response in mtDNA low, 51% response in mtDNA high, Table 4) indicates that our power is currently 45%. The objective response, which does not include patients with stable disease, but only those with

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Table 4. Overall response rate of patients receiving either FAC/FEC or CMF first-line chemotherapy for their recurrent disease (advanced cohort)

	mtDNA low *n* (%)	mtDNA high *n* (%)	OR (95% CI)	*P*
First-line FAC/FEC				
Nonresponders	10 (27.8%)	20 (48.8%)	2.48 (0.97–6.61)	0.06
Responders	26 (72.2%)	21 (51.2%)		
First-line CMF				
Nonresponders	14 (58.3%)	8 (40.0%)	0.48 (0.14–1.57)	0.2
Responders	10 (41.7%)	12 (60.0%)		

complete and partial remission or progressive disease and thus only takes into account the two extremes, in logistic regression analysis for the mtDNA low group compared with the mtDNA high group gives an OR of 4.11 (95% CI, 1.27–15.17, $P = 0.02$) in the FAC/FEC group, and an OR of 0.6 (95% CI, 0.12–3.00, $P = 0.23$) in the CMF group; however, these patient numbers are very small (Supplementary Table S3). A total number of 166 patients will be necessary to elucidate with 80% power if there is a difference in overall response to first-line FAC/FEC in the mtDNA low and mtDNA high groups.

Several markers have been described to predict benefit from adjuvant anthracycline-based chemotherapy, including *ERBB2* (HER2; ref. 32), TIMP1 (29), and *TOP2A* (33). None of these three markers showed the postulated association with outcome in our cohorts of adjuvant or first-line FAC/FEC-treated patients (Supplementary Table S10), neither did we observe a correlation between mtDNA content and these markers (Supplementary Table S11).

The retrospective nature of this study did not allow us to include patients receiving taxane-based chemotherapy, nowadays often used to treat breast cancer as well. Similar to the methotrexate-based regimen, taxanes induce only low levels of oxidative stress (16) and do not work directly at the DNA level. Thus, we do not expect differences in efficacy in tumors with low or high mtDNA content. Specifically, we reason that tumor cells with low mtDNA content possess a vulnerability to drugs affecting mitochondria. It would be interesting to assess the outcome of patients with low or high tumor mtDNA content when treated with regimen containing chemotherapeutics that induce either mild (such as taxanes) or moderate oxidative stress (such as platins or alkylating agents), or the sequential combination of regimen. But given the common use of taxanes in breast cancer patients and the retrospective nature of our study, validation of our findings as well as exploring the impact of mtDNA content on outcome of taxane-treated patients is warranted.

In conclusion, where it was previously shown that a low mtDNA content in primary breast cancer tumors is associated with a worse prognosis (14), our study here in two well-defined cohorts indicates that breast cancer patients with low mtDNA content in their primary tumor have better outcome from anthracycline-containing chemotherapy, in the adjuvant as well as in the advanced setting. We suggest that the frequently observed decrease in mtDNA content in breast tumors may be exploited by guiding chemotherapeutic regimen decision-making. Larger (prospective) cohorts of uniformly treated patients are necessary to validate our results and to determine the clinical relevance of mtDNA content quantification in cancer.

Table 5. Univariable and multivariable Cox regression analysis for PFS of patients receiving FAC/FEC first-line chemotherapy for their recurrent disease (advanced cohort)

		Univariable			Multivariable	
Characteristic	**Events/*N***	**HR (95% CI)**	***P***	**Events/*N***	**HR (95% CI)**	***P***
Age (at recurrence)						
≤45	19/24	1		17/21	1	
>45–55	26/30	1.15 (0.64–2.09)	0.6	24/28	1.74 (0.77–3.93)	0.2
>55	18/23	0.97 (0.51–1.85)	0.9	17/21	0.85 (0.25–2.88)	0.8
Menopausal status (at recurrence)						
Pre	37/44	1		33/39	1	
Post	26/33	0.85 (0.51–1.40)	0.5	25/31	0.90 (0.35–2.35)	0.8
Dominant relapse site						
Soft tissue	5/5	1		5/5	1	
Bone	8/10	0.13 (0.04–0.42)	<0.001	7/9	0.07 (0.02–0.28)	<0.001
Visceral	50/62	0.18 (0.06–0.48)	<0.001	46/56	0.17 (0.05–0.55)	0.003
Disease-free interval						
≤1 year	25/26	1		24/25	1	
>1–3 years	28/37	0.73 (0.42–1.26)	0.3	24/32	1.06 (0.55–2.04)	0.9
>3 years	10/14	0.65 (0.31–1.36)	0.3	10/13	0.85 (0.33–2.15)	0.7
ER status						
Negative	38/43	1		35/40	1	
Positive	25/34	0.58 (0.35–0.96)	0.036	23/30	1.21 (0.56–2.63)	0.6
PR status						
Negative	45/52	1		42/48	1	
Positive	18/25	0.69 (0.40–1.20)	0.2	16/22	0.85 (0.33–2.14)	0.7
HER2 status						
Balanced	44/55	1		44/54	1	
Amplified	14/16	1.31 (0.71–2.39)	0.4	14/16	1.07 (0.52–2.17)	0.9
Hormonal consolidation therapy						
No	51/53	1		46/48	1	
Yes	12/23	0.21 (0.11–0.40)	<0.001	12/22	0.16 (0.07–0.36)	<0.001
mtDNA						
High	36/41	1		35/40	1	
Low	27/36	0.60 (0.36–1.00)	0.048	23/30	0.49 (0.27–0.90)	0.022

NOTE: In the multivariable model, analysis was limited to 70 patients (58 events) with no missing values.

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Disclosure of Potential Conflicts of Interest

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Authors' Contributions

Conception and design: M.J.A. Weerts, J.A. Foekens, S. Sleijfer, J.W.M. Martens
Development of methodology: M.J.A. Weerts
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Sieuwerts, J.A. Foekens, S. Sleijfer, J.W.M. Martens
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.J.A. Weerts, A. Hollestelle, S. Sleijfer, J.W.M. Martens
Writing, review, and/or revision of the manuscript: M.J.A. Weerts, A. Hollestelle, A.M. Sieuwerts, J.A. Foekens, S. Sleijfer, J.W.M. Martens
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.M. Sieuwerts
Study supervision: S. Sleijfer, J.W.M. Martens

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