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The experiments were based on the following interrelated hypotheses: a) Exposure of the mammary epithelium (ME) to chronic oxidative stress (OxS) underlies the high breast cancer (BC) incidence associated with Western industrialized environment/lifestyle and b) that there is a positive correlation between BC incidence within a population and the prevalence within that population of protein adducts of the well-documented marker of chronic, OxS 4-hydroxy-2-nonenal (4HNE) that is a major breakdown product of lipid peroxidation caused by OxS. The PI had available for this research; a) archived forensic breast tissue from women without BC from two populations in SW USA with an over three-fold difference in BC incidence and; b) reduction mammoplasty specimens from women representative of the high BC incidence population served by her institution. A qualitative survey of tissue sections from 247 women from the three populations confirmed the pilot observation of a higher prevalence of 4HNE immunostaining of mammary epithelial (ME) in high BC risk populations and that 4HNE adducts are present in the ME already in many teenagers in that populations. However, the marked heterogeneity in extent, localization and intensity of the 4HNE immunostaining proved an obstacle to achieving the research goals by the proposed, conventional semiquantitative scoring method for assessing immunostaining. Hence, in order to obtain a more objective and quantitative measure of immunostaining, effort was directed towards adapting to the task recently developed cytometric methods, specifically; a) the application of densitometric image analysis to mammary ductal units extracted from digitized images of immunostained tissue sections and; b) multiplex immunoblotting of proteins transferred from tissue sections, a method that allows for assessing levels of multiple epitopes normalized to total protein. These methods promise to provide a relatively high-throughput approach to obtaining quantitative information on levels and localization of both 4HNE and of specific redox-responsive proteins in specific cell populations in tissue sections.

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Introduction

Subject: The research focuses on the question of the mechanisms operating at the level of the mammary epithelium (**ME**) that are responsible for the high breast cancer (**BC**) incidence associated with Western industrialized environment/lifestyle. It is based on evidence implicating a state of chronic oxidative stress (**OxS**) in this high BC incidence, and on the demonstrated potential of many known or suspected environmental risk factors for BC to generate electrophilic reactive oxidizing species (**ROxS**) that, in addition to causing oxidative cellular and DNA damage, can affect cellular homeostatic regulatory mechanisms. Importantly, while mutations resulting from DNA damage are central to the carcinogenic process, for cancer to develop requires additional changes in cellular functions, such as might be caused by ROxS. A well-known consequence of OxS is lipid peroxidation. Hence, electrophilic breakdown products of organic hydroperoxides generated by lipid peroxidation can serve as markers of OxS. *Together, this evidence led to the PI to postulate that there is a correlation between incidence of BC in a population and the prevalence of markers of lipid peroxidation in ME and associated stroma of women without BC who are representative of women of that population.* In order to test this hypothesis 4-hydroxy-2-nonenal (**4HNE**) was chosen as the marker of chronic OxS. This choice was informed by a body of evidence that; **a)** 4HNE is quantitatively a major electrophilic breakdown product of lipid hydroperoxides generated by lipid peroxidation caused by ROxS; **b)** when 4HNE is generated in an amount in excess of that which can be inactivated it forms protein adducts that can be readily identified immunohistochemically and; **c)** 4HNE, by virtue of its electrophilic properties, can propagate OxS and affect multiple signaling pathways important for maintaining cellular homeostasis, cause oxidative DNA damage and is mutagenic.

Purpose and Scope: The principal purpose of the research carried out was to test the following inter-related hypotheses: **a)** Breast parenchyma of women representative of a population with a high BC incidence show more evidence of chronic OxS, as defined by immunostaining for 4HNE, than that of women representative of a population with a significantly lower BC incidence and; **b)** that evidence of chronic OxS in breast parenchyma increases with age, in particular in that of women representative of a population with a high BC incidence. The research was made possible by the availability to the PI of a unique collection of breast tissue obtained at forensic autopsy in the 1970s from two populations of women living in SW USA with an over three-fold difference in BC incidence³.

List of personnel (not salaries) receiving pay from the research effort: The two individuals receiving pay from this research effort were Judith Weisz, the PI, and Debra Schearer, her Senior Research Technician.

Body: Tasks

Tasks 1 and 3: Selection and preparation of tissues blocks for 4HNE immunocytochemistry.

Task 1. Selecting archived blocks for 4HNE immunocytochemistry (ICC) from the forensic tissue collection from populations with different BC incidence. As planned under SOW, the PI traveled to Albuquerque in order to meet with Dr. Susan Bartow and review with her the status of the archived collection of breast tissue that she had donated on her retirement to the University of New Mexico Health Sciences Center (UNM HSC). Together, they met with Dr. Wiggins, the Director of New Mexico Tumor Registry, University of New Mexico Cancer Research and Treatment Center, the institution that first took charge of the forensic breast tissue following Dr. Bartow's retirement. While there, the PI learnt that the collection was on the point of being transferred to the Human Tissue Repository of UNM HSC and would not become available for study until that transfer was completed. The PI then met with pathologist in charge of that collection to familiarize him with the project and to identify a researcher who might serve as a liaison and a collaborator in the project. Dr. Theresa Bocklage was identified as that individual who might fulfill that function. During the PI's visit to UNM HSC it became evident that the Bartow collection of tissue blocks and corresponding hematoxylin and eosin (H & E) stained tissue sections would require considerable reorganization and cataloging before it could be used effectively for research purposes. Clearly, to wait for these arrangements to be completed would have resulted in considerable delay in

tackling this component of the study. We were able to avoid such a delay by Dr. Bartow providing a subset of archived tissue from that collection that was retained by her.

Task 3. Selection of surgical tissue specimens from the PI's institution. Archived blocks of tissue obtained at reduction mammoplasty for simple macromastia from women of different age were retrieved from the PI's collection and from the archives of Dept. Pathology, Penn. State College of Medicine. Tissue sections stained with H & E from each block were reviewed and ones found to be contain breast parenchyma encompassing adequate ductal elements were selected for the study. Sections were reviewed also from the PI's collection of archived blocks of histologically normal breast tissue obtained by her over the years, and those that met the histological criteria, referred to above, were selected for inclusion in the study. While not originally proposed, it was considered of interest to extend the study to normal tissue from BC patients in order to testing the notion that there is a higher prevalence of marker(s) of chronic OxS in women who developed BC than in women without BC in the same population.

Source Age	NORMAL FROM MACROMASTIA (REDUCTION MAMMOPLASTY)			NORMAL FROM CANCER (MASTECTOMY)		
	JW Collection	Pathology Archives	Total	JW Collection	Pathology Archives	Total
<20	22	12	34	0		
20-30	50	11	61	1		
31-40	25	8	33	10		
41-50	15	13	28	28		
51-60	6	4	10	17		
>60	5	1	6	11		
	123	49	172	67		

Table 1. Surgical specimens screened and available for study from the PI's tissue collection (JW Collection) and from the archives of the Dept. Pathology. These archived tissues are from women representative of the population served by the PI's institution that has the high incidence of BC that characterizes the US population. The advantage of tissue specimens in the PI's collection is that fixation was carried out in the PI's research laboratory under better-controlled conditions than in the laboratory of the pathology department that are used for routine diagnostic purposes. In addition, matching flash-frozen tissue, suitable for follow-up studies, is available for the majority of formalin fixed paraffin embedded (FFPE) tissue in the PI's collection. The number of samples from this collection that have been immunostained is listed in Table 2.

Tasks 2 and 4: Immunostaining of tissue sections, analysis of immunostained sections and confirmation by immunoblot analysis.

Immunocytochemistry of 4HNE forensic and surgical specimens. Sections were obtained from the archived tissue blocks selected under tasks 1 and 3 and immunostained for 4HNE. Obtaining sections from the archived forensic tissue proved to be more time consuming than anticipated because of poor fixation of the tissues. This led to loss and fragmentations of some sections and detachment of portions of sections from the slides during immunocytochemistry. Consequently, we were unable to take advantage of all of the tissue blocks provided to us by Dr. Bartow. The number of subjects in each of the experimental groups from whom adequate immunostained tissue sections could be obtained is listed below in Table 2. Because of the marked differences in intensity of Immunostaining among the specimens tissue sections were immunostained for 4HNE using the antibody at three dilutions (1:500, 1:1000 and 1:2000).

Forensic specimens		Surgical Specimens – Normal Parenchyma	
American-Indians	Non-Hispanic Whites	Mammoplasty	Mastectomy
69	82	66	30

Table 2. Number of specimens from the four group of subjects immunostained for 4HNE and available for quantification.

Analysis of immunostained sections. To facilitate analysis of 4HNE immunostaining an Aperio ScanScope System was used to obtain digitized images (virtual slides) of all immunostained tissue sections and of corresponding H & E stained sections. This enables us to view each section in its entirety at high resolution (200-400x), thereby avoiding any bias that might be introduced by having to select areas for study based on viewing sections in small segments under a microscope at an adequate magnification, not to mention the visual fatigue and tedium associated with analyzing hundreds of sections. Importantly, extracted images from the virtual slides can be interfaced with a densitometric image analysis system needed for quantifying immunostaining. While digitizing stained sections and working with digitized images are being increasingly used and enthusiastically embraced by pathologists for diagnosis, quality control and teaching purposes, their advantages for basic research has yet to be recognized and exploited.

When we proceeded to assess the prevalence, localization and intensity of 4HNE immunostaining in tissue sections, whether under the microscope or in the digitized images, it became clear that the generally accepted approach for transforming observation under a microscope to numerical data, as proposed under SOW, would be of little value. This is because of marked differences in the extent of immunostaining of ME, whether it was restricted to a few loci or was more widespread, whether staining was predominantly in the ME, interlobular stroma or both and if the cellular localization was predominantly cytoplasmic or also involved nuclei. In sections from the forensic tissue the problem was compounded by the poor quality of the tissue caused by poor fixation. Under the circumstances, it became evident that obtaining reproducible, more objective data that would allow for comparing the prevalence, intensity and localization of 4HNE in breast parenchyma of each of the four experimental groups, and from individuals of different ages within each group, would require more than the conventional method of experienced breast pathologists assigning semi-quantitative scores to their observations. Therefore, in order to enable us to complete the proposed tasks, we directed our efforts to adapting for the purpose of testing our hypotheses, recently developed slide-based cytometric methods, specifically: **a)** the application of densitometric image analysis to digitized immunostained sections and; **b)** the method of multiplex immunoblotting of proteins transferred from tissue sections.

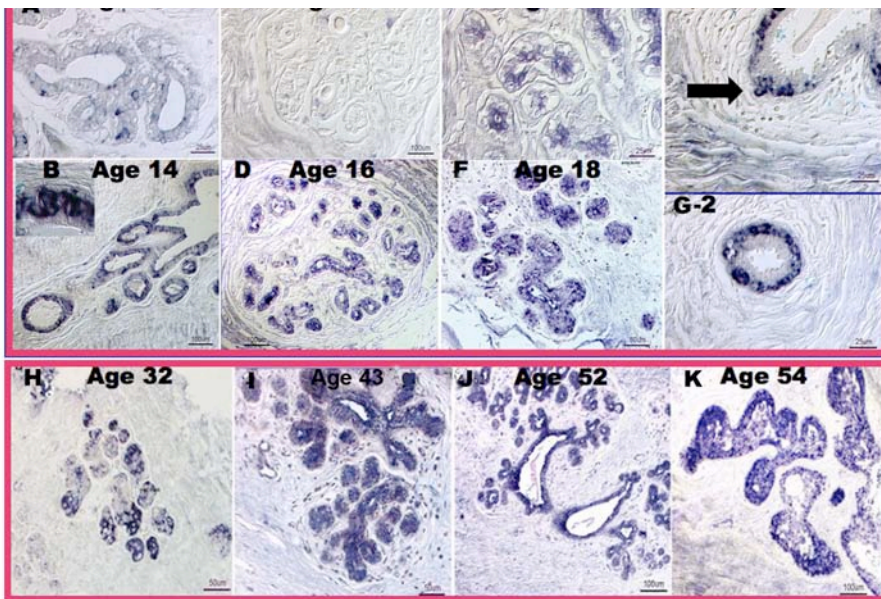
Developing/adapting methodology for quantifying immunostaining. Effort to develop/adapt methodology for quantifying immunostaining was warranted because even a qualitative assessment of the immunostained sections confirmed the prevalence of immunoreactivity for 4HNE in the high BC incidence populations and, importantly, the presence of 4HNE adducts already in teen-agers in those populations (Fig. 1 and Fig. 2, pp 7 and 8). *In fact, there was significant 4HNE immunostaining of ME cells in breast parenchyma of over half the 24 subjects between the ages of 14-20 in the population served by the PI's institution that were included in the study.* The finding is reminiscent of the finding evidence of atherosclerosis at post-mortem in very young US soldiers who died in the Korean war⁶. It was this finding that led to the realization of the need to learn about the early, silent stages in the evolution of atherosclerosis, and that led to the recognition of the need for early intervention if the progression of the disease to its potentially deadly ultimate manifestations was to be prevented.

Application of densitometric image analysis to virtual slides. From available options, we chose the recently introduced CellProfiler software developed by researchers at MIT. It is built on experience and lessons learnt from use of systems, such as NIH Image, introduced decades ago. We are finding CellProfiler to be more flexible than commercially available software and to be much more user-friendly. By using this software, we are now able to obtain histograms of staining intensity in ME in extracted images of mammary ductules and aggregates of ductules, such as in terminal ductal units (TDLU) and in the interlobular stroma (Figure 3, page 9).

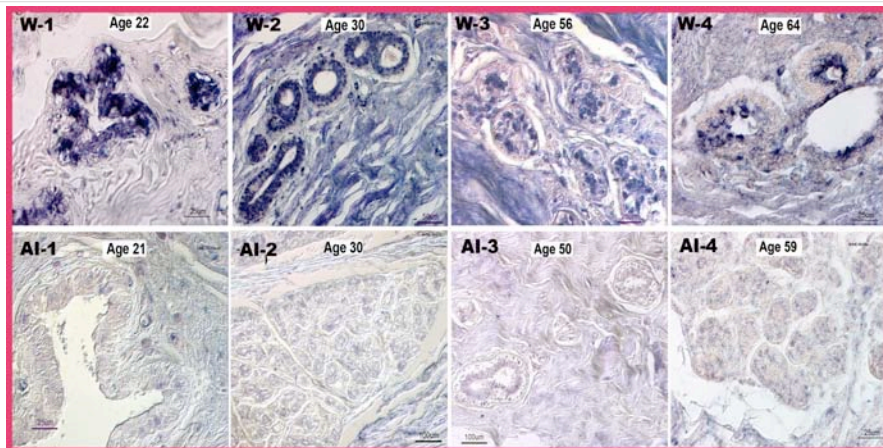
Multiplex immunoblotting of proteins transferred from sections from paraffin embedded tissue to multiple specialized membranes: An alternative method for quantifying specific antigens in tissue sections is offered by the recently introduced method of multiplex tissue immunoblotting. The method involves transfer of proteins from sections cut from FFPE tissues to a stack of up to ten specialized membranes. The membranes can then be probed individually with different antibodies for detection of specific epi-

topes and scanned by a microarray scanner. Importantly, total protein stained on each membrane provides a basis for normalizing immunostaining with each of the antibodies used (Fig. 4 page 10). Work is in progress to establish morphometric parameters to sample TDLU immunostaining in sections from different individuals in a consistent manner and to applying statistical methods for identifying significant differences in the extent and distribution of immunostaining within and between populations. An important attribute of the method is that the scanned images of immunostained membranes can be used to determine co-localization of different epitopes. We are adding this methodology to our armamentarium of cytochemical techniques with the help of Dr. Stephen Hewitt, who heads the team at NIH that developed this technique^{7, 8}. The technique will enable us to examine the tissue sections for additional markers of OxS, such as protein carbonyls and 3-nitrotyrosine (**3-NT**), as well as proteins with known functions relevant to carcinogenesis that possess nucleophilic centers and can be subject to regulation by electrophiles, including 4HNE. The importance of additional markers of oxidative stress is that they reflect some of the hierarchical nature of oxidative modification: In terms of its causative agents and its targets, 4-HNE primarily modifies cysteines, histidines and lysines, while 3-nitrotyrosine is a marker of nitrosative stress that typically occurs through the interaction of reactive oxidizing species with nitric oxide. This is in contrast to carbonylation that is rather promiscuous and fairly non-specific.

Figure 1. Photomicrographs of 4HNE immunostained sections of breast parenchyma from three populations of women without BC. Upper two panels (A-K), tissue from surgical specimens from women ranging in age from 14 to 54 living in central PA, a high BC incidence population (upper two panels). Lower pane (W-1 to W-4 and AI-1 to AI-4), tissue collected at forensic autopsy from two populations living in SW US that differed >3 fold in BC inci-



Upper two panels. 4HNE immunostained sections of breast parenchyma from surgical specimens obtained at reduction mammoplasty from women living in central PA, a high BC incidence population. The indication for reduction mammoplasty, simple macromastia, has not been found to be a risk factor for BC and incidence of occult cancer in mammoplasty specimens was reported to be similar to that in population at large¹. **A-G**, upper two rows, age 14-19; **H-K**, lower row, age 32-54. In **G-1**, arrow points to immunostaining at terminal end bud, proposed site of vulnerability to BC initiation²



Lower panel: 4HNE immunostained sections of from breast tissue obtained at forensic autopsy from two populations of women without BC living in SW USA with >3.5 fold difference in age adjusted BC incidence; **W-1 to W-4**. Non-Hispanic Caucasian (BC incidence, 89/100,00) **AI-1 to AI-4**. American Indian (BC incidence, 24.9/100,000³⁻⁵).

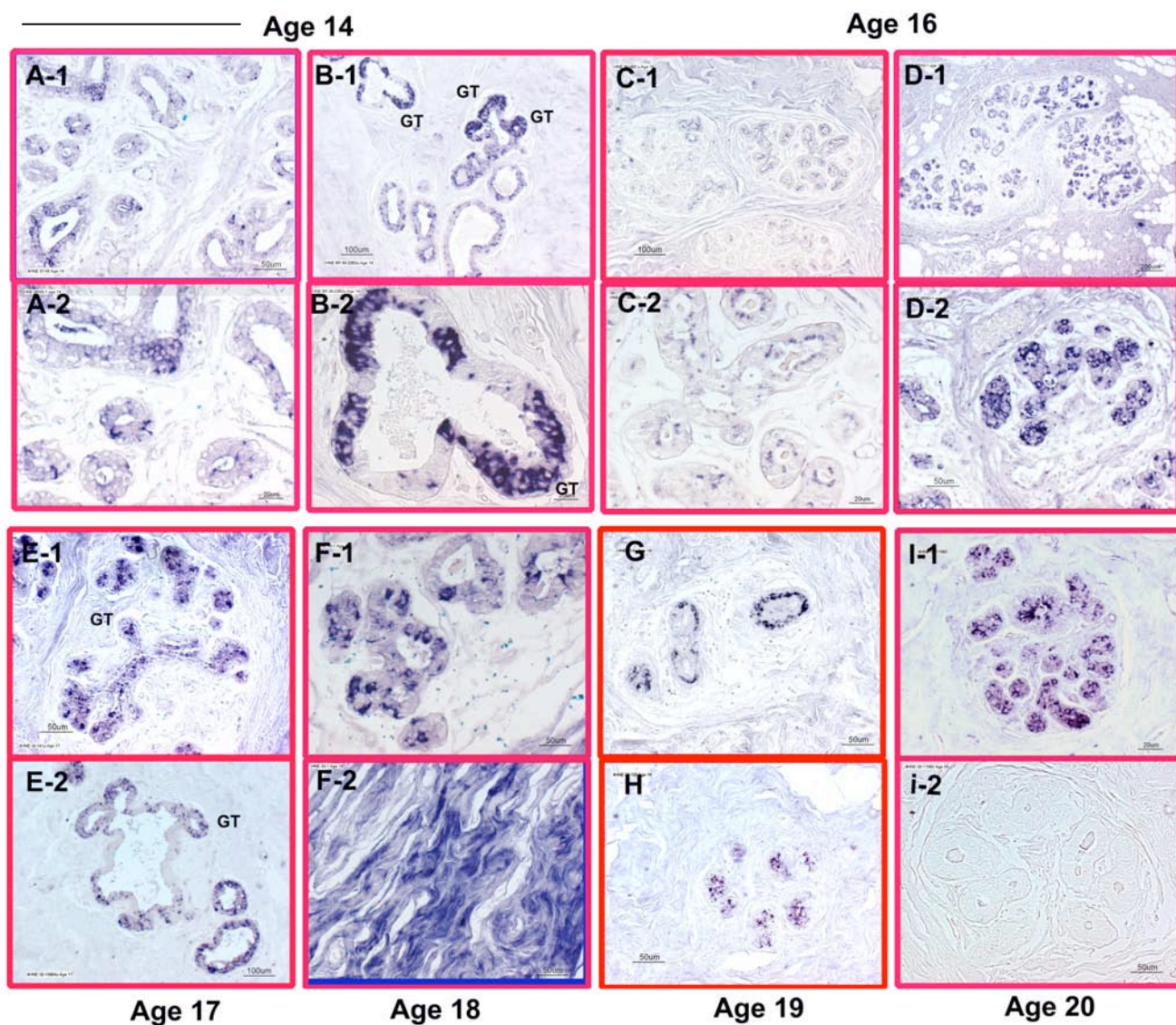


Figure 2. Photomicrographs of 4HNE immunostained tissue sections of breast parenchyma from mammoplasty specimens from nine donors, ranging in age from 14-20, from the high breast cancer-incidence population served by the PI's institution. Tissue sections (5 µM) were immunostained with a monoclonal antibody developed against oxidized low-density lipoproteins using Vector Purple™ as reporter. Sections were not counterstained. A-1 through F-2 from six subjects aged 14-18. Lower panel shows a portion photographed at higher power than that shown in the upper panel. G and H, micrographs from two 19 yr old subjects. I-1 shows photomicrograph from a 20 yr old subject, and I-2 a secondary antibody only control from an adjacent section. In micrographs of sections from six of the nine subjects there were patches of strongly 4HNE immunopositive ME cells. In three of the subjects (C, G and H) immunostaining is minimal and largely limited to isolated cells. F-2 shows strongly positive immunopositive collagen in breast parenchyma of an 18 yr old subject. In all other specimens, stromal/collagen immunostaining was minimal or absent. This is in contrast to strongly immunopositive collagen seen in many older subjects. B-1, B-2 and E-2 shows 4HNE immunopositive cells concentrated in what appear to be growing tips of mammary ducts (marked GT). Oxidative damage to cells in this locus could be especially relevant to carcinogenesis because of evidence that that this is the site of greatest vulnerability to carcinogens, where cells are actively dividing, where myoepithelial basement membrane layer is deficient and where long-lived (label retaining), presumptive stem cells are concentrated².

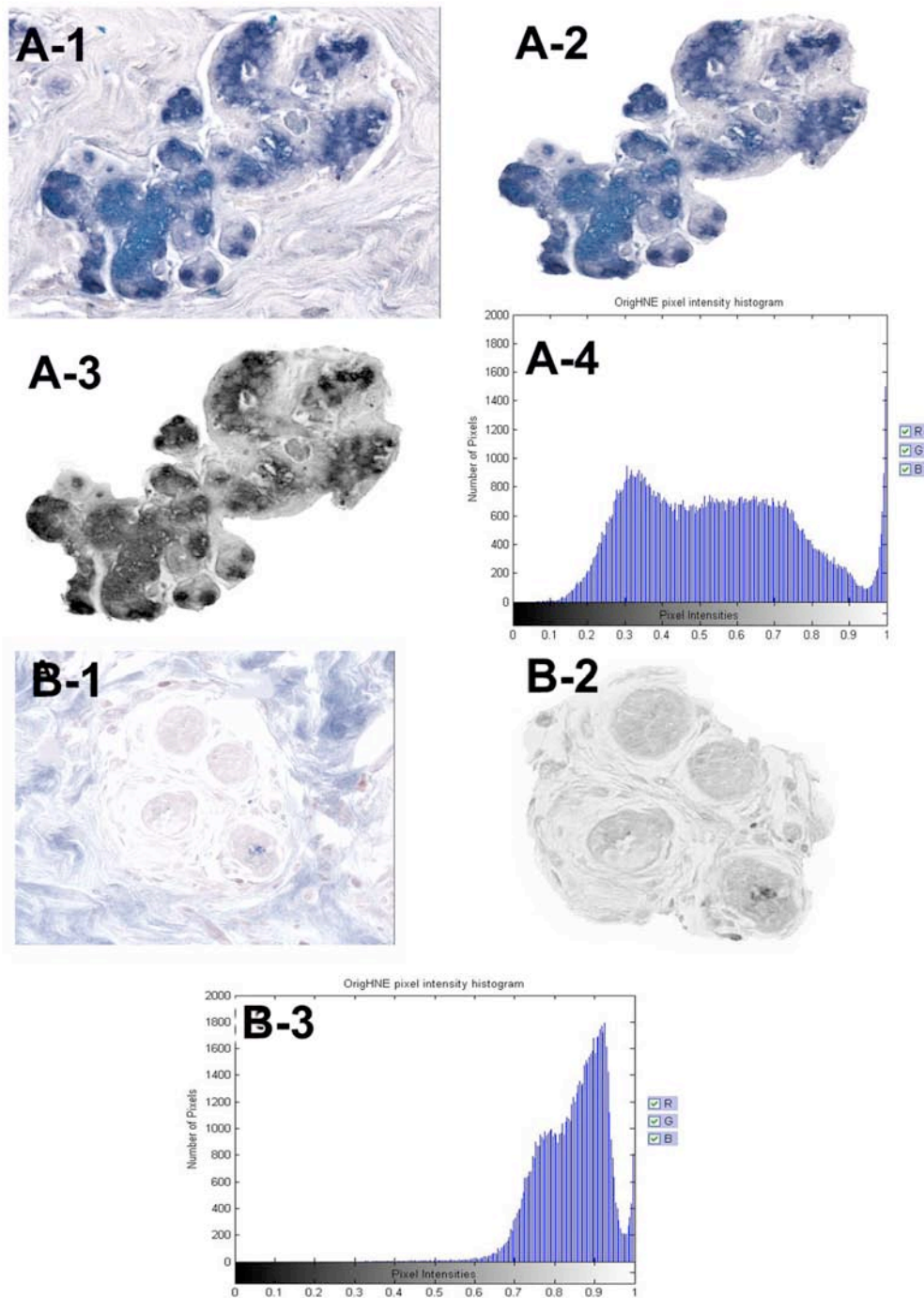


Figure 3, Application of densitometric image analysis to virtual (digitized) virtual images to quantify 4HNE immunostaining in tissue sections. A-1 through A-4, sequence of steps leading to generation of histogram (A-4) of immunostaining intensity in a strongly 4HNE immunopositive terminal lobular ductal unit (TDLU). A-1, image of TDLU with surrounding stroma transferred to from ScanScope generated virtual slide to Photoshop. A-2 through A-3, image of TDLU extracted from surrounding stroma and transformed to gray-scale in Photoshop. A-4, Histogram of pixel intensity generated by CellProfiler. B, corresponding images for a TDLU with minimal 4HNE immunostaining: B-1 colored image transferred from virtual slide to Photoshop. B-2. Gray scale image of extracted TDLU. B-3, histogram of pixel intensities generated in CellProfiler.

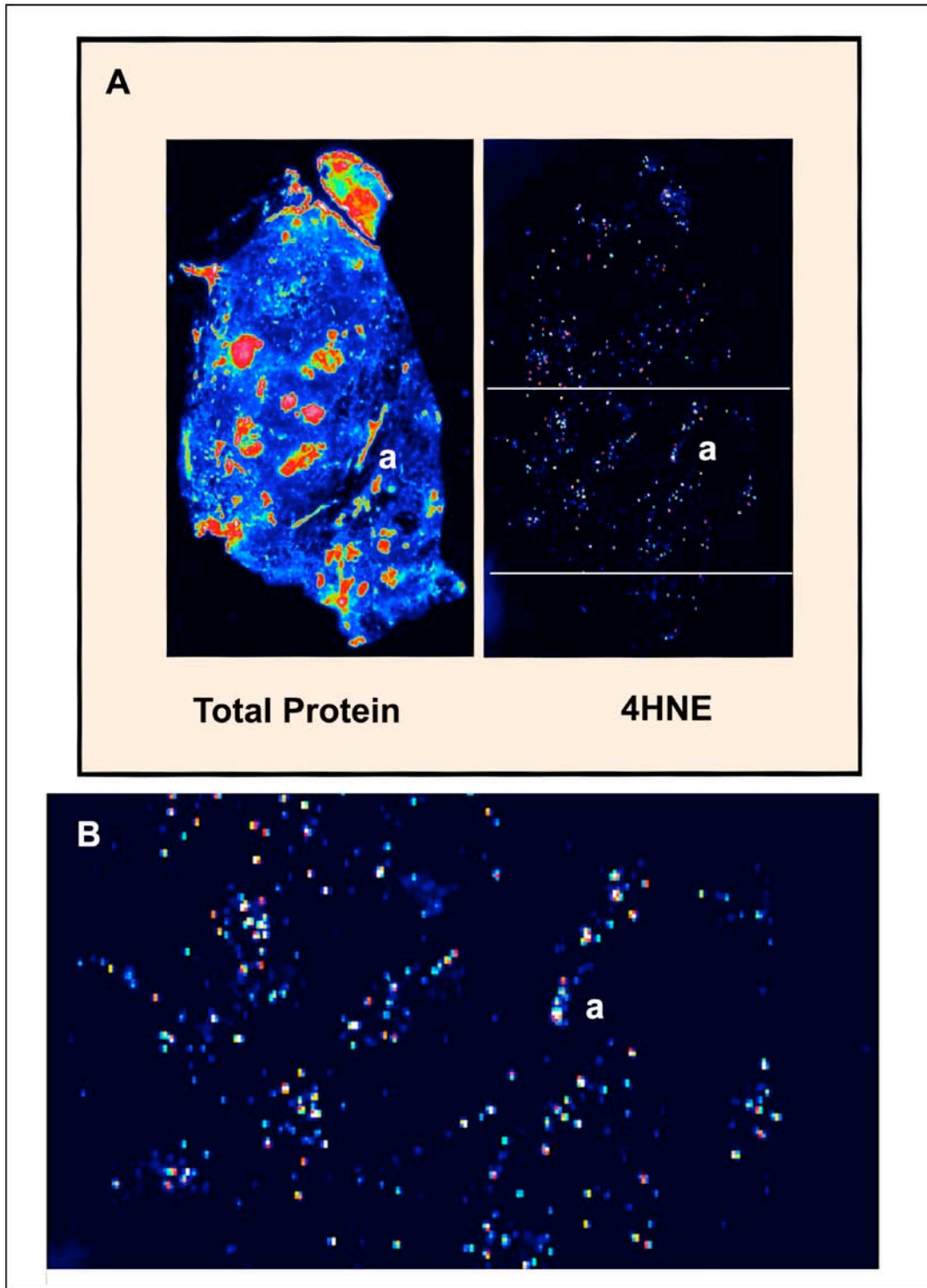


Figure 4. Application of multiplex immunoblotting to localization and quantifying 4HNE protein in tissue sections. Scans of one of ten membranes to which protein was transferred from a tissue section (5 μ m) immunostained for 4HNE and for total protein according to the protocol of Chung et al.,^{7,9}. Streptavidin linked Cy5 was used as reporter for total protein and FICT conjugated secondary antibody as reporter for 4HNE. Membranes were imaged with a microarray scanner. Signal intensities obtained are represented by pseudocolors, white>red>yellow>green>blue>black. **A** scans of the membrane immunostained for total protein (left panel) and for 4HNE (right panel), **B**. shows portion of the image between white lines in A at higher magnification. Fluorescence attributable to 4HNE is associated with mammary ductal elements. Marking "a" next to a duct leading to a TDLU provides a point of reference.

Functional consequences of a state of chronic oxidative stress. In the SOW the PI proposed to examine whether there was a correlation between 4HNE immunostaining and that of inflammatory cytokines. This proposition was based on evidence linking OxS with inflammatory responses and evidence suggesting a cancer chemopreventive role for anti-inflammatory agents, such as inhibitors of cyclooxygenase-2. Since submitting the proposal, there have been new technical developments in the field of proteomics that allow for assessing potential functional consequences of OxS by identifying proteins that are modified by electrophiles. These include methods that makes it possible to compare the profile of reduced and reversibly oxidized proteins in small tissue samples using 2-D differential gel electrophoresis (DIGE) and CyDyes as reporters¹⁰. The relevance of these methods to the topic addressed by this research is underscored by mounting evidence for electrophiles' role in physiological regulation and in pathophysiology by causing post-translational modification of proteins involved in diverse functions including ones relevant to carcinogenesis^{11, 12}. Importantly, there is a substantial literature on the potential of 4HNE to affect the functioning of proteins associated with the inflammatory response and ones linked to carcinogenesis^{13, 14}. Therefore, instead of embarking on characterizing cytokine profiles of breast tissue from women without BC in our high-risk population, we have enlisted the help of researchers with proteomic expertise to adapt the method of Bety et al. to the fundamental problem addressed in our research, that is, the functional consequences of chronic OxS in the high BC risk associated with Western industrialized environment/lifestyle. Using DIGE technology will enable us to correlate levels of 4HNE with profile of reversibly and irreversibly oxidized proteins.

Preliminary data obtained through this collaboration and by the use of the cytochemical methods outlined above, provided the basis for a Synergistic Idea Grant application we recently submitted to the DOD. The application received a score of 1.6 that, regrettably, was still outside the funding range. However, we will be able to pursue this line of investigation with some funds awarded the PI for this purpose by the Pennsylvania State Department of Health (starting date, July 1, 2008).

Problems Encountered:

- *Access to the forensic tissue collection* proved less user-friendly than anticipated. To make it useful for research purposes will require cataloging and organizing it. This became evident when a student working with the PI spent a month at the UNM HSC in Albuquerque attempting to identify additional blocks to be included in the study. The number of tissue samples provided Dr. Bartow should, however, should be sufficient to identify differences in immunostaining between the non-Hispanic White and American Indian donors. Fixation artifacts may still prove an obstacle to obtaining robust quantitative data on levels of 4HNE immunostaining in these forensic tissue specimens, even with the more sophisticated cytometric methods.
- *Inadequacy of the method originally proposed for quantifying immunostaining* and the steps taken to overcome this problem are detailed in the body of the report.

Key Research Accomplishments.

- The first essential step in achieving the goal of the research has been accomplished. Preparation of tissue sections of histologically characterized (normal) breast tissue immunostained for 4HNE from three populations of women without BC from populations with different known BC incidence and from a group of women with BC has been completed. We have now on hand a comprehensive set of immunostained slides ready for analysis once the cytometric methods outlined in body of the application are fully standardized.
- A systematic effort to adapt and standardize methodology that will make it possible to assess differences in 4HNE immunostaining among the experimental groups and the effect of age on this marker of chronic OxS has been initiated. This essential step towards achieving the goal of the research could not be completed within the time frame and funds provided by the Concept Award. It has, however, made it possible to obtain pilot data for seeking and obtaining additional funds to pursue the goal of determining if adducts of 4HNE, a recognized marker of chronic OxS is a marker of BC risk. As indicated above, some funding to support this initiative has already been obtained and additional funds are being sought.

Reportable Outcome

The study provides evidence supporting the proposition that the ME of women, including young women under the age of 20, living in our high BC risk environment is subject to chronic OxS. Whether there is a correlation between presence of 4HNE adducts, a surrogate marker of chronic OxS, and BC incidence will have to await application of methods for obtaining quantitative data on the levels of immunoreactive 4HNE. Findings from this research will be presented at this year's Era of Hope meeting

Conclusions

The data, although only descriptive, provide evidence supporting the hypothesis on which this proposal is based. They provide evidence of the high prevalence of adducts of 4HNE in mammary epithelium of women living in or high BC risk environment and, importantly, for the presence of this well-documented marker of chronic OxS mammary epithelium of many girls as young as 14. They also provide evidence of the potential of novel cytometric methods to provide more robust, quantitative data of the type needed to assess the effects of environmental factors at the molecular level on human breast parenchyma. Such methods will make possible take full advantage of the unique contribution that cytochemical characterization of tissues can offer and to make better use of the vast collection of archived tissue from subjects with known outcomes. Together, our findings provide the rationale and lay the foundation for studies, such as those outlined above, and the combined use of technical advances in cytometry and proteomics, to begin to assess the functional consequences of chronic OxS and their role in breast carcinogenesis.

Recommended changes or future work to better address the research topic. The research topic addresses a critical problem in the BC field, the lack of progress in the area of BC prevention. Acquisition of a malignant genotype/phenotype by mammary epithelial cells requires the accumulation of mutations and/or aberrant expression of many of the diverse genes controlling cell proliferation, differentiation and death, as well as their relationship to neighboring cells and structures. Therefore, some changes in mammary epithelial cells and associated stroma relevant to the process of carcinogenesis can be expected to be present in a significant percentage of "normal" individuals in a country such as the US in which one on eight can be expected to develop BC in her lifetime. Hence, the need to challenge the tacit acceptance as "normal", breast tissue obtained from women without BC living in an environment associated with such a high BC incidence. Instead, we should use tissue that we now call "normal controls", as an opportunity to identify changes relevant to carcinogenesis. To develop effective, stage-specific interventions for prevention will require tracking progression towards cancer within the breast tissue itself during the long latent period that precedes the appearance of sporadic BC. To accomplish this will require:

- Establishing repositories of breast tissue from women of different ages without BC who live in an high BC risk environment, such as the USA. Currently, tissue banks focus on collecting neoplastic tissue, tissue with histologically identifiable pre-neoplastic changes and histologically normal tissue from women who have already developed BC.
- Establishing repositories of breast tissue from women of different ages without BC living in an environment with significantly lower incidence of BC, and to do so before "Westernization" causes that incidence to rise. In this regard, it is worth noting that in SW USA the incidence of BC in American Indian women is still significantly lower than that of non-Hispanic Whites living in the same area. This is in contrast to NW USA where the incidence of BC in the American Indian population is reported to have risen and now approaches and even exceeds that of the US population^{15, 16}.
- Ensuring that such tissues are collected and stored in a manner that makes it possible to use them for cytochemical as well as molecular biological studies.
- Further encourage the development of methods designed specifically for studies of archived tissue at the cytological, cytometric and molecular biological level.

Finally, if a new concept was found intriguing/promising enough to invest in why not offer additional year(s) funding if the review of the findings after one year indicate that the research is on the right track. One year is just not enough to lay chart a course for a new direction, obtain enough preliminary data to apply to more conventional sources of funds.

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