

Effect of Cytotoxic Chemotherapy on Markers of Molecular Age in Patients With Breast Cancer

Hanna K. Sanoff, Allison M. Deal, Janakiraman Krishnamurthy, Chad Torrice, Patrick Dillon, Jessica Sorrentino, Joseph G. Ibrahim, Trevor A. Jolly, Grant Williams, Lisa A. Carey, Amy Drobish, Brittaney-Belle Gordon, Shani Alston, Arti Hurria, Karin Kleinhans, K. Lenhard Rudolph, Norman E. Sharpless, Hyman B. Muss

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Correspondence to: Hyman B. Muss, MD, Division of Hematology/Oncology, University of North Carolina School of Medicine, 170 Manning Dr, CB# 7305, Chapel Hill, NC 27599-7305 (e-mail: hyman_muss@med.unc.edu) and Norman E. Sharpless, MD, Departments of Medicine and Genetics, Lineberger Comprehensive Cancer Center, 21-225, University of North Carolina School of Medicine, CB# 7295, Chapel Hill, NC 27599-7295 (e-mail: nes@med.unc.edu).

Background Senescent cells, which express *p16INK4a*, accumulate with aging and contribute to age-related pathology. To understand whether cytotoxic agents promote molecular aging, we measured expression of *p16INK4a* and other senescence markers in breast cancer patients treated with adjuvant chemotherapy.

Methods Blood and clinical information were prospectively obtained from 33 women with stage I to III breast cancer at four time points: before anthracycline-based chemotherapy, immediately after anthracycline-based chemotherapy, 3 months after anthracycline-based chemotherapy, and 12 months after anthracycline-based chemotherapy. Expression of senescence markers *p16INK4a* and *ARF* mRNA was determined using TaqMan quantitative reverse-transcription polymerase chain reaction in CD3⁺ T lymphocytes, telomere length was determined by Southern analysis, and senescence-associated cytokines were determined by enzyme-linked immunosorbent assay. Findings were independently assessed in a cross-sectional cohort of 176 breast cancer survivors enrolled a median of 3.4 years after treatment; 39% previously received chemotherapy. All statistical tests were two-sided.

Results In prospectively analyzed patients, expression of *p16INK4a* and *ARF* increased immediately after chemotherapy and remained elevated 12 months after treatment. Median increase in $\log_2 p16INK4a$ was 0.81 (interquartile range = 0.28–1.62; Wilcoxon signed-rank $P < .001$), or a 75% absolute increase in expression, equivalent to the increase observed over 14.7 years of chronological aging. *ARF* expression was comparably increased ($P < .001$). Increased expression of *p16INK4a* and *ARF* was associated with dose-dense therapy and hematological toxicity. Expression of two senescence-associated cytokines (VEGFA and MCP1) was durably increased by adjuvant chemotherapy. Telomere length was not affected by chemotherapy. In a cross-sectional cohort, prior chemotherapy exposure was independently associated with a \log_2 -increase in *p16INK4a* expression of 0.57 (repeated measures model, $P < .001$), comparable with 10.4 years of chronological aging.

Conclusions Adjuvant chemotherapy for breast cancer is gerontogenic, inducing cellular senescence in vivo, thereby accelerating molecular aging of hematopoietic tissues.

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With the aging of the American population, the incidence of new cancer diagnoses is projected to increase 45% from 2010 to 2030 (1). Coupled with the growing proportion of cancer patients who are cured (2), we face a new challenge: a large population of aging cancer survivors (3). Long-term survivors of childhood and adult cancer can exhibit substantial late sequelae, including endocrine dysfunction, cognitive impairment, cardiovascular morbidity, secondary neoplasms, and neuromuscular impairment (4–8). Little is known about how chemotherapy causes long-term adverse effects and whether it alters the pace of physiologic aging.

Human aging is characterized by a steady decline in organ function, which leads to loss of physiologic reserve and frailty (9).

This loss of function is characterized by a decline in the replicative capacity of certain self-renewing cells and the accumulation of cells that have undergone cellular senescence (10,11). Cellular senescence is triggered by the activation of tumor-suppressor mechanisms in response to varied cellular stresses such as oncogene activation, tissue injury, telomere dysfunction, and persistent DNA damage. Senescence is strongly associated with activation of the *INK4/ARF* (*CDKN2a*) locus on human chromosome 9p21.3, which encodes the p16^{INK4a} and ARF tumor suppressor proteins. Several lines of evidence suggest senescence influences mammalian aging: 1) expression of *p16INK4a* increases exponentially with chronological aging (12–14) and causes reduced replicative capacity of some cell

types (15–19); 2) regulatory polymorphisms of senescence regulators (eg, *CDKN2a* and *TERT*) have been linked through unbiased genome-wide studies with many age-associated conditions such as cancer, pulmonary fibrosis, atherosclerosis, and type II diabetes (20); and 3) therapies to decrease the production of or increase the clearance of senescent cells in mice ameliorate certain age-associated phenotypes (21–23).

Because of the intimate links between senescence and aging, markers of cellular senescence, including leukocyte telomere length (LTL), expression of senescence-associated (SA) cytokines such as interleukin 6 (IL-6), and expression of *INK4a/ARF* transcripts, have been tested as potential biomarkers of molecular aging. Decreased LTL has been linked with chronological age and age-promoting stressors such as cigarette smoking in several studies in human populations (24–26). Senescent cells elaborate a host of potent cytokines (ie, the senescence-associated secretory phenotype) (27), which promote a proinflammatory tissue microenvironment. Expression of SA-cytokines, such as IL-6, has been reported to increase with aging and to predict age-associated morbidities and mortality (28–32). More recently, expression of *p16^{INK4a}*, and to a lesser extent *ARF*, in defined tissues such as peripheral blood T cells (PBTLs) have been described as biomarkers of aging. Using the *p16^{INK4a}* assay, we have shown that smoking, physical inactivity, and chronic human immunodeficiency virus infection accelerate expression of this biomarker of molecular age in the PBTL compartment (14,33). Given the apparent long-term toxicities of DNA-damaging agents, we sought to determine whether cytotoxic chemotherapy given with curative intent accelerates molecular aging in humans.

Methods

Patients

Human studies were approved by the University of North Carolina Institutional Review Board (08-0823; 09-2344). All patients gave written informed consent before undergoing any study-related procedures. The adjuvant cohort (LCCC0810) (Figure 1A) consisted of women with newly diagnosed American Joint Committee on Cancer 6th edition (34) stage I to III breast cancer planning to receive either neoadjuvant or adjuvant anthracycline-containing chemotherapy. Women were consented before treatment to undergo phlebotomy before chemotherapy, at count recovery immediately after chemotherapy, and at their 3- and 12 month regularly scheduled visits. Patients with prior pelvic radiation or a clonal bone marrow disorder were excluded.

The survivor cohort (LCCC0924) (Figure 1B) consisted of women aged 50 years and older with a history of a surgically resected stage I to III breast cancer. Those who had received chemotherapy needed to enroll at least 3 months from completion of chemotherapy. Those taking endocrine therapy were eligible after 3 months of treatment. Women with recurrent cancer, a clonal bone marrow disorder, or a life expectancy less than 12 months were excluded. A single sample was obtained from women in the survivor cohort at time of consent. Women in both cohorts received standard-of-care chemotherapy regimens or treatment on institutional review board–approved clinical trials. Standard-of-care chest wall radiotherapy and adjuvant hormonal therapy were

given as clinically indicated. Health behaviors and demographics were evaluated by patient questionnaire. Medical history and treatment were extracted from the medical record. For each time point, a single lavender (EDTA) tube (5–10 mL) was collected and placed immediately on ice. Molecular and serologic analyses were performed by investigators blinded to patient data, and investigators collecting clinical information were blinded to laboratory results until data collection was complete.

Analysis of *INK4a/ARF* Expression, Telomere Length, and SA-Cytokine Expression

Analysis of *p16^{INK4a}* and *ARF* expression in CD3⁺ PBTLs was performed as described (14,33). In brief, on the day of collection, PBTLs were isolated using anti-CD3 microbeads and an AutoMACS^{PRO} separator (Miltenyi Biotec, San Diego, CA). Total RNA and cDNA were prepared (14,33), followed by TaqMan quantitative reverse-transcription polymerase chain reaction specific for *p16^{INK4a}* (exon 1alpha-exon 2; custom order ID: AIII1L5T; Life Technologies USA, Carlsbad, CA) or *ARF* (exon 1beta-exon 2; custom assay order ID: AIKAKB1; Life Technologies USA) with normalization to *18S* housekeeping gene (order number 4352655; Life Technologies USA). Using this method, 15.4% of samples in the adjuvant cohort and 16.2% of samples in the survivor cohort failed analysis because of sorting failure, insufficient nucleic acid yield, poor quality RNA, or failure to pass a data replicate filter (Figure 1; see also [Supplementary Methods](#), available online, for details). There was no statistically significant difference in age between patients whose samples failed analysis and patients with results who were included in either cohort. Expression of *p16^{INK4a}* with aging in reporter mice was assayed as described (see also [Supplementary Methods](#), available online) (35).

Analysis of senescence-associated cytokines and LTL was performed on patient sera collected before and 12 months after chemotherapy in the adjuvant cohort (in 3 patients without a 12-month sample, the 3-month sample was used). Analysis of vascular endothelial growth factor A (VEGFA; R&D systems DVE00, Minneapolis, MN), IL-6 (R&D systems LUH206), interleukin 7 (IL-7; R&D systems HS750), interleukin 8 (IL-8; R&D systems LUH208) and monocyte chemotactic protein-1 (MCP1, also referred to as chemokine CCL2; R&D systems DCP00) was performed by enzyme-linked immunosorbent assay. IL-6 was detectable before and after chemotherapy in only three patients; therefore it was not further analyzed. LTL was measured by Southern analysis as described (36).

p16INK4a Expression With Reporter Mice

All murine studies were done under a protocol approved by the University of North Carolina Institutional Animal Care and Use Committee. Albino hairless SKH1-E *p16⁺/Luc* mice developed in the Sharpless lab at the University of North Carolina as described in Burd et al. (35) were serially housed in the University of North Carolina LCCC Mouse Phase I Unit. A cohort of 32 female mice were aged (6, 23, 55, and 73 weeks) and imaged. This was repeated five times with different sets of mice from this cohort. Animals were imaged using an IVIS LUMINA imaging system (PerkinElmer, Waltham, MA). The image shown is a 2-minute exposure taken 8 minutes after luciferin injection. For the full protocol, see Burd et al. (35).

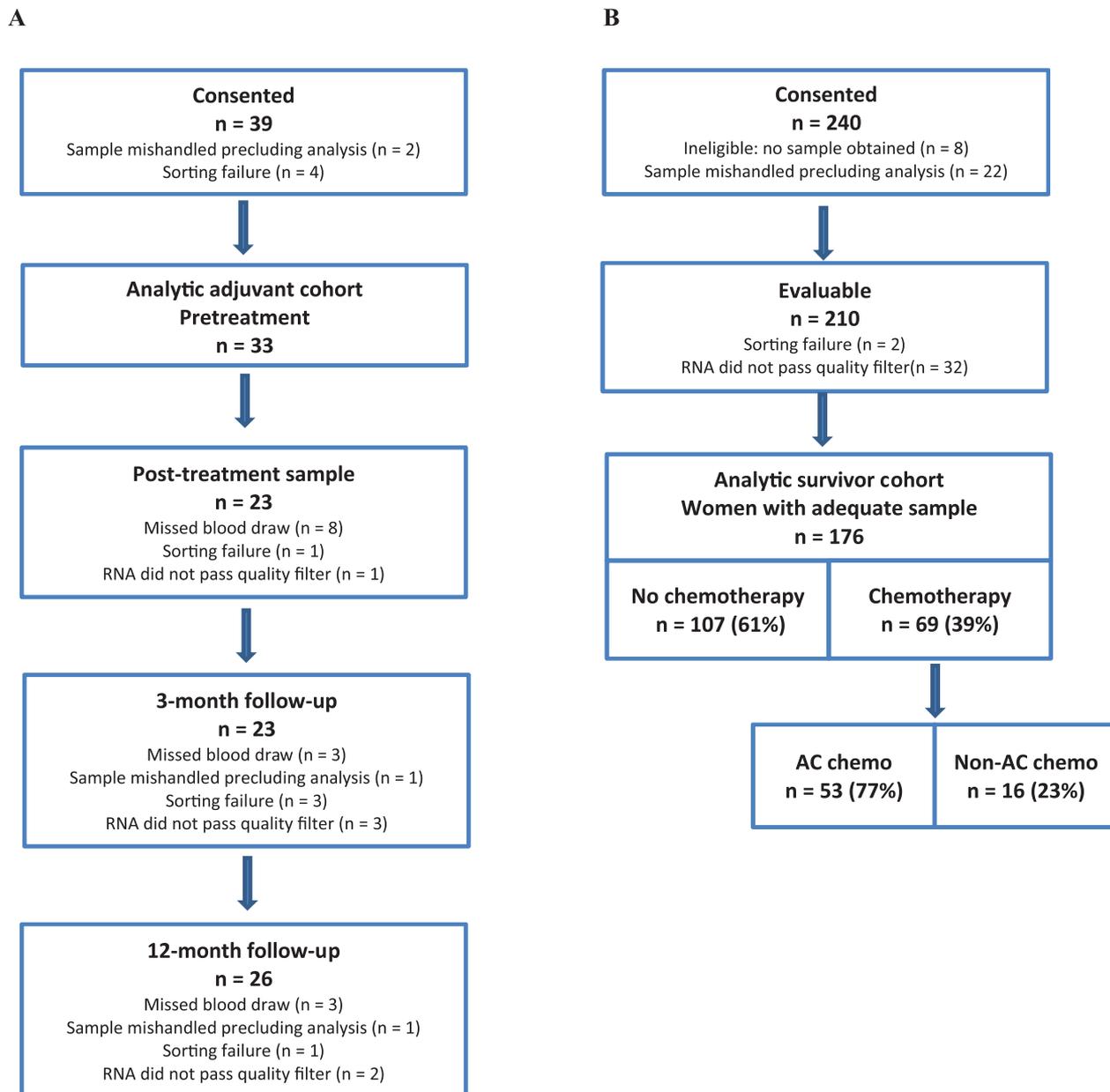


Figure 1. Cohort assembly. The consort diagram for LCCC0810, the adjuvant cohort (A) shows number of evaluable patients at each time point. Thirty-nine women consented to participation. Results from the baseline sample were not available for six of the 39 patients. These six could not be included in the paired analysis; thus the analytic cohort is comprised of the 33 women for whom baseline results are available. All 33

women had at least one post-treatment sample collected. Fourteen had results at all post-treatment points, 11 were missing one time point, and 8 were missing two time points. The consort diagram for LCCC0924, the survivor cohort (B) shows 34 of 210 evaluable patients were excluded because of sorting or RNA failure (see [Supplementary Methods](#), available online). AC = doxorubicin and cyclophosphamide.

Statistical Analysis

In brief (see [Supplementary Methods](#), available online, for additional details), because $p16^{INK4a}$ and ARF expression increase exponentially with age (14,37), results were logarithmically transformed. For the adjuvant cohort, change in $INK4a/ARF$ expression, SA-cytokine, and LTL from baseline to post-treatment time points were compared overall and across age strata using the Wilcoxon signed-rank, rank-sum, and Kruskal-Wallis tests. Because each woman had at least one post-treatment sample, to maximize sample size we used the average post-treatment $p16^{INK4a}$ and ARF expression to test for association of clinical factors with magnitude of change in expression. In the survivor cohort, $p16^{INK4a}$ was measured twice

in all patients. Rather than arbitrarily choose a single or mean value for each patient, we assumed that measures within a batch were not independent, and thus multivariable repeated measures models, which account for this nonindependence, were used to evaluate the association between clinical factors and $p16^{INK4a}$ expression. ARF expression was measured in a single batch and analyzed by linear regression. Predicted $p16^{INK4a}$ for age according to prior treatment is shown graphically. In both cohorts, the association between age, race, and key clinical factors with $p16^{INK4a}$ expression was evaluated. Results for the Adjuvant cohort are shown in [Supplementary Table 1](#) (available online). In the survivor cohort, few clinical factors were associated with expression; therefore only factors either

statistically significantly associated with expression or of key clinical relevance were included in multivariable models. Data were analyzed by H. K. Sanoff, A. M. Deal, and J. G. Ibrahim using SAS version 9.2 (SAS, Cary, NC) and STATA version 12 (StataCorp, College Station, TX). To quantitate the age-promoting effects of chemotherapy, the fold-change in $p16^{INK4a}$ expression induced by chemotherapy was compared with the effect of chronological aging on $p16^{INK4a}$ in healthy donors from previously published, independent cohorts (14,35).

All tests of statistical significance were two-sided. *P* values of .05 or less were considered statistically significant.

Results

To test the hypothesis that chemotherapy accelerates the activation of cellular senescence in vivo, we measured expression of senescence markers in two cohorts of women with breast cancer treated with curative intent.

The prospective adjuvant cohort consisted of 33 women, with a median age of 49 years (range = 32–69) (Table 1; Supplementary Table 1, available online). The women had few comorbid conditions, although nearly half were obese (body mass index ≥ 30 kg/m²; *n* = 15; 45.5%). The majority (*n* = 29) of women received adjuvant doxorubicin and cyclophosphamide and a taxane. Most women (*n* = 27; 81.8%) received adjuvant doxorubicin and cyclophosphamide in a dose-dense fashion (38). Baseline $p16^{INK4a}$ and *ARF* expression were not associated with age in this small sample with narrow age range (Supplementary Table 1, available online).

In the adjuvant cohort, chemotherapy led to a statistically significant and persistent rise in both $p16^{INK4a}$ and *ARF* expression at all post-treatment time points (Figure 2; Supplementary Table 2, available online). Expression of $p16^{INK4a}$ decreased with chemotherapy in only one patient, who was later found to have congenital p53 deficiency (Li-Fraumeni syndrome; see Supplementary Methods, available online). In the entire cohort, a median increase in the $\log_2 p16^{INK4a}$ expression of 0.81 (interquartile range [IQR] = 0.28–1.62; Wilcoxon signed-rank *P* < .001) and in the $\log_2 ARF$ expression of 0.89 (IQR = 0.29–1.27; Wilcoxon signed-rank *P* < .001) was seen from baseline to the average post-treatment value. In non-log-transformed terms, this reflects median increases of 75% and 85% in $p16^{INK4a}$ and *ARF*, respectively. Using a murine reporter system (Supplementary Figure 1, available online) and in prior studies of normal human volunteers (14,35), we have noted a characteristic exponential increase in $p16^{INK4a}$ expression with aging that is associated with species' lifespans. In independent cohorts of healthy human donors, $\log_2 p16^{INK4a}$ exhibits a mean increase of 0.055 units per year (or 4% per year) (14,35). Therefore, the increase in PBTL $p16^{INK4a}$ expression associated with adjuvant breast cancer chemotherapy is equivalent to increases seen with 14.7 years of chronological aging.

Cytotoxic chemotherapy also increased the in vivo levels of other markers of senescence. Plasma concentrations of two SA-cytokines, VEGFA and MCP1/CCL2 (27,39), were statistically significantly increased in postchemotherapy compared with prechemotherapy samples (median VEGFA = 118 vs 80 pg/mL, Wilcoxon signed-rank *P* = .001; median MCP1/CCL2 = 166 vs 117 pg/mL, Wilcoxon signed-rank *P* < .001). Expression of other

Table 1. Patient and treatment characteristics*

Patient and treatment characteristics	Adjuvant cohort (n = 33)	Survivor cohort (n = 176)
Age, y, median (range)	49 (32–69)	67 (50–93)
<50	17 (51.5%)	0
50–64	12 (36.4%)	63 (35.8%)
65–74	4 (12.1%)	80 (45.5%)
≥ 75	0	33 (18.8%)
Race/ethnicity		
White	26 (78.8%)	148 (84.1%)
Black	7 (21.2%)	26 (14.8%)
Asian/Other	0	2 (1.1%)
Comorbidities		
Diabetes	5 (15.2%)	24 (13.9%)
Coronary heart disease	0	6 (3.5%)
Stroke	0	4 (2.3%)
BMI, kg/m ² , median (range)	29 (18–50)	28 (18–58)
<18.5, underweight	1 (3.0%)	1 (0.6%)
18.5–24.9, normal	6 (18.2%)	59 (33.5%)
25.0–29.9, overweight	11 (33.3%)	55 (31.3%)
≥ 30 , obese	15 (45.5%)	61 (34.7%)
Smoking		
Never	20 (62.5%)	102 (58.3%)
Former	7 (21.9%)	63 (36.0%)
Current	5 (15.6%)	10 (5.7%)
AJCC summary stage†		Not collected
Stage I	6 (18.2%)	—
Stage II	18 (54.5%)	—
Stage III	9 (27.3%)	—
ER or ER/PR positive	20 (60.6%)	—
HER2 positive	7 (21.2%)	—
Triple-negative	9 (27.3%)	—
Adjuvant radiotherapy	25 (75.8%)	103 (58.5%)
Adjuvant hormonal therapy	20 (60.6%)	124 (70.5%)
Adjuvant chemotherapy	33 (100%)	69 (39.2%)
AC	3 (9.4%)	12 (17.4%)
AC → T	29 (87.9%)‡	41 (59.4%)§
TC	0	9 (13.0%)
CMF	0	3 (4.3%)
Other	1 (3.0%)	4 (5.8%)
Years from chemotherapy, median (range)	NA	3.4 (0.3–18.8)

* Data are No. (%) unless otherwise noted. AC = doxorubicin and cyclophosphamide; AJCC = American Joint Committee on Cancer; BMI = body mass index; CMF = cyclophosphamide, methotrexate, 5-fluorouracil; HER2 = human epidermal growth factor receptor 2; ER = estrogen receptor; PR = progesterone receptor; T = paclitaxel; TC = docetaxel and cyclophosphamide.

† Ten women were treated neoadjuvantly; summary stage is pretreatment clinical stage. For the 23 adjuvantly treated women, summary stage is the pathological stage. Stage, ER/PR, and HER2 could not be confirmed from pathology reports for the survivor cohort.

‡ One woman received nab-paclitaxel.

§ One woman received 2 AC/2 TC; one woman received docetaxel instead of paclitaxel; two women received capecitabine after completing AC-T; one woman received post-AC+T bevacizumab and metronomic chemotherapy.

|| One woman in the adjuvant cohort received fluorouracil, epirubicin, cyclophosphamide. One woman in the survivor cohort received navelbine only, and three women in the survivor cohort received paclitaxel only.

senescence markers (eg, IL-7, IL-8, and LTL) (Supplementary Figure 2, available online) did not statistically significantly change after chemotherapy. These data suggest that cytotoxic chemotherapy increases expression of transcriptional senescence markers in

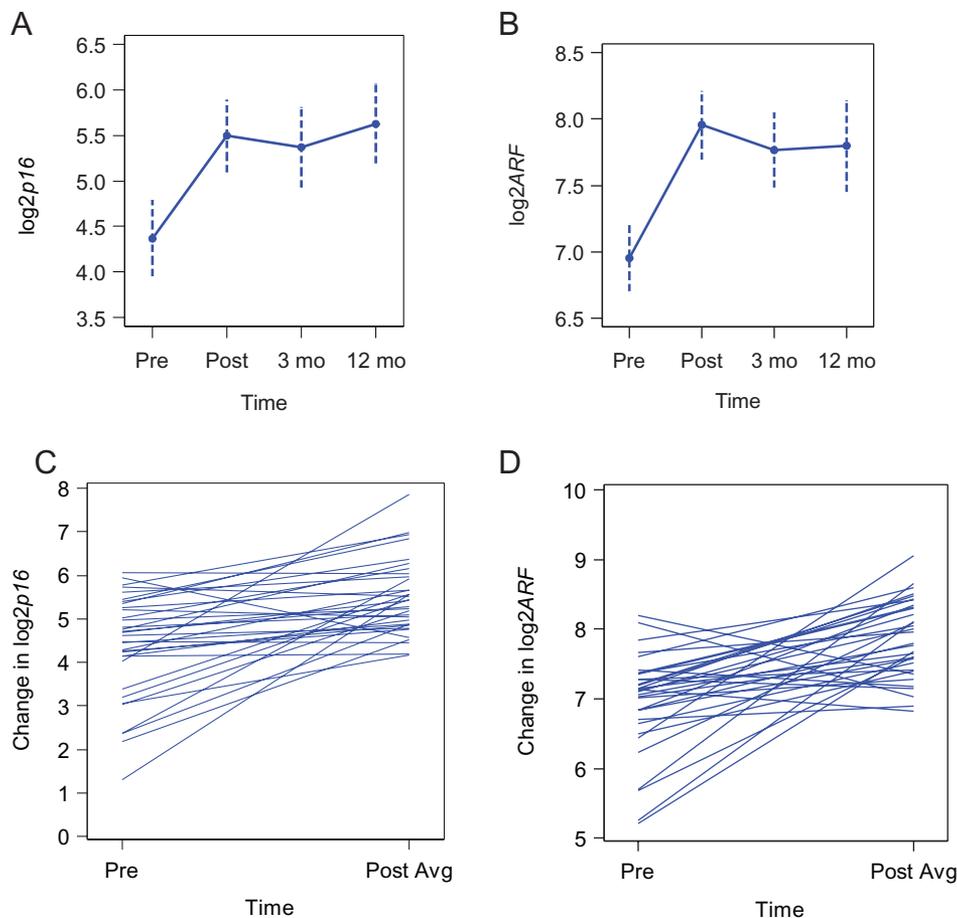


Figure 2. Chemotherapy-induced increase in *p16INK4a* and *ARF* expression in the prospective adjuvant cohort. Mean values (dots) with 95% confidence intervals (dotted lines) of $\log_2 p16INK4a$ (A) and $\log_2 ARF$ (B) from pretreatment through three post-treatment time points: immediately upon count recovery after chemotherapy and 3 months and 12 months after chemotherapy. The change

from pretreatment (Pre) to the average post-treatment (Post avg) value is shown for individual patients for $\log_2 p16INK4a$ (median change = 0.81; interquartile range [IQR] = 0.28–1.62; Wilcoxon signed-rank $P < .001$) (C) and $\log_2 ARF$ (median change = 0.89; IQR = 0.29–1.27; Wilcoxon signed-rank $P < .001$) (D). All statistical tests were two-sided.

PBTLs, as well as serologic levels of two well-described secreted markers of senescence activation.

We also considered as secondary endpoints the effect of other treatment-related factors on change in PBTL *p16INK4a* expression with cytotoxic chemotherapy. Neither adjuvant chest wall radiotherapy ($n = 25$) nor taxane use ($n = 29$) was independently associated with change in *p16INK4a* expression, although caution is warranted given the small number of patients. Dose-dense (every 2 weeks) chemotherapy was associated with a greater rise in $\log_2 p16INK4a$ than a standard schedule of adjuvant doxorubicin and cyclophosphamide every 3 weeks (median = 1.13 vs 0.32; Wilcoxon rank-sum $P = .04$) (Supplementary Figure 3, available online). A trend toward greater median change in *p16INK4a* expression was observed in women experiencing grade 3 and 4 hematologic toxicity than in women without severe hematologic toxicity (median = 1.31 vs 0.57; Wilcoxon rank-sum $P = .07$). These results suggest that more intensive chemotherapy and more severe myelosuppression are associated with a greater acceleration of molecular age in hematopoietic tissues.

Given the statistically significant induction of *p16INK4a* for up to 1 year after chemotherapy (Figure 2A), we examined the effect of cytotoxic chemotherapy on *p16INK4a* expression at later time points

in an independent cross-sectional survivor cohort (Figure 1B and Table 1). Of the 176 evaluable breast cancer survivors, 69 (39.2%) had received adjuvant chemotherapy. In this cohort, smoking, comorbidity, and body mass index were not independently associated with *p16INK4a* expression. Age was statistically significantly associated with *p16INK4a* expression, with an estimated *p16INK4a* doubling time of 16.7 years (ie, a 0.06 unit per year increase in $\log_2 p16INK4a$; repeated measures model $P < .001$). In adjusted analyses, $\log_2 p16INK4a$ expression in chemotherapy-treated women was 0.57 higher (repeated measures model $P < .001$) than in women not treated with chemotherapy. Compared with the increase seen in independent cohorts of healthy human donors [0.055 per year (14, 35)], this increase is equivalent to 10.4 years of chronological aging (Figure 3). *ARF* expression was not statistically significantly associated with prior chemotherapy use in the survivor cohort. As in the adjuvant cohort, chest wall radiotherapy and adjuvant hormonal therapy were not independently associated with *p16INK4a* expression (Supplementary Table 3, available online). Because patients in this cohort were a median 3.4 years from the completion of chemotherapy (some up to 18 years from treatment), these results suggest that the sharp increase in *p16INK4a* expression observed after

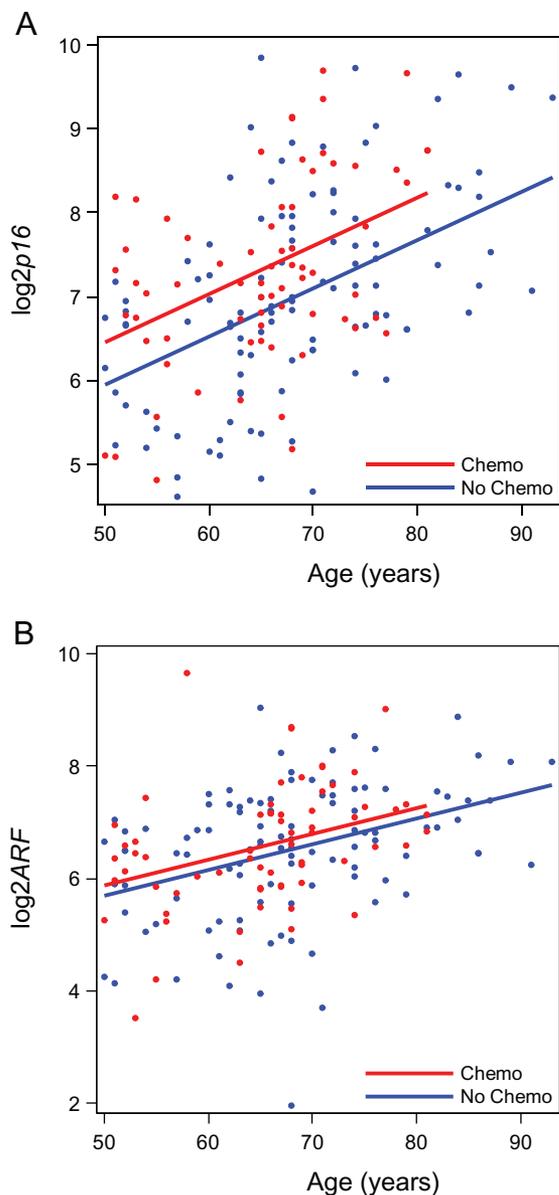


Figure 3. Expression of *p16INK4a* and *ARF* by age and prior chemotherapy exposure in the cross-sectional survivor cohort. $\text{Log}_2p16INK4a$ (A) and log_2ARF (B) at each age according to receipt of adjuvant therapy. Dots represent the average value for each patient among the three batches. Lines represent the predicted values for chemotherapy-treated (red) and non-chemotherapy-treated (blue) patients based on the results of the repeated measures models. $\text{Log}_2p16INK4a$ is statistically significantly higher in chemotherapy-treated vs non-chemotherapy-treated patients (repeated measures model $P < .001$). Log_2ARF is not statistically significantly higher in chemotherapy-treated patients vs non-chemotherapy-treated patients (linear regression model $P = .10$). All statistical tests were two-sided.

chemotherapy persists for at least several years, if not indefinitely, after chemotherapy exposure.

Discussion

We have shown that adjuvant breast cancer chemotherapy leads to a substantial increase in molecular markers of cellular senescence, including *CDKN2a* expression in PBTLs, as well as serologic levels of two senescence-associated cytokines. In these prospectively

followed patients treated with standard adjuvant chemotherapy, a near doubling of *p16INK4a* and *ARF* expression was seen in T cells after adjuvant doxorubicin and cyclophosphamide chemotherapy, an increase comparable to 14.7 years of normal chronological aging (14,35). More aggressive dose-dense chemotherapy schedules and greater hematologic toxicity were associated with a greater in vivo induction of markers of cellular senescence. The effect of chemotherapy on *p16INK4a* expression was confirmed in an independent, cross-sectional cohort of long-term breast cancer survivors. In aggregate, results from these two analyses demonstrate that adjuvant cytotoxic chemotherapy durably promotes expression of markers of cellular senescence in breast cancer patients.

The principal limitation of this study is the reliance on markers that can be only easily assayed in the peripheral blood. Although data from both cohorts showed an increase in *p16INK4a* expression after systemic chemotherapy, we can only conclusively state that chemotherapy promotes expression of senescence markers in $CD3^+$ lymphocytes. Age-related increases in *p16INK4a* expression occur across a wide variety of mammalian tissue compartments (Supplementary Figure 1, available online) (see also [12,13,35,40]), yet the amplitude of age-related change varies by tissue compartment as does its response to interventions that modify aging such as caloric restriction (13). Likewise, the tissue source of increased senescence-associated cytokines after chemotherapy is unknown. Thus, it is uncertain whether measurement of senescence-associated cytokines or *p16INK4a* expression in a hematopoietic compartment can serve as a proxy for generalized organismal age, and our data do not speak to the pro-aging effects of chemotherapy in non-hematopoietic compartments.

We were able to reproducibly show that breast cancer chemotherapy leads to a durable increase in PBTL *p16INK4a* expression, but we were unable to show an effect of chemotherapy on LTL and IL-6. Both of these biomarkers have been extensively studied as aging biomarkers, and unequivocally change (decrease for LTL and increase for IL-6) across the lifespan when measured in large populations (24–26,41). However, neither assay is well suited for individual risk prediction: the absolute level/length vary substantially among same-aged individuals, and the absolute change is relatively small over an individual's lifespan (24,32,41–43). Longitudinal studies of LTL have shown an expected annual decrease in length of 25 to 45 base pairs, although 15% to 20% of prospectively followed people demonstrated telomere elongation over the studied period (41). Such variation has also been observed in studies of patients receiving chemotherapy (44,45). This wide variation in individual LTL over time in concert with the finding that administration of granulocyte colony stimulating factor may increase LTL through upregulation of telomerase (46,47), suggest LTL is unlikely to be a reliable biomarker of the effect of chemotherapy on molecular aging. Further, others have shown that the chemotherapy-induced cellular senescence in a variety of cancer cell lines and murine models is independent of telomere length and telomerase activity, suggesting LTL is a poor marker of chemotherapy-induced aging [reviewed in (48)]. Likewise IL-6 is suboptimal for this purpose because baseline expression is low but shows substantial age-independent confounding from circadian variation and induction by viral infection and other stressors (32,49). We believe that *p16INK4a* expression in PBTLs offers new potential as a diagnostic marker

of senescence induction in vivo given its very large dynamic range (approximately 16-fold change over a human lifespan), ease and low cost of measure, and strong correlation with chronological age (R^2 with chronological age is approximately 0.6 for $p16^{INK4a}$ vs <0.2 for LTL or IL-6) (14,26,41,42).

Based on murine analyses, we believe the PBTL increase in $p16^{INK4a}$ expression likely reflects heritable damage to hematopoietic stem cells (17,50) but may also reflect damage to the thymic milieu (21) or direct damage to self-renewing, post-thymic T cells (18). Adjuvant anthracycline-based chemotherapy unequivocally saves lives, markedly decreasing the relative risk of breast cancer recurrence by 27% and death by 21% (51). Therefore, we believe the finding of durable, age-promoting effects of anthracyclines and alkylating agents must be weighed against these established benefits with regard to relapse risk. The ability to serially measure markers of molecular age in patients may provide a means to identify beneficial adjuvant approaches that are less “pro-aging.” For example, these results suggest that dose-dense therapy may induce greater long-term hematologic damage, whereas taxanes may be associated with less long-term risk. Further work to more directly quantify the contribution of each component of adjuvant therapy on certain known long-term sequelae may allow for more balanced treatment decisions and perhaps greater use of less gerontogenic regimens for low-risk cancers.

In summary, we have shown that cytotoxic chemotherapy potently induces the expression of markers of cellular senescence in the hematologic compartment in vivo, comparable with the effects of 10 to 15 years of chronologic aging in independent cohorts of healthy donors. We are currently enrolling in two prospective trials: one evaluating whether changing PBTL $p16^{INK4a}$ expression is a biomarker of acute toxicity of various chemotherapy regimens (LCCC1027, NCT01305954); and a second focused exclusively on older patients evaluating the combination of a validated geriatric assessment and PBTL $p16^{INK4a}$ for prediction of treatment-related toxicity and change in function (NCT01472094). Although refining the clinical utility of this biomarker of molecular aging will take further study, we have shown that chemotherapy accelerates molecular aging in the hematopoietic compartment of breast cancer survivors.

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Affiliations of authors: Lineberger Comprehensive Cancer Center (HKS, AMD, JK, CT, JS, JGI, LAC, AD, B-BG, SA, NES, HBM), Department of Medicine (HKS, TAJ, GW, LAC, NES, HBM), Department of Genetics (JK, CT, NES), and Department of Biostatistics (JGI), University of North Carolina, Chapel Hill, NC; Department of Medicine, University of Virginia, Charlottesville, VA (PD); Department of Medical Oncology and Therapeutics Research, City of Hope Cancer Center, Duarte, CA (AH); Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena, Germany (KK, KLR).