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Obesity increases DNA damage in the breast epithelium

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Abstract

Obesity is a modifiable risk factor for breast cancer. Yet, how obesity contributes to cancer initiation is not fully understood. The goal of this study was to determine if the body mass index (BMI) and metabolic hallmarks of obesity are related to DNA damage in normal breast tissue. In a mouse model of diet-induced obesity, weight gain was associated with elevated levels of DNA double-strand breaks in the mammary gland. We also found a positive correlation between BMI and DNA breaks in the breast epithelium of premenopausal women (but not postmenopausal women). High BMI was associated with elevated systemic and tissue-level oxidative DNA damage across the lifespan, and we propose that the breast epithelium undergoing menstruous proliferation waves is particularly prone to the generation of DNA breaks from oxidative lesions. Ancestry was an important modulator of the obesity-DNA break connection. Compared to non-Hispanic Whites, women identifying as African Americans had higher levels of DNA breaks, as well as elevated leptin and IGF-1. In 3D cultures of breast acini, both leptin and IGF-1 caused an accumulation of DNA damage. The results highlight a connection between premalignant genomic alterations in the breast epithelium and metabolic health modulated by obesity and ancestry. They call for attention on biological determinants of breast cancer risk disparities.

Keywords Breast cancer risk, Obesity, Cancer health disparities, Molecular markers, DNA double-strand breaks, 3D cell culture, Leptin, IGF-1

Background

Obesity is a highly prevalent and modifiable breast cancer risk factor [1, 2]. Growth factors and cytokines produced by adipocytes stimulate tumor cell proliferation, migration, and invasiveness, leading to poorer prognoses for patients with high BMI [3–6]. The mechanisms by which obese microenvironments promote cancer initiation are however less understood than those fueling tumor progression.

DNA damaging exposures and DNA damage repair defects are drivers of breast cancer. Several breast cancer susceptibility genes function in the repair of DNA double-strand breaks, and studies have shown reduced DNA repair potential in breast cancer patients compared to

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cancer-free controls [7, 8]. Non-conservative (mutagenic) DNA repair pathways were also more active in breast cancer patients and in women with familial breast cancer risk compared to the general population [9]. Exposure to ionizing radiation increases breast cancer risk, for example among atomic bomb survivors in Japan [10], in bone marrow transplantation patients subjected to total body irradiation [11–13], and for women who received extensive chest irradiation to treat hematologic malignancies [14], emphasizing the connection between DNA breaks and breast cancer.

Breast cancer health disparities affecting African American/Black (AA) women are well documented in the United States. Although breast cancer incidence is overall lower in AA women compared to non-Hispanic White (NHW) women, there is a higher prevalence of aggressive cancers in young women of African descent compared to NHW [15]. Despite progress in early detection and targeted therapies, 5-year survival for AA women is 10% points lower than for NHW women [16]. The reasons for this disparity are multiple, interrelated and include social inequalities [17]. Whereas these factors commonly affect different racial/ethnic populations in the US, some cancer disparities are specific to certain groups, indicating that genetic differences (and/or differences in gene-environment interactions) also contribute to the different cancer risk and outcomes.

The potential link between obesity and DNA damage in the breast epithelium is still poorly understood. Some studies suggest that overweight/obesity may increase DNA damage and/or reduce DNA repair capabilities while others found no association (reviewed in [18]). A limitation of most studies performed so far is that lymphocytes rather than breast epithelial cells were analyzed. Specific tissue contexts may influence DNA damage and repair outcomes. Here, we quantified DNA breaks directly in breast epithelial cells, using a mouse model of diet-induced obesity, human tissue samples and 3D cell culture models. We focused on DNA double-strand breaks since these lesions are highly toxic and defects in their repair are intimately linked to breast cancer.

Methods

Animals

Animal experimentation was approved by the Animal Care and Use Committee of the Wake Forest School of Medicine (protocol #A18-136). Four-week-old female C57BL/6 mice were purchased from Jackson. The animals were placed on a low-fat control diet (control; TD.08806) or on a lard-based obesity-inducing diet (HF; 60% of total calories from fat; TD.06414), both from Envigo (Teklad diets). Animal weights were recorded weekly. Mammary glands were used for immunostaining and serum was collected for adipokine measurements (see below). A second, independent cohort of C57BL/6 mice was used to confirm DNA damage results by comet assay (see below). The animals were fed the control or HF diets for 12 weeks. The dietary intervention on BALBc mice is described in Tenvooren et al. [19].

Procuration of human breast tissue and serum samples

Non-cancerous breast tissue specimens were obtained from reduction mammoplasty surgeries performed at the Atrium Health Wake Forest Baptist Medical Center (Winston-Salem, NC, USA) between 2021 and 2022. Matching breast tissue samples from the left and right breasts were snap-frozen and formalin-fixed/paraffin-embedded (FFPE) for future analysis. This tissue collection was approved by the Institutional Review Board (IRB) of the Wake Forest School of Medicine (Protocol #IRB00074778) and was done in accordance with Health and Human Services (HSS) regulations for the protection of human research subjects.

Frozen needle biopsies of normal human breast tissues were obtained from the Susan G. Komen Tissue Bank (KTB; IU Simon Cancer Center, IN, USA). Collection of data and biospecimen by the KTB was approved by the IRB of Indiana University (Protocol #1011003097). The tissue samples were donated between 2009 and 2017. Selection of the donors was based on BMI, age and race/ethnicity. Additional selection criteria were presence of epithelium in digitalized H&E-stained tissue sections, consulted using the Virtual KTB (https://virtualtissuebank.iu.edu), availability of frozen serum samples, no hormone replacement therapy (HRT) usage, no alcohol consumption, non-smoker donor, and pre-menopausal status. HRT users, smokers, and alcohol consumers were excluded to avoid possible risk confounding factors. From the 42 donors selected, 33 biopsies had sufficient epithelium for analyses. Characteristics of KTB donors can be found in ref [20].

To validate DNA damage detection, we used normal breast tissue explants from mastectomies (normal-adjacent tissue) and from reduction mammoplasties, collected at the IU Health Arnett Hospital (Lafayette, IN). This tissue collection was approved by the Purdue University IRB (protocol #1206012467). Specimens were placed in RPMI, minced to approx. 4 mm cubes, irradiated (3 Gy; Nordion Gammacell 220 irradiator), and left to recover for one hour in a cell culture incubator before freezing in optimal cutting temperature compound. Controls were mock-irradiated.

Plasmas from healthy women were supplied by the Établissement Français du Sang (EFS; Convention N°CPDL-PLER-2022-029).

Analyses of serum samples, breast tissue, and cell lysates by ELISA

Levels of 8-OHdG (8-hydroxy-2'-deoxyguanosine) in human serum, human tissue, and cell lysates were quantified by ELISA (AbCam, cat# 201734). Levels of 40 cytokines and adipokines were quantified in human serum samples using the Quantibody Human Obesity Array 3 kit (RayBiotech). Adipokines were measured in mouse serum by ELISA, using kits from Bertin Pharma (leptin) and RayBiotech (adiponectin). Reactive oxygen species were measured in cell lysates with the Human ROS ELISA kit (Novatein, cat# BG-HUM220964). γH2AX levels were measured in cell lysates and serum with the Human Phospho-Histone H2AX (S139) ELISA kit (R&D Systems, cat# DYC2288).

Immunohistochemistry

Analysis of the Ki67 proliferation marker in FFPE tissue was performed as described previously [21], using Ki67 antibodies from Cell Signaling Technology (cat# 12202). DAB (3, 3'-diaminobenzidine) staining was imaged at 20x magnification and quantified using a Mantra Quantitative Pathology Imageing System (PerkinElmer).

Cell culture and treatments

Non-neoplastic breast epithelial cells were cultured at 37 °C with 5% CO_2 in a humidified incubator. HMT-3522 S1 cells were propagated between passages 54 and 60 in H14 medium [22]. Epithelial differentiation was achieved with 3D culture on top of a thin layer of Matrigel (Corning, cat# 354234) for ten days in chambered slides (MilliporeSigma, cat# PEZGS0896), as described [22]. MCF10A cells were cultured in DMEM/F-12 (Life Technologies, cat# 21331046) supplemented with 1% penicillin/streptomycin, 1% glutamine, 500 ng/ml hydrocortisone (Sigma-Aldrich, cat# H0888), 100 ng/ml cholera toxin (Sigma-Aldrich, cat# C8052), 10 µg/ml insulin (Sigma-Aldrich, cat# I1882) and 20 ng/ml epidermal growth factor (EGF, Peprotech, cat# AF-100-15). Routine mycoplasma tests were all negative. Cells were treated with human recombinant leptin (Protein Laboratories Rehovot [PLR], cat# Lep-5), leptin receptor antagonist (PLR, cat# SLAN-2), recombinant human IGF-1 (PeproTech, cat# 100 – 11), and the IGF-1R inhibitor picropodophyllin (PPP; Santa Cruz Biotechnology, cat# sc-204008 A), as indicated in the results section. DNA damage was induced by gamma irradiation (3 Gy, Gammacell 220 irradiator) or with bleomycin (20 mU/ml for 2 h; Cayman Biochemicals).

Immunofluorescence and imaging

Frozen sections of human breast tissue and C57BL/6 mammary glands (5 µm thick) were thawed at room temperature and areas with tissue were delineated using a hydrophobic pen. Sections of paraffin-embedded mammary glands from BALBc mice (3 µm thick) were deparaffinated and rehydrated with successive washes with xylene (3 times), 100% ethanol (twice), 95% ethanol (twice), and MiliQ water (twice). The antigen retrieving solution (10 mM sodium citrate, 0.05% Tween) was preheated to 97 °C and incubated on the slides for 30 min at this temperature. Slides were allowed to cool to room temperature and rinsed three times with MiliQ water before immunostaining. Cultured acini were stained in 8-well chambered slides (Millipore). Samples were fixed in formalin, permeabilized with TX-100, washed in PBS-glycine, and incubated 2 h in blocking buffer (10% goat serum in immunofluorescence buffer [IF; 130 mM NaCl, 13.2 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 0.1% bovine serum albumin, 0.05% NaN_3, 0.2% Triton X-100, and 0.05% Tween 20]). Antibodies were diluted in blocking buffer and incubated on samples overnight at 4 °C. Samples were washed three times with IF buffer, incubated with fluorescently labeled secondary antibodies (1 h at room temperature), washed again with IF and stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, 0.5 µg/ml; 10 min). Stained sections were mounted with coverslips using ProLong Gold Antifade reagent (Invitrogen). Antibodies were against 53BP1 (Abcam, cat# Ab36823, 5 µg/ml), γH2AX (Millipore, clone JBW301, 2 µg/ml), Ki67 (Thermo Fisher Scientific, PA5-19462, 1 µg/ml), and NuMA (clone B1C11, a gift from Dr. Jeffrey Nickerson, UMass, Worcester, USA). Secondary antibodies conjugated with Alexa Fluor dyes (AF488, AF568, or AF647; ThermoFisher) were used at 1:500 dilutions. Immunofluorescent signals in breast tissue sections were imaged using a Zeiss LSM880 confocal microscope equipped with a 63x water immersion objective (NA = 1.2). Mouse mammary gland sections were imaged with an Olympus IX83 epifluorescence microscope, using a 40x objective (NA = 0.95) and a sCMOS camera (Hamamatsu ORCA-Flash 4.0). Cultures of acini were imaged with a Zeiss LSM710 confocal microscope, using a 63x oil objective (NA = 1.4).

Image analysis

Repair foci were enumerated by visual scoring. For DNA damage quantification in human breast tissue, approx. 100 nuclei were evaluated. For DNA damage quantification in mouse mammary glands, a minimum of 120 nuclei were evaluated. For DNA damage quantification in cultured acini, 150–400 nuclei were analyzed for each treatment replicate. For validation, automated foci count was performed using a custom macro in FIJI. Briefly, nuclei were segmented with the StarDist deep learning tool [23], using a model trained on the TissueNet dataset [24]. Nuclei on the image border or with sizes outside user-defined boundaries (45–550 μm^2) were removed

from the analysis. Repair foci were segmented using the Laplacian of Gaussian detector as implemented in the Trackmate plugin [25]. For each processed image, one image showing DAPI and foci immunostaining overlaid with segmented nuclei contours and one image showing foci immunostaining overlaid with nuclei contours and detected repair foci were generated for visual inspection of the results.

Comet assay

To analyze cultured breast acini [26], the structures were released from Matrigel using dispase (Corning, cat# 354235; 10 min at 37 °C), suspended in low-melting point agarose and processed according to the neutral comet assay protocol from Trevigen. For the analysis of mouse mammary glands and human breast samples, fresh tissue was cut in 5 mm cubes, flash-frozen in liquid nitrogen, and thawed on ice in mincing solution (Hank's balanced salt Solution free of magnesium, calcium, and phenol red, supplemented with 10% (v/v) DMSO and 20 mM EDTA pH 7.5). Tissue was minced with fine scissors to release cells and cell suspensions were strained through a 40 µm mesh before performing neutral comet assays. The Olive tail moment was determined using the CometScore 2.0 software.

Statistics

Statistical analyses were performed using Prism 10 (GraphPad). The D'Agostino & Pearson omnibus normality test was used to test for normality. Nonparametric tests were used if the data did not pass the normality test (at alpha = 0.05). Statistical tests are indicated in figure legends. All statistical tests were two-sided. A *P* value < 0.05 was considered significant. Unless indicated otherwise, dots in graphs represent the values of individual biological replicates.

Results

Diet-induced obesity increases DNA break levels in the mouse mammary gland

To explore the relationship between breast epithelial DNA damage and obesity, we analyzed mammary glands from C57BL/6 mice fed for 20 weeks a control diet or a lard-based high-fat (HF) diet. As reported previously [20], animals on the lard diet gained significantly more weight than the control group (Fig. 1A). Frozen

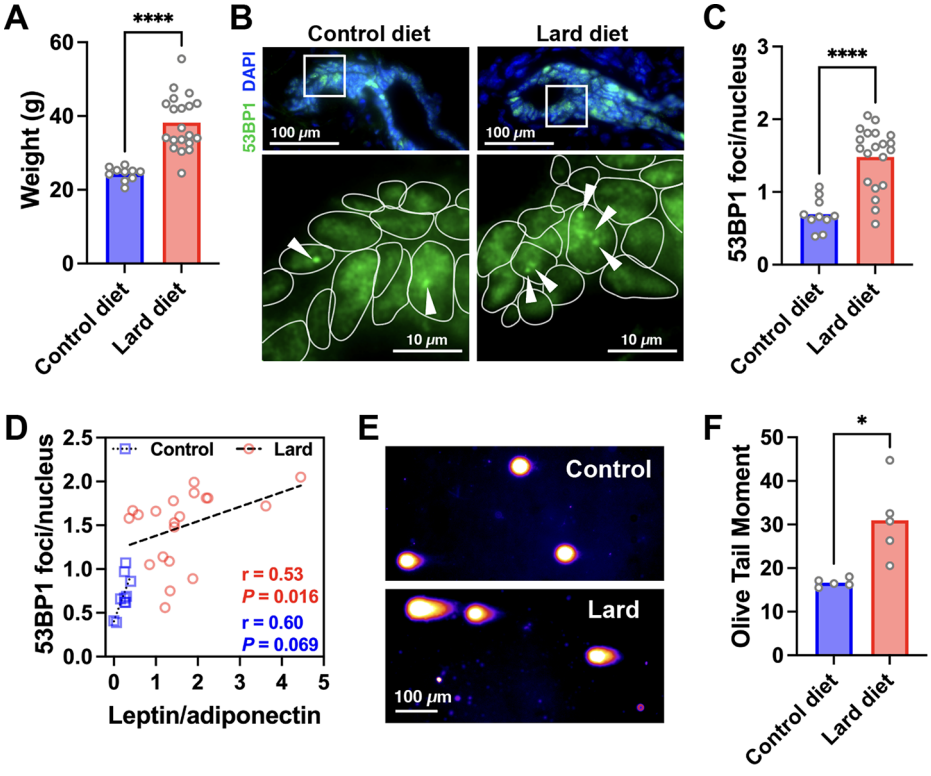

Fig. 1 Effect of diet-induced obesity on DNA damage in the mammary gland. **A** Weight of C57BL/6 mice after 20 weeks on a control or lard-based high-fat diet. **** $P < 0.0001$ (t test with Welch's correction). **B** 53BP1 damage foci Immunodetection (arrowheads) in the mammary gland from mice fed a control or high-fat diet. **C** Quantification of 53BP1 damage foci in mammary epithelial cells. **** $P < 0.0001$ (t test with Welch's correction). **D** 53BP1 foci as a function of the serum leptin-to-adiponectin ratio in mice fed control or high-fat lard diets. r, Spearman's correlation coefficients. **E** Comet assay on mouse mammary glands. **F** Quantification of comet Olive tail moment. * $P < 0.05$ (t test with Welch's correction). Symbols on the graphs represent different animals

mammary gland sections from these animals were immunostained for the p53 binding protein 1 (53BP1) to quantify DNA double-strand breaks (Fig. 1B). This protein forms distinct foci at DNA damage sites [27, 28], which are straightforward to enumerate in mouse and human tissues [29]. Unlike DNA damage-induced chromatin modifications such as H2AX phosphorylation (γH2AX), where absence of signals can be interpreted either as lack of damage or lack of staining, cells without DNA damage have pan-nuclear 53BP1 signals. In normal tissue, 53BP1 foci generally overlapped with γH2AX (Suppl. Figure S1A). The number of 53BP1 foci was significantly higher in samples irradiated ex vivo (Suppl. Figure S1B) and there was good agreement in 53BP1 foci enumeration between independent scorers (Suppl. Figure S1C), validating the use and quantification of this marker in normal mammary glands.

Based on 53BP1 immunostaining, tissue levels of DNA breaks were significantly higher in mammary glands from mice on the HF diet compared to controls (Fig. 1C). In correlation analyses, an association between 53BP1 damage foci and serum leptin-to-adiponectin ratio, a metabolic hallmark of obesity, was detected (Fig. 1D). Elevated levels of DNA double-strand breaks in mammary glands from mice on a HF diet were confirmed by neutral comet assay performed on a second cohort of animals (Fig. 1E-F).

The high-fat and control diets were quite distinct, with notably 5.7 times higher cholesterol and a 1.6 times higher n-6:n-3 poly-unsaturated fatty acid ratio in the HF diet. To distinguish between obesity and dietary effects, we analyzed mammary glands from BALB/c mice which are resistant to diet-induced obesity [30]. Whereas in the C57BL/6 strain, the body weight of mice fed the HF diet was 56% higher than mice on the control diet, the same HF diet only led to a 14% increase in body weight in the BALB/c strain [19]. The amounts of 53BP1 damage foci were not different in mammary glands from BALB/c mice on the HF and control diets (Suppl. Figure S2), suggesting obesity and its metabolic consequences rather than specific dietary components as potential drivers of genomic instability.

High body mass index is associated with increased DNA double-strand breaks in breast tissue

To assess if the link between obesity and DNA breaks translates to humans, we analyzed breast tissue from reduction mammoplasties by comet assay (Fig. 2A). Tissue was collected from both the right and left breasts, enabling both inter- and intra-individual comparisons. Although there was a significant bilateral correlation in DNA damage (Suppl. Figure S3A), the lack of a perfect left-right agreement indicates local differences in genome integrity which may be compounded with measurement errors. Therefore, when data was available from both sides (for 53 out of 61 patients), patient averages were used. There was no correlation between age and DNA damage (Suppl. Figure S3B). Overall, DNA damage was positively correlated with BMI (Pearson's $r = 0.29$; $P = 0.025$). Interestingly, this effect was driven by premenopausal patients, and was not observed in postmenopausal women (Fig. 2B). We also noticed that the correlation between BMI and DNA damage in younger women was restricted to non-Hispanic White patients. It was not significant in women self-identifying as African American/Black (Suppl. Figure S3C).

Breast tissues from reduction mammoplasties are not considered fully normal. They are indeed more similar to benign breast disease than normal tissue in terms of histological abnormalities and proliferation [31]. To further assess if obesity is associated with DNA damage levels in the breast epithelium, we analyzed normal breast tissue samples donated to the Komen Tissue Bank by women with different BMI, this time using 53BP1 immunostaining to focus the analysis on breast epithelial cells (Fig. 2C). There was again no association between the age of the donors and DNA damage in the breast epithelium (Suppl. Figure S3D). Overall, there was a small (24%), non-significant increase in DNA damage for donors with BMI ≥ 30 compared to the lean group (Fig. 2D). However, matched comparisons (age and race/ethnicity) showed significantly more DNA breaks in donors with obesity compared to donors with normal weight (Fig. 2E). Taken together, the results suggest that obesity causes an accumulation of DNA double-strand breaks in the breast, and that covariates influence DNA damage levels.

Serum leptin correlates with systemic levels of DNA breaks

To assess the connection between obesity and DNA damage at a systemic level, we obtained serum samples from healthy women banked at the Établissement Français du Sang. We quantified leptin, a robust metabolic marker of obesity [19, 32, 33] and γH2AX from cell-free circulating histones (circ-γH2AX) to estimate systemic levels of DNA double-strand breaks. Circ-γH2AX was not associated with the age of the donors (Fig. 3A) but positively correlated with leptin (Fig. 3B), indicating that the association between obesity and DNA breaks is not restricted to the mammary gland.

Leptin induces DNA breaks in breast epithelial cells

As mentioned above, elevated leptin is a metabolic hallmark of obesity. It is also an independent molecular marker of breast cancer risk [34–36]. To test if leptin modifies DNA damage levels in the breast epithelium, we cultured mammary epithelial cells (HMT-3522 S1) in the presence of reconstituted basement membrane, leading to the differentiation of acini resembling mammary gland

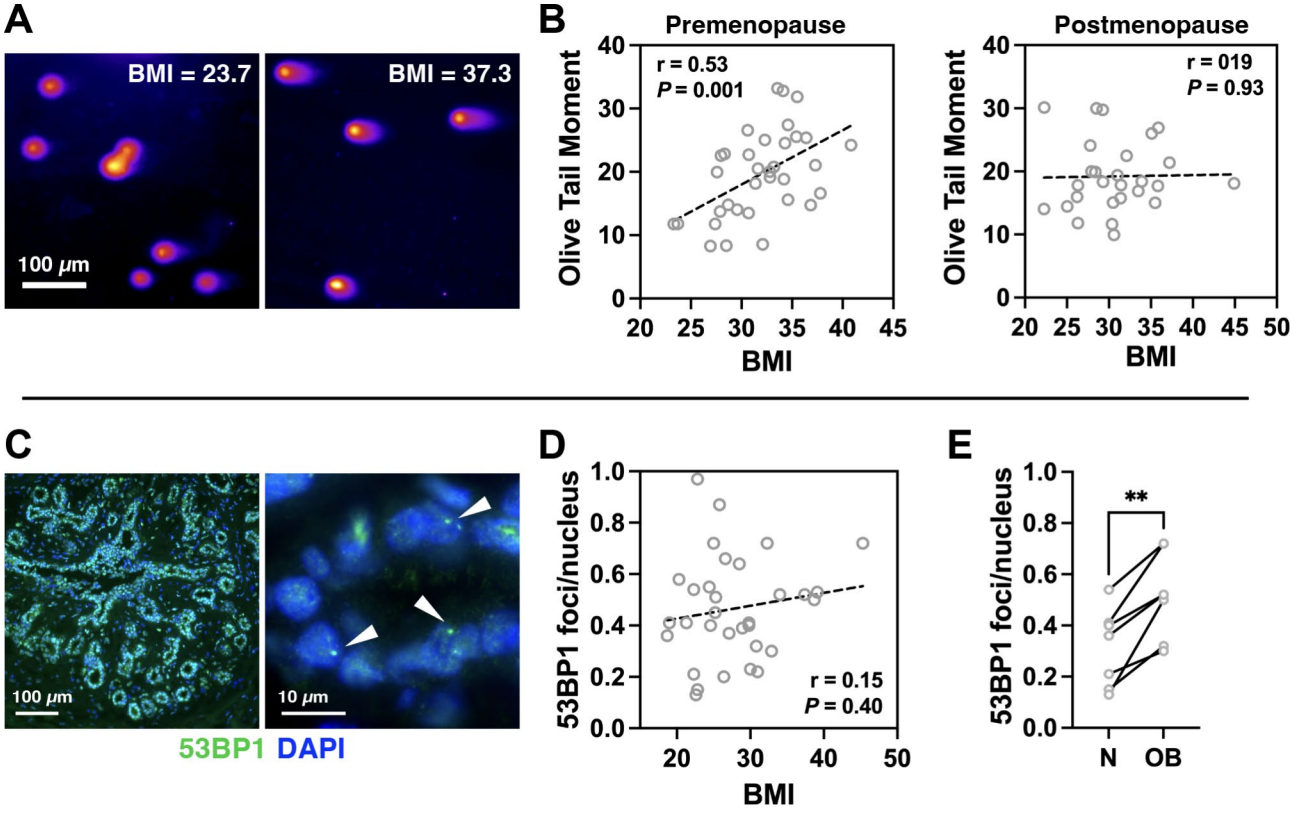

Fig. 2 DNA double-strand breaks in normal breast epithelia as a function of the body mass index. **A** Representative images from neutral comet assays performed on human breast tissue from reduction mammoplasties (premenopausal patients with contrasted BMIs). **B** Comet Olive tail moment as a function of BMI in samples from premenopausal ($N=36$) and postmenopausal ($N=28$) patients. r, Pearson's correlation coefficients. **C** Illustration of immunodetection of 53BP1 in normal breast tissue samples from the Komen Tissue bank. Arrowheads indicate DNA damage foci. **D** Association between BMI and 53BP1 foci numbers in normal breast tissue. r, Pearson's correlation coefficient. **E** 53BP1 foci in donors with normal weight (N) or obesity (OB). The lines indicate matched comparisons. ** $P=0.002$ (paired t-test)

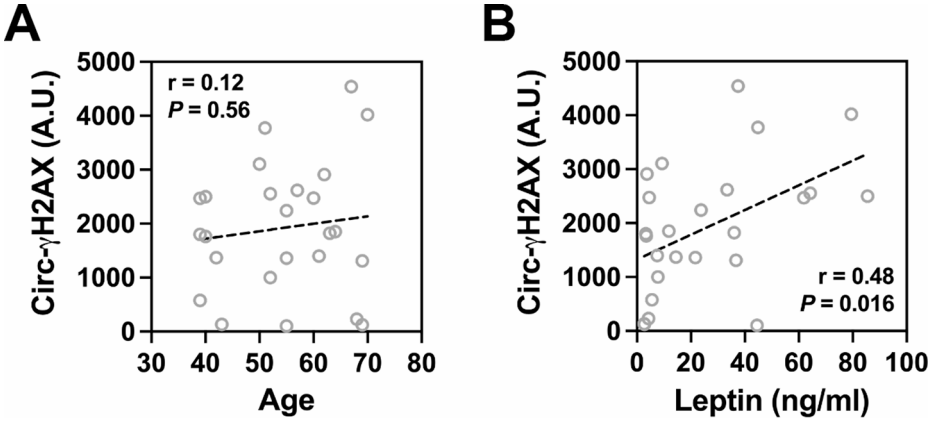

Fig. 3 Systemic levels of DNA double-strand breaks. Association between age (**A**) or leptin (**B**) and cell-free phosphorylated H2AX (circ-γH2AX) in serum samples from healthy French women. r, Pearson's correlation coefficient. Symbols on the graphs represent individual blood donors

units [22] (Fig. 4A). These acini were treated with leptin for three days and used to analyze DNA breaks. Exposure to leptin levels found in obesity (100 ng/ml) led to a 30% increase in the number of 53BP1 foci (Fig. 4A-B and Suppl. Figure S4A-C). Similar observations were made for breast epithelial cells cultured as monolayers, with ~20% increase in DNA damage foci in leptin-treated cells. This suggests that glandular morphogenesis is not a key mediator of leptin's effect on genome integrity (Suppl. Figure S4D-E).

We also observed higher amounts of DNA damage foci in leptin-treated acini that were irradiated and

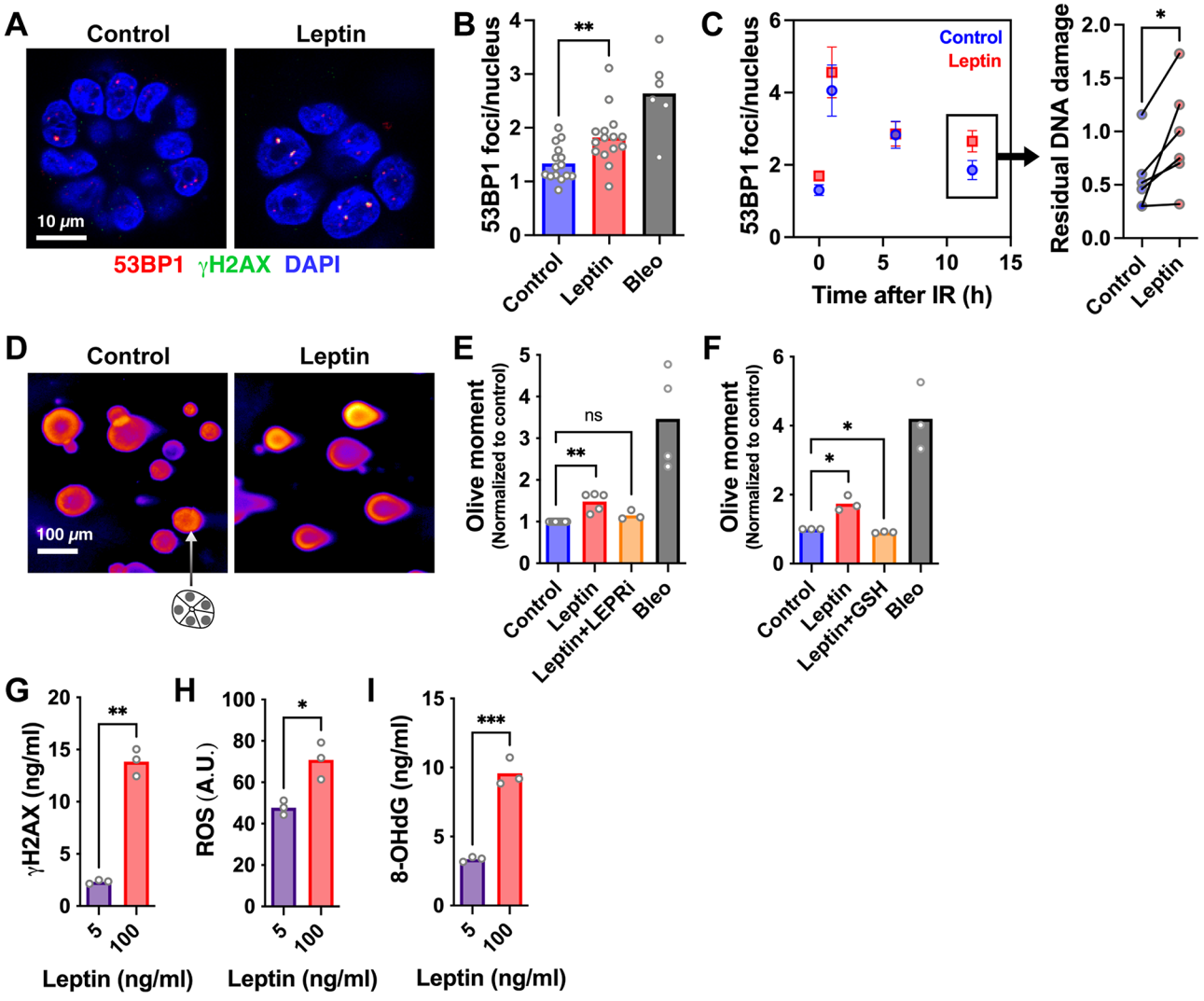

Fig. 4 Induction of DNA double-strand breaks by leptin in breast epithelial cells. **A** Immunostaining for 53BP1 and γH2AX in differentiated HMT-3522 S1 acini treated with leptin (100 ng/ml, 72 h) or untreated (control). **B** Enumeration of 53BP1 foci in S1 cell acini treated as in A. Bleomycin (Bleo) was used as positive control for DNA damage induction. ** $P < 0.005$ (Mann-Whitney). **C** Quantification of 53BP1 repair foci in acini treated or not with leptin. Cells were irradiated (IR), then left to recover. Data are mean ± SEM ($N = 6$). Residual DNA damage (after 12 h recovery; right) was calculated as the difference between IR-induced damage and DNA damage post-recovery with the formula: (IR/1 h - nonIR) - (IR/1 h - IR/12 h). * $P < 0.05$ (t-test). **D** Representative comet images of S1 acini treated as in A. **E-F**Comet Olive tail moments from untreated acini and from acini treated with leptin, in the absence or presence of a leptin receptor antagonist (LEPRi; 1 μg/ml; **E**) or of glutathione (GHS; 0.1 mM; **F**). Bleomycin was used as positive control. * $P < 0.05$; ** $P < 0.01$, ns not significant (one sample t-test). Symbols on the graphs represent independent experiments. **G-I** Quantification of γH2AX (**G**), reactive oxygen species (ROS, **H**), and 8-OHdG (**I**) by ELISA in lysates from MCF10A cells treated for 72 h with 5 or 100 ng/ml of leptin. *, $P = 0.014$; **, $P = 0.0037$; ***, $P = 0.0005$ (unpaired t-tests)

incubated to recover for 12 h, suggesting a lower DNA repair activity (Fig. 4C). DNA double-strand break induction by leptin was confirmed by the neutral comet assay (Fig. 4D-E). In these experiments, leptin's DNA damaging effect was blocked by the administration of a leptin receptor antagonist (Fig. 4E). Our group and others have shown that leptin induces reactive oxygen species (ROS) in breast epithelial cells [20, 37, 38]. The addition of low doses of the antioxidant glutathione prevented DNA damage induction by leptin in breast acini (Fig. 4F). DNA double-strand break levels in the leptin-glutathione combination treatment were slightly lower than in the control. We note that higher levels of the antioxidant increased DNA damage levels, even as single treatments (Suppl. Figure S5). This relates to the observations that multiple DNA damage response factors are regulated by oxidative modifications [39]. We confirmed by ELISA in a different breast epithelial cell line that exposure to 100 ng/ml of leptin significantly increased γH2AX compared to a 5 ng/ml of leptin treatment, the latter corresponding to serum value for normal weight (Fig. 4G). This effect was accompanied by ROS accumulation (Fig. 4H), and

a marked increase in oxidative DNA damage (Fig. 4I). Hence, prolonged leptin exposure alters the redox balance in the mammary gland and impacts genome integrity.

Obesity results in systemic and tissue-level oxidative DNA damage

While it is well-established that obesity causes a chronic state of low-grade inflammation which may affect genome integrity, human studies have produced conflicting results regarding the effect of obesity on oxidative DNA damage (reviewed in [18, 40]). We therefore quantified 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, in the serum samples from the Établissement Français du Sang and from the Komen Tissue Bank. In both cohorts, 8-OHdG levels were not associated with age (Suppl. Figure S6A-B). In the French cohort, serum leptin and 8-OHdG were significantly correlated (Fig. 5A), indicating an association between systemic oxidative DNA damage and metabolic derangements in obesity. In the US serum samples, serum 8-OHdG levels were slightly (12%) higher in obese compared with lean women (Fig. 5B). Serum 8-OHdG was not associated with epithelial DNA damage ($P = 0.48$). Finally, tissue levels of 8-OHdG were measured in breast reduction mammoplasty samples. Overall, 8-OHdG correlated with BMI (Spearman $r = 0.47$, $P = 0.002$). Unlike DNA breaks, both pre- and postmenopausal obese patients had higher 8-OHdG than non-obese patients (Fig. 5C). Accordingly, oxidative DNA damage strongly correlated with DNA breaks in pre- but not in postmenopausal women (Fig. 5D). The repair of 8-OHdG and other oxidative lesions involves the formation of DNA nicks by the base excision repair process, which may get converted into double-strand breaks during S-phase in proliferating cells. The breast epithelium undergoes menstrual proliferative cycles until menopause, and higher levels of the Ki67 proliferation marker were detected in breast epithelial cells from pre- compared to postmenopausal women (Fig. 5E-F). The results suggest that systemic oxidative DNA damage may predict systemic but not breast tissue levels of DNA breaks, whereas breast tissue levels of oxidative damage and double-strand breaks are linked in younger women, but not postmenopause.

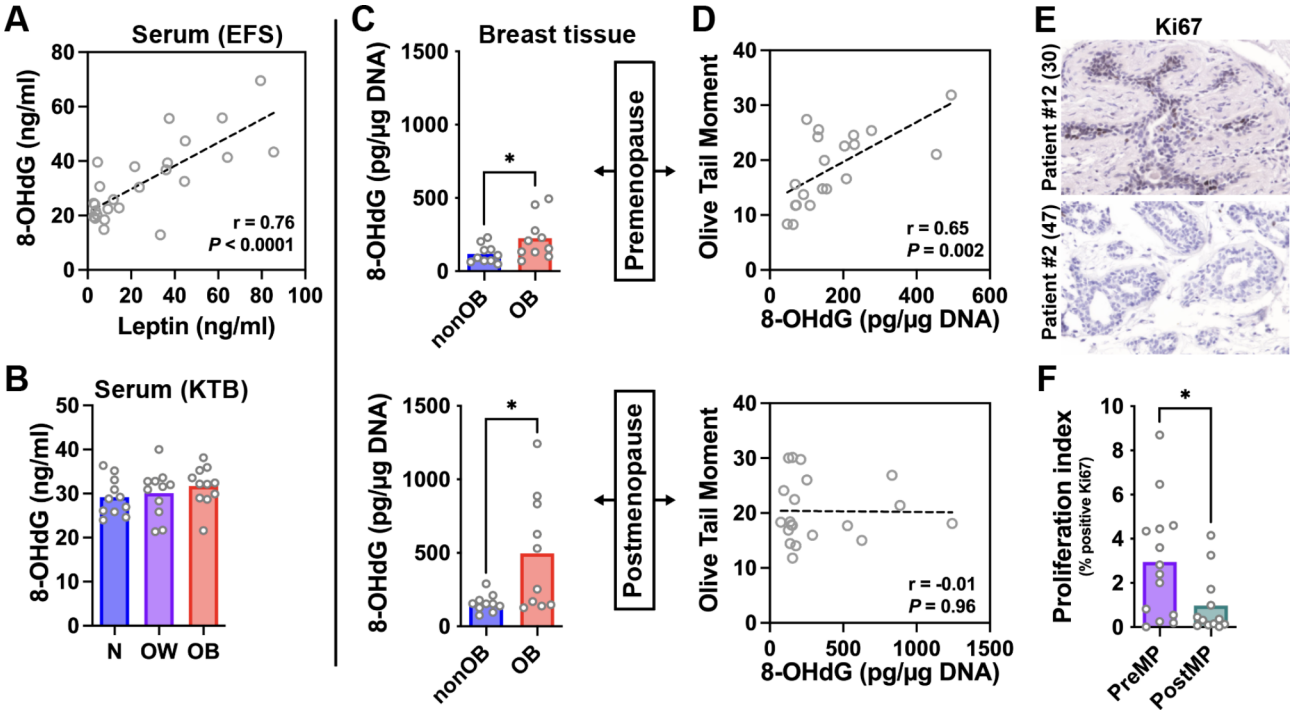

Fig. 5 Obesity results in systemic and tissue-level oxidative DNA damage. **A** 8-OHdG levels as a function of leptin concentration in serum samples from the Établissement Français du Sang (EFS). r, Pearson's coefficient. **B** Serum 8-OHdG levels in Komen Tissue Bank (KTB) donors with normal weight (N), overweight (WO), and obesity (OB). **C** Breast tissue levels of 8-OHdG in premenopausal (top) and postmenopausal (bottom) patients, classified as non-obese and obese based on BMI. *, $P < 0.05$ (t-test). **D** DNA breaks (Olive tail moment) as a function of oxidative DNA damage in breast tissue from pre- and postmenopausal patients. r, Pearson's coefficients. Symbols on the graphs represent individual donors. **E** Illustration of breast tissue staining for the proliferation marker Ki67. Patient age is indicated. **F** Quantification of Ki67 immunohistochemistry signals in the breast epithelium from premenopausal (PreMP) and peri/postmenopausal (PostMP) patients. *, $P < 0.05$ (Mann-Whitney)

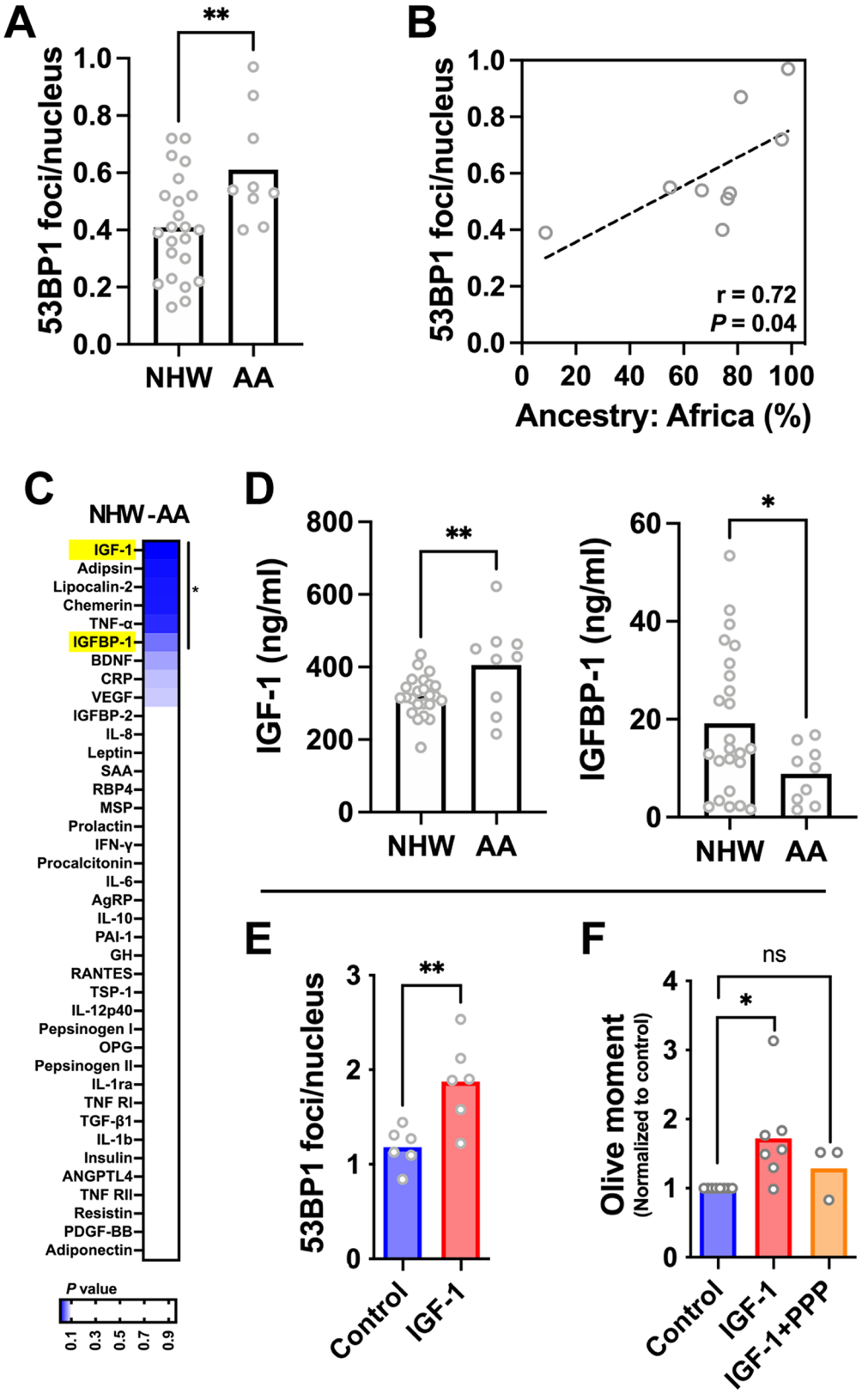

Fig. 6 (See legend on next page.)

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Fig. 6 IGF-1 is elevated in African American women and induces DNA breaks in breast acini. **A** Comparison of 53BP1 foci in the breast epithelium of non-Hispanic White (NHW) and African American/Black (AA) donors. ** $P=0.009$ (unpaired t-test). **B** Correlation between 53BP1 foci and the percentage of African ancestry of the donors. Donors with > 5% African ancestry were included in the analysis. r, Spearman's correlation coefficient. **C** Differences in cytokine/adipokine in serum from NHW ($N=24$) vs. AA ($N=9$) women. * $P<0.05$ (t-test). **D** IGF-1 and IGFBP-1 levels in serum from NHW and AA women. * $P<0.05$, ** $P<0.01$ (t-test). **E** 53BP1 repair foci in acini treated with IGF-1 (100 ng/ml, 72 h) or untreated (control). ** $P<0.01$ (t-test). **F**Comet assay with S1 acini treated with IGF1, in the absence or presence of an IGF1R inhibitor (PPP; 0.01 μM). * $P<0.05$, ns not significant (one-sample t-test). Symbols on the graphs represent individual donors (A-D) and independent experiments (E-F)

IGF-1 is elevated in African American women and induces DNA breaks in breast epithelial cells

In normal breast tissue, an effect of obesity on DNA break frequency was evident for matched donor comparisons (Fig. 2E). Since age and race/ethnicity were considered for matching, and age did not affect DNA damage outcomes, we compared DNA damage in non-Hispanic White and African American/Black donors. We note that, at the time this analysis was made, the Komen Tissue Bank had relatively few donations from Latina/Hispanic women and other minority groups, who could therefore not be included in our analyses. DNA damage was significantly higher in AA compared to NHW ($P=0.009$; Fig. 6A). By design, BMI and age were not different between the two racial groups. Socioeconomic indicators were similar between the NHW and AA donors in this study, with no difference in categorized income and slightly higher education levels in the AA compared to the NHW group (Suppl. Figure S7). Single-nucleotide polymorphism ancestry genotyping is available for a subset of donors, enabling admixture assessments. Assessing these donors, we found that the percentage African ancestry was significantly associated with the number of damage foci in breast epithelial cells (Fig. 6B). The results suggest that the association between obesity and DNA double-strand breaks in normal breast epithelial cells is modulated by genetic ancestry.

Leptin levels were higher in AA compared to NHW donors (29.4 ± 16.9 vs. 20.1 ± 12.4 ng/ml) and this difference was significant after controlling for BMI ($P=0.025$). To identify additional mediators of the difference in tissue-level DNA damage measured between African American/Black and non-Hispanic White women, we compared systemic levels for a panel of 40 cytokines, adipokines, and growth factors (Fig. 6C). Among serum factors significantly different in the two groups were the insulin-like growth factor 1 (IGF-1; 27% higher in AA vs. NHW) and the insulin-like growth factor-binding protein 1 (IGFBP-1; 2-fold lower in AA vs. NHW) (Fig. 6D). As observed with leptin, nuclei of cultured breast acini treated with IGF-1 had elevated numbers of 53BP1 DNA damage foci compared to the controls (Fig. 6E). Increased DNA damage in IGF-1-treated cells was confirmed by comet assay (Fig. 6F). There was a significant increase in DNA damage in acini treated with IGF-1, but not in acini treated with IGF-1 and picropodophyllin, an IGF1 receptor antagonist. Whereas IGF-1 induces proliferation of cancer cells in the breast and other organs, differentiated S1 acini do not resume a proliferative state when exposed to this growth factor [19]. Accordingly, there was no correlation between serum IGF-1 levels and the proportion of Ki67-positive breast epithelial structures in breast tissues, and no difference in the proportion of Ki67-positive structures between the NHW and AA groups (Suppl. Figure S8). Hence, the connection between IGF-1 and DNA damage is likely independent from cell proliferation.

Finally, we evaluated breast tissue DNA breaks as a function of serum leptin and IGF-1, the two factors that were significantly higher in AA compared to NHW women and that elicited DNA double-strand breaks in vitro. Individually, serum levels of IGF-1 and leptin were not (or weakly) associated with DNA double-strand breaks. However, women for whom both leptin and IGF-1 were elevated had significantly higher 53BP1 damage foci (Suppl. Figure S9). This effect was more pronounced for NHW who had a broader distribution of DNA damage outcomes than AA. In summary, inter-individual variations in adipokines and growth factors may contribute to the difference in tissue-level DNA damage in the breast. In particular, the IGF-1 signaling axis may partially explain the racial disparity in DNA damage measured in our study.

Discussion

In this study, we combined analyses of a mouse model of diet-induced obesity, human breast tissue samples, and breast acini cultures to address the connection between obesity, metabolic heath and DNA damage in mammary cells. A BMI > 30 significantly increased breast tissue levels of DNA double-strand breaks. We also present evidence that this effect is systemic, with circulating γH2AX correlating with leptin. Increased DNA damage in the breast epithelium is likely to disproportionally impact women with defective DNA repair, such as *BRCA1* and *BRCA2* mutation carriers. In fact, a recent report shows that DNA damage levels correlate with BMI in normal breast epithelial cells carrying *BRCA* mutations [41].

We found no correlation between the age of the donors and the frequency of DNA double-strand break in breast epithelial cells. Aging is largely driven by the consequences of DNA damage, including mutations and epigenetic alterations to the genome [42]. Previous studies with lymphocytes [43] and normal breast epithelium [41] found associations between DNA damage and age

over the lifespan. This discrepancy is likely due to differences in human subject characteristics. In particular, patients/donors in our study had no known *BRCA1 /-2* mutations, in contrast to the study of Bhardwaj et al. [41] which focused on *BRCA* mutation carriers. Reproductive factors have a strong influence on breast epithelial cells. Although we cannot exclude an effect of these factors on DNA break levels measured in this study, key reproductive characteristics, including age at menarche, parity, and breast-feeding choices, were similar for KTB tissue donors across BMI categories (Suppl. Figure S10). It is therefore unlikely that these factors caused the difference in DNA breaks measured in donors with obesity vs. normal weight.

Changes in tissue organization may contribute to genomic instability conferred by obesity. Apical-basal cell polarity, which is a hallmark of normal epithelia, is compromised by metabolic derangements characterizing obesity. We and others reported previously that leptin disrupts apical polarity complexes and cortical actin networks in breast epithelial cells [19, 20, 44–46]. Epithelial polarity may be important for genome maintenance. We previously found that cell-ECM communication is necessary for an efficient DNA damage response [26]. Moreover, a key regulator of apical junctional complexes (Par3) is necessary for genome maintenance [47, 48]. Hence, disruption of epithelial polarity by proinflammatory cytokines may contribute to an increase in DNA damage by limiting the DNA repair capacity. Our in vitro data indicate that elevated leptin may indeed reduce DNA repair in breast acini. It will be important to address if obesity impacts on DNA double-strand break repair efficacy and fidelity *in vivo.*

A striking result of our study is that the connection between obesity and DNA damage in the breast seems to be restricted to premenopausal women. Cross-talks between leptin and estrogen signaling are well-documented: estradiol modulates the expression of the leptin receptor and, reciprocally, leptin and leptin receptor expression correlate with estrogen receptor expression [35]. Although these cross-talks have been documented in breast cancer cell lines and breast cancer patients, they may account for hormone-dependent DNA break induction in normal breast tissue from women with high BMI. We also propose that mammary cells have different sensitivities pre- and postmenopause. Excess ROS from pro-inflammatory cytokines can lead to oxidative DNA lesions which, when left unrepaired, may degenerate into double-strand breaks, notably during DNA replication [18, 40, 49]. Our result show that obesity increases oxidative DNA lesions in the breast irrespective of age. Yet these lesions may be more consequential in premenopausal where breast epithelial cells go through repeated waves of proliferation corresponding to the menstrual cycle [50]. It may seem paradoxical that DNA breaks are associated with obesity in younger women since obesity is a well-established risk factor for breast cancer postmenopause [51]. However, overweight/obesity has been linked with higher breast cancer incidence in high-risk premenopausal women [52]. Early-onset breast cancers tend to be triple-negative and often display mutations in *BRCA1* and other DNA damage response genes, highlighting an important connection between the DNA damage response and breast cancer in younger women.

There is a parallel between our results and previous work based on MMTV-PyMT mice, which develop spontaneous mammary tumors. A high-fat diet consistently increases tumorigenesis in this model [53–55]. This dietary effect was however restricted to animals with intact ovaries and was not observed in ovariectomized (OVX) animals, a postmenopausal breast cancer model [55]. As mice have limited peripheral aromatase expression, the lack of tumorigenic effects of the high-fat diet in OVX MMTV-PyMT mice suggests reliance on hormonal effectors.

An important finding of this study, which deserves further attention, is that African genetic ancestry may modulate DNA damage in breast epithelial cells. Our findings relate to a recent report documenting higher expression of DNA double-strand break repair genes in breast tissue from Black vs. White women, both in tumors and normal tissues [56]. A possible interpretation is that this differential DNA repair gene expression reflects different tissue levels of DNA double-strand breaks [57]. Both observations parallel the higher prevalence of aggressive early-onset breast cancers in African American women [5, 15, 58]. Compared to Whites, a lower proportion of TNBC from women of African descent have *BRCA1* mutations with known deleterious effects [15], suggesting distinct biology for TNBC in different racial groups. Our study hints at differences in breast tissue DNA damage in different racial groups. In reduction mammoplasties, the correlation between BMI and DNA damage was restricted to non-Hispanic White women and was not found in African Americans. Yet, DNA damage levels were not different in NWH and AA patients. In contrast, in normal tissue, steady-state levels of DNA damage were distinct in different racial groups. The relative contribution of biology and disparities as drivers of breast cancer risk and determinants of survival remains unclear. We measured similar serum levels of 8-OHdG in African American and non-Hispanic White women (data not shown). Similarly, previous studies that compared oxidative DNA lesions in African Americans and Whites found either no difference [59] or slightly more oxidative damage in Whites [60]. Hence systemic oxidative stress may not explain the difference in DNA damage in the breast epithelium.

Women of African descent in the Komen Tissue Bank cohort had higher serum levels of leptin compared to Whites. This difference has been documented previously [61–63] and may, at least partly, explain the disparities in DNA damage between racial groups. In the same cohort, IGF-1 levels were also significantly higher in AA compared to NHW women, which confirms previous reports [64–68]. The cancer connection of IGF-1 is substantial. High serum IGF-1 levels are associated with increased risk for premenopausal breast cancers [69] and individuals with Laron dwarfism (who have a mutation in the growth hormone receptor and very low serum IGF-1) have virtually no (breast) cancers [70]. The effect of IGF-1 on the DNA damage response is likely context-dependent. While most in vitro studies have shown that IGF-1 stimulates DNA repair and promotes cell survival to DNA damaging agents, in vivo studies are scarce and inconsistent [71]. Noticeably, developmental IGF-1 deficiency leads to increased DNA repair capabilities in preclinical models [72, 73]. In cancer cells, the IGF-1 axis stimulates cell proliferation [74]. Yet, in our study, expression of the proliferation marker Ki67 was not different between racial groups, nor did it correlate with IGF-1. The lack of association between IGF-1 and proliferation in normal breast epithelial cells is consistent with our previous analyses of breast acini cultures, where prolonged IGF-1 treatment did not increase Ki67 positiveness [19]. Moreover, Mazumder et al. [56] found no difference in the expression of the Ki67 gene (*MKI67*) in normal breast tissue from Black vs. White women.

We also found lower IGFBP-1 levels in AA compared to NHW women. Although the role of IGFBP-1 in breast cancer is poorly understood (in particular in non-neoplastic contexts), preclinical studies showed that this factor reduces growth and migration of breast cancer cells by antagonizing IGF-1 and modulating a5/b1-integrin signaling [75, 76]. Unlike the pro-survival effects of IGF-1, IGFBPs generally promote apoptosis [77]. Therefore, we speculate that imbalance in IGF-1/IGFBPs may compromise apoptotic responses in breast epithelial cells, enabling survival of cells with DNA damage and with weaker genome maintenance mechanisms.

Other serum factors that significantly differed between AA and NHW donors in our study include the tumor necrosis factor alpha (TNF-α). Little is known on the impact of race on TNF-α. One study found increased TNF-α bioavailability in response to lipopolysaccharides in fetal membranes of African American vs. White women [78]. In breast epithelial cells and other cell types, TNF-α stimulates the synthesis of the proinflammatory adipokine chemerin [79, 80], which was detected at higher levels in AA compared to NHW donors in our analysis. Previous studies have shown that TNF-α triggers DNA damage and genomic instability [81, 82]. Imbalance in this cytokine may therefore influence DNA break levels in the breast epithelium.

This study has several limitations. BMI was used to define comparison groups. Although this proxy for obesity correlated well with metabolic markers, BMI is influenced by body composition which varies between individuals as well as between racial/ethnic groups [83]. In addition, the timing of weight gain may be determinant for genotoxic outcomes in the breast, and this information was not available for the human subjects in this study. Not all serum factors relevant to obesity (and, more generally, metabolic health) were included in our analyses. For instance, sex hormones were not considered for our in vivo analyses. Pinheiro et al. [66] reported higher estrogen levels in African American compared to White women and evidence suggest that elevated estrogens may induce DNA damage in the breast epithelium [41, 84, 85]. Therefore, multiple factors, including IGF, TNF, and sex hormones, may contribute to racial disparities in genome damage/maintenance of breast epithelial cells. This study highlights the need to increase the diversity of pre-clinical models in order to better represent human populations [86].

Supplementary Information

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Supplementary Material 1

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Author contributions

PAV and KLC conceived the study, MG, JH, CL, AQ, IT, HT, AW, VP, CD, and GBC performed experiments; TP wrote computer code for image analysis; GBC obtained, stored, and prepared EFS plasma samples; AP, MD, and AJK procured breast reduction mammoplasty samples; MG, JH, CL, AQ, CD, HT, PFC and PAV analyzed and/or interpreted the data; PAV wrote the first draft of the manuscript. KLC, MG, PFC, and TP edited the manuscript.

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Data availability
The data generated during this study are included in this published article and its supplementary information files. Original microscopy images are available from the corresponding author on reasonable request.

Declarations

Ethics approval
Frozen needle biopsy samples of normal human breast tissues and matching serum samples were obtained from the Susan G. Komen Tissue Bank (KTB) at the IU Simon Cancer Center (IN, USA). Data and biospecimen collection by the KTB has been approved by the Institutional Review Board of Indiana University (Protocol #1011003097). Collection of surgical breast tissue specimens at the IU Health Arnett Hospital (Lafayette, IN) was reviewed and approved by the Institutional Review Board of Purdue University (protocol #1206012467). Collection or breast reduction mammoplasties was approved by the Institutional Review Board (IRB) of the Wake Forest School of Medicine (Protocol #IRB00074778). Animal experimentation was approved by the Animal Care and Use Committee of the Wake Forest School of Medicine (IACUC protocol #A18-136).

Competing interests
The authors declare no competing interests.

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