# The Role of Glucocorticoid Receptor in the Racial/Ethnic Disparity of Aggressive Breast Cancer

BY

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Thesis

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## DEDICATION

This thesis is dedicated to my daughter Ines, the sunshine of my life and my greatest achievement. I am eternally grateful to my dear husband, Edouard, and my parents Abdallah and Waliya.

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I would like to thank my thesis committee for their support and patience. It is impossible to do any work without the invaluable help of colleagues and collaborators. I performed the genetic study with the help of Ken Bati and Ebony Shah from Rick Kittle laboratory. All the tissue microarray and pathology work was done by Dr. Abeer Mohamud in collaboration with Peter Gann. I would like to acknowledge the support I received from colleagues, Abigail Silva, Judith Garber, Caryn Peterson, and Joelle Hallack. Finally, Faith Davis, Melinda Stolley and Marian Fitzgibbon from R25 Cancer Education and Career Development Program. Also, the numerous people who helped me through this journey.

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## LIST OF ABBREVIATIONS

**A:** Adenine

**ADIPOQ:** Adiponectin

**AJCC**: American Joint Committee on Cancer

**AIMs**: Ancestry Informative Markers

**ANOVA:** Analysis of Variance

**ASCO/CAP**: American Society of Clinical Oncology/College of American Pathologists

**BCCC**: Breast Cancer Care in Chicago

**Bcl-2**: B-cell lymphoma 2

**BMI:** Body Mass Index

**C**: Cytosine

**CAT**: Catalase

**CC1**: Cell Conditioning Solution 1

**CDC:** Centers for Disease Control and Prevention

**CI**: Confidence Interval

**CK5/6**: Cytokeratin 5/6

**DAB**: 3,3'-diaminobenzidine

**DBD**: DNA Binding Domain

**dNTP:** Deoxynucleotide

**ER**: Estrogen Receptor

**FFPE**: Formalin-Fixed Paraffin-Embedded

**FKBP5**: FK506 Binding Protein 5

**G**: Guanine

## LIST OF ABBREVIATIONS (continued)

**GCR**: Glucocorticoid Receptor

**GRE**: Glucocorticoid Response Element

**LBD**: Ligand Binding Domain

**LEPR**: Leptin Receptor

**HER2:** Human Epidermal Growth Factor Receptor 2

**$H_2O$**: Hydrogen Dioxide

**HIER**: Heat-Induced Epitope Retrieval

**HPA axis**: Hypothalamus-Pituitary-Adrenal Axis

**HCF**: Histology Core Facility

**Hsp**: Heat Shock Protein

**HWE**: Hardy-Weinberg Equilibrium

**IHC**: Immunohistochemical Staining

**IL-6:** Interleukin-6

**MA**: Minor Allele

**MAF**: Minor Allele Frequency

**MALDI-TOF**: Matrix-Assisted Laser Desorption/Ionization

**MC**: Medical Center at Chicago Clinical Reference Surgical Pathology laboratory

**MnSOD**: Manganese Superoxide Dismutase

**mRNA:** Messenger Ribonucleic Acid

**nH Whites**: Non-Hispanic Whites

**nH Blacks**: Non-Hispanic Blacks

**NR3C1**: Nuclear Receptor Subfamily 3, Group C , Member 1

## LIST OF ABBREVIATIONS (continued)

**OR**: Odds Ratio

**PCR**: Polymerase Chain reaction

**PR**: Progesterone Receptor

**SD**: Standard Deviation

**SES:** Socioeconomic Status

**SGK1**: Serum and Glucocorticoid-Regulated Kinase 1

**SNP**: single nucleotide polymorphism

**T:** Thymine

**TMA**: Tissue Microarray Array

**TSS:** Transcriptional Start Site

**UIC**: University of Illinois at Chicago

## SUMMARY

Breast cancer is the most common malignancy and the second leading cause of cancer-related death in women worldwide. There is a well-documented variation in breast cancer incidence and mortality across nations and among racial/ethnic groups within these nations. In the United States, the incidence of breast cancer is lower among African American and Hispanic women when compared with White women, yet, as a group African American and Hispanic women have a more aggressive disease at diagnosis and worse survival outcomes. The reasons for racial disparity in breast cancer mortality are largely unknown but likely multifactorial involving environmental and biological factors.

A number of epidemiological studies have shown that the cellular alterations resulting from chronic psychosocial stress may increase breast cancer development and progression. One of the primary mediators of stress is glucocorticoid. Glucocorticoid is a steroid hormone with a physiological and pathological role in the body; it acts via its cytoplasmic receptor, the glucocorticoid receptor (GCR). Upon binding to glucocorticoid, GCR is activated and released from a chaperone complex. Activated GCR travels to the nucleus to regulate a myriad of physiological processes such as mammary development and differentiation, inflammation, apoptosis as well as glucose and fatty acid metabolism-processes that have been associated with breast cancer development and progression. The main hypothesis is that alterations in the level or localization of GCR might interfere with the glucocorticoid response, resulting in aberrant downstream cellular responses such as decreased apoptosis and chronic inflammation that might contribute to aggressive breast cancer. And if these characteristics vary by race/ethnicity then this may play a role in the pathogenesis of the racial/ethnic disparity of breast cancer.

The overarching theme of this research is to understand the role of GCR in breast cancer and its potential involvement in racial/ethnic disparities. To answer our research question, we used data from the Breast Cancer Care in Chicago (BCCC), a large, multiethnic population of incident breast cancer cases between the ages of 30 and 79 with stored biological samples and linked clinical, genetic ancestry and sociodemographic data. Our specific aims are as follows:

**Specific Aim 1**: Candidate single nucleotide polymorphism of GCR and GCR-related genes associations with breast cancer in BCCC cohort.

We examined the association between breast cancer characteristics and genetic variations of GCR and GCR-related genes, including candidate genes that regulate GCR function or are involved in the GCR response. We examined if those associations vary by race/ethnicity. A potential explanation for racial/ethnic differences in breast cancer aggressiveness is the differences in inherited genetic risks in candidate genes in the glucocorticoid-response pathway such as GCR signaling, inflammation, oxidative stress and apoptosis. We have genomic DNA from 656 cases (67% of the total cohort) with linked clinical, genetic ancestry and sociodemographic data to assess variance in our candidate genes. Common genetic variants were selected a priori based on a minor allele frequency greater than 5% and previous association with GCR function or downstream responses. We hypothesized that genetic variations in the candidate genes are associated with breast cancer and these genetic variations might be overrepresented in non-White cases.

**Specific Aim 2**: A tissue-microarray based immunohistochemical (IHC) analysis to quantify the level and subcellular localization of the GCR in the invasive component of breast tumors.

We performed a tissue-microarray based IHC analysis to quantify the level and subcellular localization of GCR in the invasive component of breast tumor. We examined if the level and subcellular localization of GCR was associated with markers of aggressive breast cancer and whether the level and subcellular localization of GCR varied by race/ethnicity. Our hypothesis is that the GCR-mediated response is associated with aggressive breast cancer and this dysregulation could be a result of aberrant GCR subcellular localization. We have formalin-fixed paraffin-embedded (FFPE) breast tissue from a subset of the BCCC cohort with linked clinical, genetic ancestry, sociodemographic, and questionnaire data, which were used to construct tissue-microarrays and perform the IHC study. We hypothesized that the level and location of GCR will be associated with breast cancer characteristics.

**Specific Aim 3:** A tissue-microarray based IHC analysis to quantify the level of GCR-targets serum/glucocorticoid regulated kinase 1 (SGK1) and B-cell lymphoma 2 **(**Bcl-2) in the invasive component of breast tumors.

We performed a tissue-microarray-based IHC analysis to quantify the level of two apoptotic markers SGK1 and Bcl-2 in the invasive component of breast tumor. We used the tissue microarray constructed for specific aim2 to stain for SGK1 and Bcl-2. We hypothesized that the level of these two markers would be associated with GCR and breast cancer characteristics.

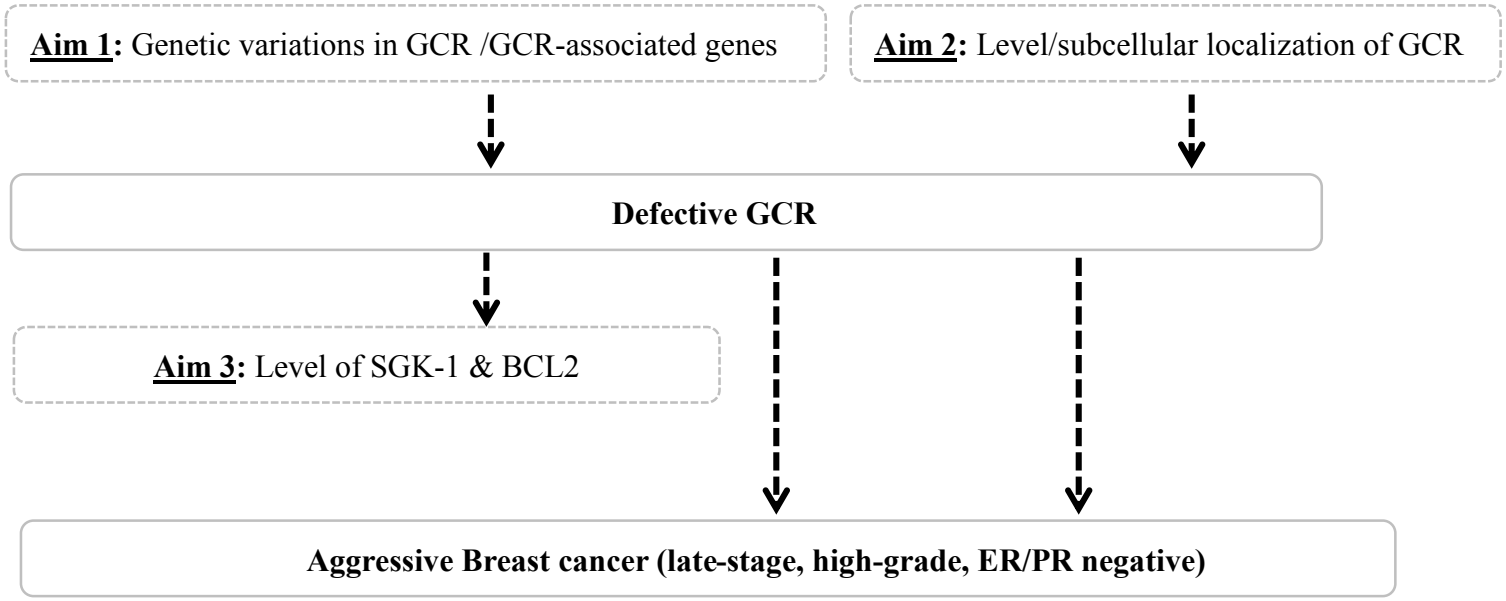



Figure 1: Hypothesized relationship between GCR and breast cancer and specific aims.

# I. SPECIFIC AIM 1

**Background:** Psychosocial stress has been hypothesized to affect breast cancer progression and survival. The glucocorticoid receptor (GCR) gene is one of the main mediators of the hypothalamus-pituitary-adrenal axis (HPA) response to stress. Glucocorticoid receptor-mediated signaling may play a role in breast cancer development and progression. The purpose of this study was to examine the association between breast cancer and genetic variations of GCR and GCR-related genes, including candidate genes that regulate GCR function or are involved in the GCR response. **Methods:** We used a sample of breast cancer patients from an urban, multiracial cohort to assess the association between breast cancer characteristics and genetic variants of functionally important single nucleotide polymorphisms (SNPs) in GCR (30SNPs), FK506 binding protein 5 (FKBP5 ,17 SNPs), serum and glucocorticoid-regulated kinase 1 (SGK1, rs9493857), Interleukin 6 (IL-6 ,4 SNPs), Adiponectin (ADIPOQ, 2 SNPs), Leptin receptor (LEPR, 2 SNPs), manganese superoxide dismutase (MnSOD rs4880), Catalase (CAT rs100179), and B-cell lymphoma 2 (BcL-2 rs2279115). Genomic DNA was isolated from peripheral blood and genotyped with Sequenom's Mass ARRAY MALDI-TOF iPlex platform. Odds ratio (OR) with 95% confidence intervals (CI) were estimated by logistic regression models. Genetic ancestry was used in the regression model to adjust for population stratification. **Results:** Several SNPs in GCR, FKBP5, ADIPOQ, IL-6, and BcL-2 were associated with breast cancer characteristics, but statistical significance was lost when the p values were adjusted for multiple measures. **Conclusion:** We observed associations between specific SNPs in the GCR gene with breast cancer stage, grade, and ER/PR receptor status, before adjustment for multiple comparisons.

## A. Candidate Single Nucleotide Polymorphism of the Glucocorticoid Receptor

Breast cancer is the most common malignancy and the second leading cause of cancer-related death in women worldwide. It is estimated that 232,670 women will be diagnosed with and 40,000 women will die of cancer of the breast in 2014 (http://www.breastcancer.org). There is a well-documented variation in breast cancer incidence and mortality across nations and among racial/ethnic groups within these nations. The incidence of breast cancer is lower among African American women when compared with White women, yet deaths among African Americans are higher (Whitman et al., 2011).

African American women are more likely to present at an earlier age, with advanced stage disease and more aggressive tumor types such as hormone-receptor-negative and higher grade (Carey et al., 2006; Barcenas et al., 2010; Cunningham et al., 2010; O'Brien et al., 2010). Socioeconomic factors cannot fully explain these variations in disease incidence and outcome. African American race has been associated with decreased overall and breast cancer-specific survival even after adjustment for prognostic factors and socioeconomic status (Newman et al., 2006; Albain et al., 2009). Similar epidemiological and molecular characteristics of breast cancer were observed in other populations of African descent in the Caribbean and the United Kingdom (Wild et al., 2006; Bowen et al., 2008).

Psychosocial stress has been hypothesized to play a role in the etiology of breast cancer and prior prospective and retrospective epidemiological studies have been carried out to examine this relationship with conflicting findings (Roberts et al., 1996; Lillberg et al., 2001; Helgesson et al., 2003; Schernhammer et al., 2004; Nielsen et al., 2005; Michael et al., 2009; Surtees et al., 2010).

Although further downstream signals converting psychosocial stress into cellular dysregulation and finally into breast cancer is still unknown, several animal and in vitro studies have implicated glucocorticoid in this process (Strange et al., 2000; Stark et al., 2002; Antonova and Mueller, 2008; Hermes and McClintock, 2008; Sloan et al., 2010; Pan et al., 2011).

Glucocorticoid production follows a circadian rhythm in the body by peaking in the early morning and then declining throughout the remainder of the day (DeSantis et al., 2007). Differences in the glucocorticoid diurnal rhythm across racial/ethnic groups were observed. African Americans and Hispanics exhibit a lower waking cortisol level and a flatter diurnal cortisol rhythm, which has been linked to chronic stress and perceived discrimination (Cohen et al., 2006; DeSantis et al., 2007; Fuller-Rowell et al., 2012).

Glucocorticoid signaling is mediated through the functional isoform, glucocorticoid receptor alpha (GCR$\alpha$). The human gene coding for the GCR or the Nuclear Receptor Subfamily 3, Group C, Member 1 (NR3C1) and is located on chromosome 5q31–q32. The GCR is expressed in almost all human tissues including immune, epithelial, and mesenchymal cells in cell-specific manner (Reichardt et al., 2001; Vaidya et al., 2010). It has nine promoters each associated with an alternative transcription start site (TSS), seven of which are found in an upstream CpG island (Turner et al., 2008). These alternative promoters regulate tissue-specific expression (Cao-Lei et al., 2011). The GCR has 9 exons: exon 2 codes for the N-terminal transactivation domain of the protein, exons 3 and 4 for the DNA binding domain, and exons 5-9 for the C-terminal ligand binding domain.

The GCR reside predominantly in the cell cytoplasm in an inactive form in association with a chaperone complex, consisting of several heat-shock proteins (Hsp90/Hsp70) and FK506-binding proteins (FKBP5) that serve to stabilize the unbound receptor (Pariante and Miller,

2001). Upon binding to glucocorticoid, the complex dissociates allowing GCR to be phosphorylated and translocated to the nucleus where it binds to glucocorticoid response elements (GRE) in target genes and regulates gene transcription in a cell-specific manner (Wiench et al., 2011) (Figure 2).

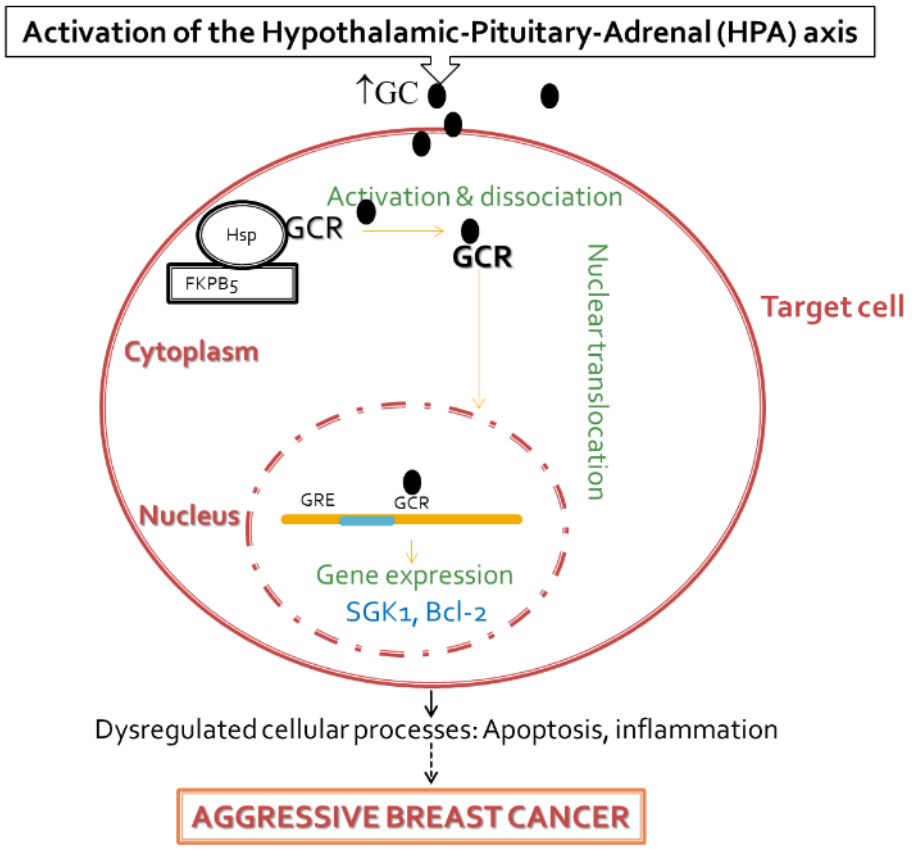



Figure 2. Overview of the suggested pathways connecting chronic stress and breast cancer involving the glucocorticoid receptor.

The GCR activity is modulated by its level, subcellular localization. There are more than 2,000 SNPs identified in the human GCR gene (http://www.ncbi.nlm.nih.gov). Many of these

SNPs have been associated with decreased or increased GCR-mediated response have been associated with altered susceptibility to sporadic breast cancer among Caucasian women with a highly polymorphic dinucleotide repeat, D5S207, located within 200 kb of the GCR (Curran et al., 2001), metabolic disorder (Rosmond, 2002), cardiovascular disease (Walker, 2007), rheumatoid arthritis (Donn et al., 2007), and depression (Spijker and van Rossum, 2009). This variability could also explain differential individual or racial/ethnic sensitivity to the effects of glucocorticoid.

We hypothesize that dysregulation of GCR-mediated response may lead to alteration in several cellular processes such as the immune response and apoptosis leading to tumor development and progression. We used data from the BCCC, a large, multiethnic population of incident breast cancer cases with stored biological samples and linked clinical, genetic ancestry and sociodemographic data to examine the association between breast cancer and genetic variations of GCR and GCR-related genes, including candidate genes that regulate GCR function or are involved in the GCR response.

## B. Materials and Methods

### 1. The Breast Cancer Care in Chicago study

Patients and samples for this study are from the BCCC study, a population-based cross-sectional study of breast cancer cases with primary invasive breast cancer diagnosed between October 1, 2005 and February 29, 2008 in the Chicago area. This study was conducted by the University of Illinois at Chicago (UIC) Center for Population Health and Health Disparities (NCI grant5 P50 CA 106743) and approved by the UIC Institutional Review Board (IRB#2010-0519). Details of this study have been previously published (Rauscher et al., 2010).

## 2. Clinicopathological data and biological samples

Of the patients who were interviewed, 86% (N=849) consented to a review of their medical record. As a result of medical record abstraction, we have stage at diagnosis, histologic grade, estrogen receptor, progesterone receptor, and HER2/neu test results for 803, 766, 739, 739, and 479 patients, respectively. Eighty-three percent of patients (N=813) consented to allow UIC researchers to obtain a sample of the tumor from the diagnosing hospital, and to provide blood samples. Genomic DNA was extracted from peripheral white blood cells using the Puregene DNA (Gentra System) and stored in liquid nitrogen until further use.

## 3. Polymorphisms selection and genotyping

We genotyped 59 functionally important SNPs and tagging SNPs in GCR and GCR-associated genes. The SNPs were selected based on a minor allele frequency greater than 5% and previous association with GCR activity, breast cancer, or downstream-related pathways such as inflammation and apoptosis. Genotyping was performed at Dr. Rick Kittle's laboratory with iPLEX Gold assay on a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass-spectrometer (MassArray system) according to manufacturer's recommendations. The iPLEX™ assays were designed using the Sequenom Assay Design software. Polymerase Chain reaction (PCR) was performed using 1.2 ng μl of genomic DNA, 25 mM MgCl, 5U/μl of Taq polymerase, 25 mM dNTP Mix, 10X PCR buffer, and 100 nmol of each PCR primer. The PCR reactions were treated with shrimp alkaline phosphatase to neutralize unincorporated dNTPs and a post-PCR single base extension reaction was performed for each multiplex reaction. Extension primer concentrations were adjusted to increase signal heights. Reactions were diluted with 20 μl of $H_2O$ and fragments were purified with resin, spotted onto Sequenom SpectroCHIP™ microarrays and scanned by MALDI-TOF mass spectrometry. Individual SNP

genotype calls were generated using Sequenom TYPER™ software, which automatically calls allele specific peaks according to their expected masses. Genotyping quality control for all SNPs was assessed using blinded duplicate genotyping for 60 DNA samples. A genotype concordance rate of 99% was observed for all markers. Genotyping call rates exceeded 98.5% for all individuals included in the analyses.

4. **Self-reported race/ethnicity**

Race/ethnicity was defined through separate self-identification of Hispanic ethnicity (yes/no) and race (European American/African American). Ethnicity was defined as Hispanic if the patient self-identified as Hispanic, reported a Latin American country of origin, or reported a Latin American country of origin for both biological parents. Racial/ethnic groups were categorized as follows: non-Hispanic European American, non-Hispanic Black, and Hispanic.

5. **Genetic ancestry with ancestry informative markers**

Variations in the distribution of single nucleotides polymorphisms (SNPs), called ancestry informative markers (AIMs) were used to estimate continental ancestry. The AIMs panel consisted of carefully selected autosomal markers that were previously identified and validated for estimating continental ancestry information in admixed populations (Kosoy et al., 2009; Nassir et al., 2009; Torres et al., 2013). The genetic ancestry proportions assigned to each individual, as opposed to membership of one racial group, served as a proxy for the genetic background of the individual and allowed us to adjust for population stratification. Global genetic ancestry for the BCCC cohort was previously reported (Al-Alem et al., 2014). Individual admixture estimates for each study participant were calculated using a model-based clustering method as implemented in the program STRUCTURE v2.3 (Falush et al., 2003). STRUCTURE

2.3 was run using parental population genotypes from West Africans, Europeans, and Native Americans (Kosoy et al., 2009) under the admixture model using the Bayesian Markov chain Monte Carlo method (K=3, assuming three founding populations) and a burn-in length of 30,000 for 70,000 repetitions. Ten cases that self-reported as European American and had more than 70% West African genetic ancestry were excluded. After the exclusions, genotype information was available for a total of 656 cases.

6. **Statistical analysis**

Stage at diagnosis, hormone receptor status, and histologic grade were abstracted from the patient's medical records. Stage at diagnosis was categorized using the American Joint Committee on Cancer (AJCC) categories (0–4), with later stage at diagnosis defined as stage >=2 versus <=1. Histological grade was determined through the Nottingham grading system and defined as low, intermediate, and high. Higher grade was defined as grade intermediate and high versus low. Estrogen/Progesterone status was defined as positive if the tumor contained either estrogen (ER) and/or progesterone (PR) receptors, and negative in the absence of both receptor types. Histological grade was defined as low, intermediate, and high. Among the 656 with biological samples, stage at diagnosis was available for 643 cases, histological grade for 575 cases, ER and PR data was available for 600 cases. As the determination of the Human Epidermal Growth Factor Receptor 2 (HER2) status was not a standard procedure when the BCCC cases were ascertained, we have HER2 status for only 362 cases and only 60 cases with triple negative in our population. Therefore, we excluded HER2 status in the present analysis. Baseline characteristics of the population were compared across self-reported racial/ethnic groups using chi-square statistics tests for categorical variables and analysis of variance (ANOVA) for continuous variables. For each SNP, deviation of genotype frequencies from

Hardy-Weinberg equilibrium (HWE) was assessed using a $\chi 2$ test. The homozygous wild-type genotype served as the reference category.

Association analyses were performed under dominant, recessive, or additive modes of inheritance. Bonferroni adjustments for multiple testing were used. Mean genetic ancestry was estimated as the average of the individual genetic ancestry estimates within self-reported racial/ethnic group. We used logistic regression to examine the association between genotypes and breast cancer characteristics within self-reported racial/ethnic group. Separate models were run for each self-reported racial/ethnic group—non-Hispanic White (nH White), non-Hispanic Black (nH Black), and Hispanic—ancestry (European, West-African, and Native American) and tumor characteristic (later stage, higher grade, hormone-receptor-negative) to estimate ORs and 95% CIs. We performed separate analysis for each racial/ethnic group because of the potential biological and environmental differences in factors contributing to breast cancer. The regression models were adjusted for health insurance, income, education, nulliparity, and age at first and last birth. All reported p-values are two-sided. Statistical analyses were conducted using R-Studio and Stata version 11 (College Station, Texas). We performed a post hoc statistical power and sample size analysis using Quanto software version 1.2.4. (Gauderman, 2002).

## C. **Results**

### 1. **Baseline characteristics of the cohort**

Description of the BCCC full cohort has been previously reported (Rauscher et al., 2010). We have 656 cases (67% of the original cohort) with valid genetic ancestry estimates and linked clinical, sociodemographic, and epidemiological data to assess variance in our candidate genes. The tumor and demographic characteristics of the final cohort, which includes a total of 250 White, 273 Black, and 120 Hispanic women, are summarized in TABLE I.

**TABLE I**

BASELINE CHARACTERISTICS OF THE SAMPLE STRATIFIED BY SELF-REPORTED RACE/ETHNICITY

| | Total | nH Whites % | nH Blacks % | Hispanics % | P-value |
|---|---|---|---|---|---|
| **Age at Diagnosis** | | | | | |
| <50years | 224 | 32 | 34 | 38 | 0.554 |
| ≥50years | 433 | 68 | 66 | 62 | |
| Age at first birth, Mean years (SD) | 23(6) | 26(6) | 21(5) | 23(6) | <0.0001 |
| Age at last birth, Mean years(SD) | 30(6) | 31(6) | 29(5) | 31(6) | <0.0001 |
| **Genetic Ancestry** | | | | | |
| European, Mean(SD) | 50(40) | 90(11) | 20(13) | 40(20) | <0.0001 |
| West African, Mean (SD) | 40(40) | 10(10) | 80(13) | 20(20) | <0.0001 |
| Native American, Mean (SD) | 10(20) | 3(5) | 4(4) | 40(24) | <0.0001 |
| **CDC Categories of BMI (kg/m$^2$)** | | | | | |
| Normal weight (18.5–24.9) | 203 | 49 | 20 | 20 | <0.0001 |
| Overweight (25.0–29.9) | 195 | 22 | 29 | 47 | |
| Obese (≥30.0) | 257 | 29 | 51 | 33 | |
| **Education** | | | | | |
| Less than high school | 120 | 4 | 20 | 44 | <0.0001 |
| High school | 138 | 15 | 27 | 21 | |
| Some college | 397 | 81 | 53 | 36 | |
| **Annual Household Income** | | | | | |
| Less than $30,000 | 263 | 17 | 56 | 57 | <0.0001 |
| $30,000 to $75,000 | 277 | 52 | 38 | 37 | |
| Greater than $75,000 | 102 | 31 | 6 | 7 | |
| **Insurance Category** | | | | | |
| No outpatient insurance | 84 | 7 | 14 | 23 | <0.0001 |
| Public | 125 | 4 | 31 | 23 | |
| Private | 447 | 89 | 55 | 55 | |
| **Any Comorbidities** | | | | | |
| No | 286 | 49 | 37 | 48 | 0.007 |
| Yes | 370 | 51 | 64 | 52 | |
| **Nulliparous** | | | | | |
| No | 523 | 63 | 90 | 93 | <0.0001 |
| Yes | 133 | 37 | 11 | 7 | |
| **Menopausal Status** | | | | | |
| No | 133 | 17 | 20 | 27 | 0.113 |
| Yes | 519 | 83 | 80 | 73 | |
| **Family History Breast Cancer** | | | | | |
| No | 503 | 75 | 77 | 84 | 0.172 |
| Yes | 147 | 25 | 23 | 16 | |
| **Mode of Detection** | | | | | |
| Screen-detected | 336 | 60 | 46 | 45 | 0.003 |
| Symptoms and no recent prior screen | 156 | 20 | 28 | 23 | |
| Symptoms despite a recent prior screen | 164 | 20 | 27 | 32 | |

BASELINE CHARACTERISTICS OF THE SAMPLE STRATIFIED BY SELF-REPORTED RACE/ETHNICITY

| | Total | nH Whites % | nH Blacks % | Hispanics % | P-value |
|---|---|---|---|---|---|
| **Stage at Diagnosis** | | | | | |
| 0,1 (early stage) | 374 | 67 | 55 | 48 | 0.0004 |
| 2,3,4 (late stage) | 269 | 33 | 45 | 53 | |
| **Histologic Grade** | | | | | |
| Low/Intermediate | 409 | 71 | 60 | 70 | 0.001 |
| High | 208 | 29 | 40 | 30 | |
| **ER and/or PR** | | | | | |
| ER and/or PR Positive | 476 | 87 | 72 | 79 | <0.0001 |
| Double negative | 126 | 14 | 28 | 21 | |
| **HER2/Neu Overexpression** | | | | | |
| No | 305 | 90 | 78 | 86 | 0.028 |
| Yes | 57 | 10 | 22 | 14 | |

P-values for categorical variables are from $\chi 2$ tests and from ANOVA for continuous variables for differences according to self-reported race/ethnicity.

Racial/ethnic disparities in breast cancer characteristics were apparent in this population, as nH Black and Hispanic women were diagnosed at a later stage, higher grade, and with a higher proportion of ER/PR negative tumors, compared to nH Whites. In addition, a greater proportion of nH Black and Hispanic women were overweight/obese, had more comorbidities, were less likely to have their cancer detected through screening mammography, had a lower level of education and income, and less likely to have private insurance than nH Whites. The distribution of estimated West African, European, and Native American ancestry varied among the three self-reported racial groups (Figure 3). The predominant genetic ancestry proportion among White cases was the European genetic ancestry, with a mean of 90% (±SD 11%). The predominant genetic ancestry among Black cases was West African genetic ancestry, with a mean of 79% (±SD 13%). Hispanic women had a wide range of European (mean 45%), Native American (mean 37%), and West African (mean 18%) genetic ancestry representing a highly admixed group.

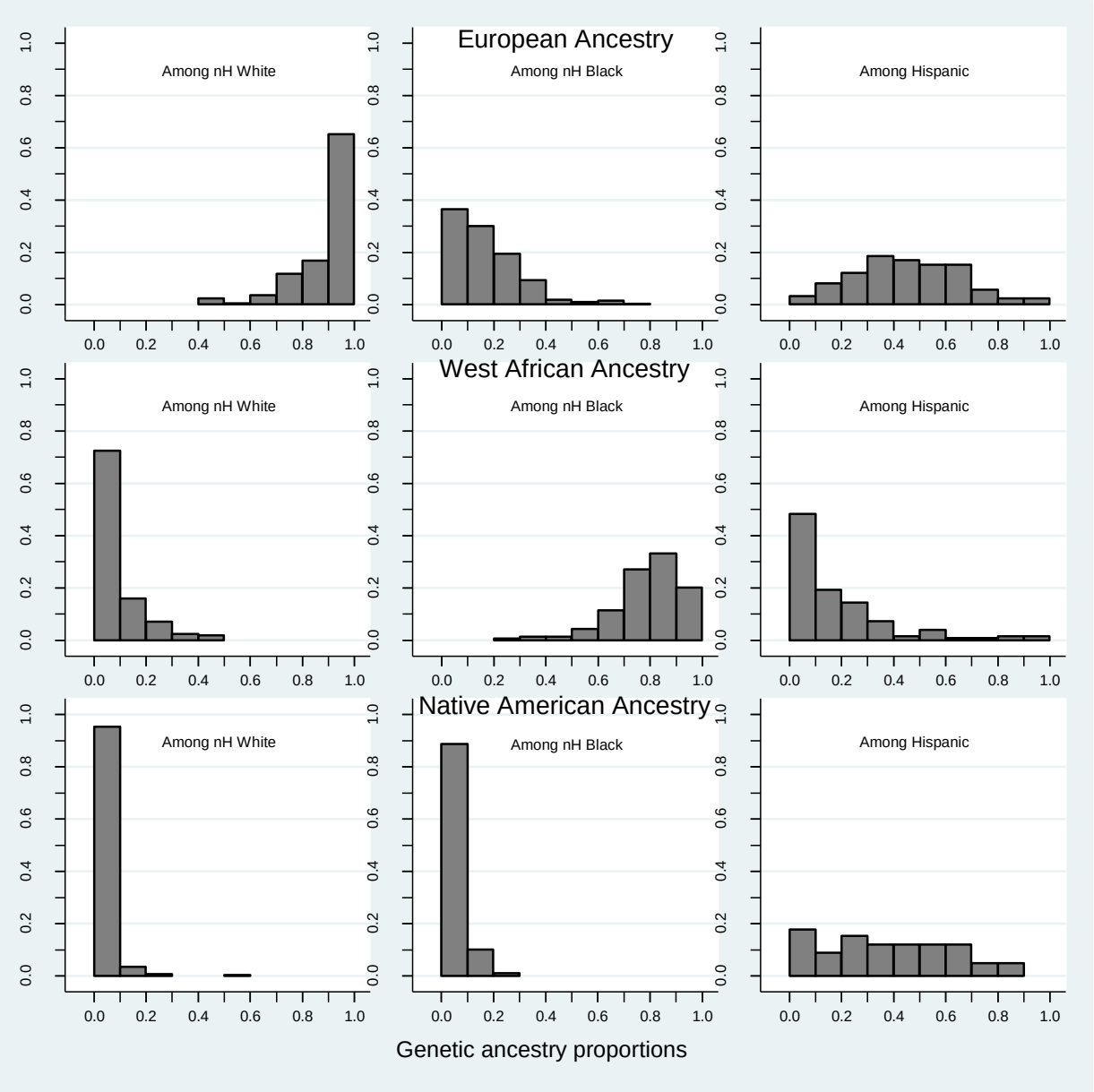



Figure 3. Histograms for European ancestry, West African ancestry, and Native American ancestry stratified on self-reported race/ethnicity.

## 7. Characteristics of studied markers

In the current analysis, we examined polymorphisms in NR3C1, FKBP5, IL6, ADIPOQ, LEPR, SGK1, MnSOD, CAT, and BcL-2. TABLE II describes the polymorphisms including the minor allele frequencies (MAF) and HWE results by self-reported race/ethnicity.

The SNPS that failed the MAF and HWE (p=.05) in each self-reported racial/ethnic group were removed. Eight failed SNP were removed for nH Black cases (rs1360780, rs6189, rs6195, rs17614642, rs72801094, rs755658, rs9380524, rs9380524), 11 failed SNPS from nH White cases (rs6189, rs6195, rs1800796, rs5871845, rs9380524, rs9493857, rs10482605, rs34866878, rs41270080, rs72801094, and rs737499), and 11 failed SNPs for Hispanic cases (rs10482605, rs6189, rs6195, rs1360780, rs17287758, rs174048, rs2918418, rs41270080, rs72801094, rs9324921, and rs1001179).

We observed different allelic frequency distribution among different racial/ethnic groups for several SNPs (GCR: rs6191, rs33388, rs9324924, rs4607376; SGK1: rs9493857; BcL-2: rs2279115; LEPR: rs1137101; MnSOD: rs4880). Our reported allele frequencies were similar to those in the SNP Database (http://www.ncbi.nlm.nih.gov/snp). A Bonferroni correction was used to account for multiple comparisons. There was a total of 52 comparisons for the nH Blacks category with a corrected alpha=0.0009615385, a total of 49 comparison for nH Whites and Hispanics with a corrected alpha=0.001020408. As Bonferroni correction is very conservative, we also looked at the proportion of the number of significant SNPs and number of comparisons.

# TABLE II
## CHARACTERISTICS OF STUDIED POLYMORPHISMS

| dbSNP_ID | Coordinates | Type | nH Blacks | | | nH Whites | | | Hispanics | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | MA | MAF | HWE | MA | MAF | HWE | MA | MAF | HWE |
| **GCR (*NR3C1*) at 5q31.1** | | | | | | | | | | | |
| rs174048 | 142650404 | upstream of NR3C1 | C | 0.167 | 1.00 | C | 0.164 | 0.48 | C | 0.09 | 1.0 |
| rs17287745 | 142655015 | Downstream gene | G | 0.135 | 0.11 | G | 0.369 | 0.10 | G | 0.26 | 0.2 |
| rs17287758 | 142657021 | Downstream gene | A | 0.080 | 0.68 | A | 0.155 | 0.63 | A | 0.08 | 1.0 |
| rs6191 | 142658156 | 3' UTR | T | 0.456 | 0.63 | G | 0.482 | 1.00 | T | 0.38 | 0.4 |
| rs17209251 | 142669223 | Intron | G | 0.055 | 0.17 | G | 0.192 | 0.68 | G | 0.17 | 0.1 |
| rs258813 | 142674690 | Intron | A | 0.306 | 0.77 | A | 0.322 | 0.47 | A | 0.18 | 0.4 |
| rs6188 | 142680344 | Intron | T | 0.272 | 0.16 | T | 0.290 | 0.005 | T | 0.14 | 1.0 |
| rs10482672 | 142692533 | Intron | T | 0.175 | 0.006 | T | 0.168 | 0.22 | T | 0.10 | 0.1 |
| rs33388 | 142697295 | Intron | A | 0.443 | 0.62 | T | 0.476 | 0.61 | A | 0.38 | 0.7 |
| rs2918418 | 142723373 | Intron | G | 0.197 | 0.44 | G | 0.170 | 0.18 | G | 0.10 | 0.6 |
| rs4912905 | 142730376 | Intron | C | 0.101 | 0.32 | C | 0.206 | 0.56 | C | 0.27 | 0.2 |
| rs2963155 | 142756004 | Intron | G | 0.279 | 0.65 | G | 0.260 | 0.19 | G | 0.15 | 1.0 |
| rs9324921 | 142767740 | Intron | A | 0.153 | 0.35 | A | 0.059 | 0.19 | A | 0.05 | 1.0 |
| rs41423247 | 142778575 | Intron | C | 0.251 | 0.52 | C | 0.353 | 1.00 | C | 0.25 | 0.1 |
| rs6195 | 142779317 | Exon | G | 0.009 | 1.00 | G | 0.026 | 1.00 | G | 0.00 | 1.0 |
| rs6189 | 142780339 | Synonymous | 0 | 0.000 | 1.00 | A | 0.036 | 0.27 | A | 0.01 | 0.004 |
| rs10482616 | 142781567 | Intron | A | 0.182 | 0.41 | A | 0.170 | 1.00 | A | 0.25 | 0.8 |
| rs10482614 | 142782402 | Intron | A | 0.204 | 0.71 | A | 0.169 | 0.11 | A | 0.12 | 1.0 |
| rs10482605 | 142783521 | Intron | C | 0.054 | 0.003 | C | 0.091 | 0.0001 | C | 0.04 | 0.1 |
| rs72801094 | 142785905 | Intron | C | 0.019 | 1.00 | C | 0.060 | 0.60 | C | 0.05 | 1.0 |
| rs10052957 | 142786701 | Intron | A | 0.273 | 0.76 | A | 0.320 | 0.67 | A | 0.20 | 1.0 |
| rs9324924 | 142792484 | Intron | G | 0.338 | 0.68 | T | 0.421 | 1.00 | T | 0.46 | 0.9 |
| rs7701443 | 142792650 | Intron | G | 0.431 | 0.11 | G | 0.406 | 1.00 | G | 0.48 | 0.7 |
| rs4244032 | 142794725 | Intron | G | 0.135 | 0.80 | G | 0.169 | 1.00 | G | 0.11 | 0.6 |
| rs4607376 | 142796532 | Intron | G | 0.241 | 0.03 | A | 0.480 | 0.16 | G | 0.44 | 0.3 |
| rs13182800 | 142801480 | Intron | T | 0.194 | 0.07 | T | 0.183 | 0.40 | T | 0.12 | 0.2 |
| rs12054797 | 142805902 | Intron | T | 0.097 | 0.02 | T | 0.248 | 0.86 | T | 0.28 | 0.5 |
| rs12656106 | 142808947 | Intron | C | 0.167 | 0.64 | C | 0.407 | 0.33 | C | 0.34 | 0.4 |
| rs12521436 | 142817607 | Upstream gene | A | 0.236 | 0.61 | A | 0.167 | 0.25 | A | 0.220 | 0.006 |
| rs4912913 | 142818306 | Upstream gene | T | 0.5 | 0.05 | T | 0.476 | 0.16 | C | 0.333 | 0.01 |
| ***FKBP5* at 6p21.3-21.2** | | | | | | | | | | | |
| rs3800373 | 35542476 | 3' UTR | G | 0.409 | 0.61 | G | 0.260 | 1.00 | G | 0.34 | 0.8 |
| rs1360780 | 35607571 | Intron | T | 0.321 | 0.00001 | T | 0.172 | 0.04 | T | 0.14 | 0.02 |
| rs17614642 | 35621921 | Intron | C | 0.023 | 1.00 | C | 0.077 | 0.16 | C | 0.06 | 0.4 |
| rs34866878 | 35544942 | Synonymous | A | 0.174 | 0.83 | A | 0.030 | 1.00 | A | 0.07 | 0.5 |
| rs3777747 | 35579002 | Intron | G | 0.389 | 1.00 | G | 0.488 | 0.70 | G | 0.47 | 1.0 |
| rs3798346 | 35562640 | Intron | G | 0.044 | 0.32 | G | 0.175 | 1.00 | G | 0.11 | 1.0 |
| rs4713916 | 3566983 | Intron | A | 0.134 | 0.28 | A | 0.270 | 0.52 | A | 0.25 | 0.1 |
| rs9296158 | 35567082 | Intron | A | 0.464 | 0.81 | A | 0.304 | 0.77 | A | 0.37 | 0.8 |
| rs9470080 | 35646435 | Intron | T | 0.435 | 0.51 | T | 0.294 | 0.19 | T | 0.37 | 1.0 |
| rs41270080 | 35542045 | 3' UTR | A | 0.170 | 0.34 | A | 0.036 | 1.00 | A | 0.07 | 1.0 |
| rs4713899 | 35569281 | Intron | A | 0.133 | 0.59 | A | 0.165 | 0.23 | A | 0.12 | 0.1 |

# CHARACTERISTICS OF STUDIED POLYMORPHISMS

| dbSNP_ID | Coordinates | Type | nH Blacks | | | nH Whites | | | Hispanics | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | MA | MAF | HWE | MA | MAF | HWE | MA | MAF | HWE |
| rs6912833 | 35617585 | Intron | A | 0.169 | 1.00 | A | 0.280 | 0.64 | A | 0.27 | 1.0 |
| rs737054 | 35575487 | Non coding exon | T | 0.078 | 0.21 | T | 0.282 | 0.88 | T | 0.29 | 1.0 |
| rs73746499 | 35578851 | Intron | G | 0.182 | 1.00 | G | 0.032 | 1.00 | G | 0.08 | 0.6 |
| rs755658 | 35549670 | 3 prime UTR | A | 0.023 | 1.00 | A | 0.085 | 0.69 | A | 0.14 | 0.7 |
| rs9366890 | 35562974 | Intron | T | 0.175 | 0.13 | T | 0.178 | 0.83 | T | 0.13 | 0.1 |
| rs9380524 | 35589070 | Intron | A | 0.025 | 1.00 | A | 0.094 | 0.14 | A | 0.09 | 1.0 |
| ***SGK1* 6q23.2** | | | | | | | | | | | |
| rs9493857 | 134530697 | Intron | G | 0.272 | 0.06 | A | 0.201 | 0.0000 | A | 0.41 | 0.2 |
| ***IL-6* 7p21** | | | | | | | | | | | |
| rs1800796 | 22766246 | Non coding exon | A | 0.130 | 0.09 | A | 0.379 | 1.00 | A | 0.21 | 1.0 |
| rs1800797 | 22766221 | Non coding exon | A | 0.091 | 0.03 | A | 0.054 | 0.42 | A | 0.18 | 0.3 |
| rs1800795 | 22766645 | Intron | C | 0.112 | 0.47 | C | 0.378 | 1.00 | C | 0.19 | 1.0 |
| rs6949149 | 22749157 | Regulatory region | T | 0.123 | 0.002 | T | 0.088 | 0.42 | T | 0.18 | 0.1 |
| ***BcL-2* 18q21.33** | | | | | | | | | | | |
| rs2279115 | 60986837 | 5' UTR | A | 0.260 | 0.002 | C | 0.451 | 0.52 | C | 0.36 | 0.1 |
| ***ADIPOQ* 3q27.3** | | | | | | | | | | | |
| rs266729 | 186559474 | Upstream gene | G | 0.119 | 0.78 | G | 0.276 | 0.15 | G | 0.26 | 0.3 |
| rs1501299 | 186571123 | Intron | A | 0.321 | 0.78 | A | 0.268 | 0.52 | A | 0.33 | 0.7 |
| ***LEPR 1p31.3*** | | | | | | | | | | | |
| rs1137100 | 66036441 | Exon | G | 0.182 | 0.41 | G | 0.230 | 0.21 | G | 0.30 | 0.8 |
| rs1137101 | 66058513 | Exon | A | 0.494 | 0.72 | G | 0.434 | 0.12 | G | 0.44 | 0.3 |
| ***MnSOD* 6q25.3** | | | | | | | | | | | |
| rs4880 | 160113872 | Missense | C | 0.489 | 0.40 | T | 0.500 | 0.38 | T | 0.38 | 0.8 |
| ***CAT* 11p13** | | | | | | | | | | | |
| rs1001179 | 34460231 | Upstream gene | A | 0.061 | 1.00 | A | 0.244 | 0.287 | A | 0.066 | 1.0 |

Abbreviations: MA: Minor allele; MAF: Minor allele frequency; HWE: Hardy-Weinberg equilibrium; UTR: untranslated region. DNA position: According to the NCBI genomic reference sequence NT_029289.11

8. **Genotypes and histological grade at diagnosis**

We examined the association between higher histological grade at diagnosis and individual SNPS. TABLE III is the summary of the significant associations ($p<.05$) prior to adjustment for multiple comparisons by self-reported race/ethnicity. Among the nH White cases, higher grade at diagnosis was associated with the IL-6 $rs1800797_{AG+AA}$ genotype (OR, 1.99: 95% CI 1.07–3.73). The GCR $rs33388_{TT}$ and $rs6191_{GG}$ genotypes were associated with a two-fold increase in the risk of high grade at diagnosis. The GCR $rsrs41423247_{GC+CC}$ genotype was associated with lower grade at diagnosis (OR 0.56: 95% CI 0.32–0.99). Among the nH Black cases, the GCR $rs10052957_{AG+AA}$, $rs258813_{AA}$, $rs2918418_{AA}$, $rs33388_{AA}$, $rs41423247_{GC/CC}$, $rs6188_{TT}$, $rs6191_{TT}$, and $rs9324924_{GG}$ genotypes were associated with higher grade. However, having a GCR $rs10482616_{GA+AA}$, $rs10482672_{TC+TT}$, or $rs7701443_{AG+GG}$ or $rs9296158_{AA}$ genotype conferred protection against higher grade. The FKBP55 $rs9296158_{AA}$ genotype was associated with decreased risk of high grade (OR 0.45: 95% CI 0.23–0.9). Among the Hispanic cases, only the GCR $rs9324924_{GT+TT}$ genotype was associated with higher grade (OR 3.14: 95% CI 0.99–10). None of the associations between those SNPs and breast cancer characteristics remained statistically significant after adjustment for multiple comparisons.

9. **Genotypes and stage at diagnosis**

We examined the association between later stage at diagnosis and individual SNPS among breast cancer cases. TABLE IV describes the significant associations ($p<.05$) prior to adjustment for multiple comparisons by self-reported race/ethnicity. None of the tested SNPs were statistically significant at $p<0.05$ level for nH White cases.

Among nH Black, the A allele of GCR rs10482614 was associated with later stage at diagnosis (OR, 8: 95% CI 2–39), but there were few cases (n=12) in this category. The nH Black

cases with FKP51 (rs3777747$_{GG}$) genotype were associated with later stage of diagnosis (OR, 2: 95% CI 0.98–4.11). However, the FKP51 genotypes rs3800373$_{GT+GG}$, rs9296158 $_{AG+AA}$, 9470080$_{CT+TT}$ were associated with nearly 50% decreased risk of later stage at diagnosis. For Hispanic cases, ADIPOQ rs1501299$_{CA+AA}$ genotype was associated with decreased risk of later stage (OR, 0.39: 95% CI 0.17–0.87), while the rs266729$_{GC+GG}$ genotype was associated with late stage (OR, 3.01: 95% CI 1.35–6.73). None of the associations remained statistically significant after adjustment for multiple comparisons.

**TABLE III**
LIST OF SNPS WITH SIGNIFICANT ASSOCIATION WITH HIGHER GRADE AT DIAGNOSIS (p<.05)

| SNP | Genotype | Low Grade (%) | High Grade (%) | Adjusted OR | P-value |
|---|---|---|---|---|---|
| **nH White[a]** | | | | | |
| **IL-6 rs1800797** | | | | | |
| | G/G | 42 | 27 | Ref | 0.079 |
| | A/G | 46 | 60 | 2.06 (1.08–3.93) | |
| | A/A | 12 | 13 | 1.73 (0.66–4.49) | |
| | A/G+A/A versus G/G | 58 | 74 | 1.99 (1.07–3.73) | 0.027 |
| **GCR rs6191** | | | | | |
| | T/T | 27 | 30 | Ref | 0.034 |
| | G/T | 53 | 37 | 0.65 (0.32–1.3) | |
| | G/G | 20 | 33 | 1.66 (0.77–3.58)) | |
| | G/G versus T/T+G/T | 20 | 33 | 2.16 (1.13–4.15) | 0.021 |
| **GCR rs33388** | | | | | |
| | A/A | 29 | 31 | Ref | 0.049 |
| | T/A | 51 | 37 | 0.68 (0.34–1.35) | |
| | T/T | 20 | 32 | 1.67 (0.78–3.55) | |
| | T/T versus A/A+T/A | 20 | 32 | 2.09 (1.09–4.01) | 0.028 |
| **GCR rs41423247** | | | | | |
| | G/G | 37 | 52 | Ref | 0.052 |
| | G/C | 51 | 34 | 0.48(0.26–0.89) | |
| | C/C | 12 | 15 | 0.91 (0.38–2.17) | |
| | G/C+C/C versus G/G | 63 | 49 | 0.56 (0.32–0.99) | 0.046 |
| **nH Black[b]** | | | | | |
| **GCR rs10052957** | | | | | |
| | G/G | 61 | 42 | Ref | 0.007 |
| | A/G | 35 | 42 | 1.56 (0.89–2.74) | |
| | A/A | 4 | 15 | 4.61 (1.64–12.97) | |
| **GCR rs258813** | | | | | |
| | G/G | 54 | 39 | Ref | 0.016 |
| | G/A | 40 | 43 | 1.39 (0.79–2.45) | |
| | A/A | 6 | 18 | 3.7 (1.48–9.28) | |
| | A/A versus G/G+G/A | 6 | 18 | 3.16 (1.32–7.59) | 0.008 |
| **GCR rs33388** | | | | | |
| | T/T | 36 | 21 | Ref | 0.005 |
| | T/A | 51 | 51 | 1.59 (0.84–3) | |
| | A/A | 14 | 28 | 3.55 (1.62–7.8) | |
| | A/A versus T/T+T/A | 14 | 28 | 2.63 (1.36–5.11) | 0.004 |
| **GCR rs41423247** | | | | | |
| | G/G | 63 | 48 | Ref | 0.033 |
| | G/C | 32 | 42 | 1.76 (1–3.09) | |
| | C/C | 5 | 10 | 2.98 (1.06–8.4) | |
| | G/C+C/C versus G/G | 37 | 53 | 1.92 (1.13–3.28) | 0.015 |
| **GCR rs10482616** | | | | | |
| | G/G | 62 | 80 | Ref | 0.015 |
| | G/A | 34 | 15 | 0.39 (0.2–0.76) | |
| | A/A | 4 | 5 | 0.98 (0.28–3.44) | |
| | G/A+A/A versus G/G | 38 | 20 | 0.46 (0.25–0.84) | 0.01 |
| **GCR rs10482672** | | | | | |
| | C/C | 66 | 81 | Ref | 0.058 |
| | T/C | 28 | 15 | 0.43(0.21–0.89) | |
| | T/T | 6 | 5 | 0.67 (0.19–2.37) | |
| | T/C+T/T versus C/C | 34 | 19 | 0.47 (0.25–0.91) | 0.021 |
| **GCR rs7701443** | | | | | |
| | A/A | 24 | 39 | Ref | 0.008 |
| | A/G | 56 | 53 | 0.56 (0.31–1.01) | |
| | G/G | 20 | 8 | 0.26 (0.1–0.65) | |
| | A/G+G/G versus A/A | 77 | 61 | 0.48 (0.27–0.86) | 0.013 |
| **GCR rs9324921** | | | | | |
| | C/C | 68 | 80 | Ref | 0.067 |
| | C/A | 28 | 17 | 0.47 (0.24–0.9) | |
| | A/A | 3 | 3 | 0.77 (0.17–3.5) | |

# LIST OF SNPS WITH SIGNIFICANT ASSOCIATION WITH HIGHER GRADE AT DIAGNOSIS (p<.05)

| **SNP** | Genotype | Low Grade (%) | High Grade (%) | Adjusted OR | P-value |
|---|---|---|---|---|---|
| | C/A+A/A versus C/C | 32 | 20 | 0.5 (0.27–0.93) | 0.025 |
| **FKBP5 rs9296158** | | | | | |
| | G/G | 27 | 30 | Ref | 0.066 |
| | A/G | 47 | 55 | 0.96 (0.52–1.78) | |
| | A/A | 26 | 16 | 0.44 (0.2–0.98) | |
| | A/A versus G/G+A/G | 26 | 16 | 0.45 (0.23–0.9) | 0.02 |
| **Hispanic cases**[c] | | | | | |
| **GCR rs9324924** | G/G | 33 | 13 | Ref | 0.075 |
| | G/T | 45 | 67 | 3.59 (1.09–11.82) | |
| | T/T | 22 | 20 | 2.22 (0.54–9.1) | |
| | G/T+T/T versus G/G | 67 | 87 | 3.14(0.99–10) | 0.036 |

[a]Adjusted for European genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth. [b]Adjusted for West African genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth. [c]Adjusted for Native American genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth.

**TABLE IV**
LIST OF SNPS WITH SIGNIFICANT ASSOCIATION WITH LATER STAGE AT DIAGNOSIS (p<.05)

| dbSNP ID | Genotype | Early stage (%) | Late stage (%) | OR[a] (95% CI) | p-value |
|---|---|---|---|---|---|
| **nH Black [a]** | | | | | |
| **GCR rs10482614** | | | | | |
| | G/G | 65 | 62 | Ref | 0.005 |
| | A/G | 34 | 29 | 0.8 (0.5–1.4) | |
| | A/A | 1 | 9 | 7.96 (1.6–38.9) | |
| **FKBP5 rs3777747** | | | | | |
| | A/A | 36 | 38 | Ref | 0.130 |
| | A/G | 52 | 43 | 0.84 (0.49–1.46) | |
| | G/G | 12 | 19 | 1.82 (0.3–3.98) | |
| | G/G versus A/A-A/G | 12 | 19 | 2.01 (0.98–4.11) | 0.054 |
| **FKBP5 rs3800373** | | | | | |
| | T/T | 30 | 42 | Ref | 0.054 |
| | G/T | 52 | 41 | 0.51(0.29–0.91) | |
| | G/G | 18 | 17 | 0.52 (0.24–1.12) | |
| | G/T-G/G versus T/T | 70 | 58 | 0.51(0.3–0.89) | 0.016 |
| **FKBP5 rs9296158** | | | | | |
| | G/G | 23 | 35 | Ref | 0.044 |
| | A/G | 56 | 44 | 0.47 (0.26–0.86) | |
| | A/A | 21 | 21 | 0.54 (0.26–1.13) | |
| | A/G-A/A versus G/G | 77 | 65 | 0.49 (0.28–0.87) | 0.014 |
| **FKPB5 rs9470080** | | | | | |
| | C/C | 24 | 38 | Ref | 0.042 |
| | C/T | 57 | 46 | 0.47 (0.25–0.87) | |
| | T/T | 19 | 16 | 0.47 (0.21–1.08) | |
| | C/T-T/T versus C/C | 76 | 62 | 0.47 (0.26–0.85) | 0.012 |
| **Hispanic [b]** | | | | | |
| **ADIPOQ rs1501299** | C/C | 37 | 57 | Ref | 0.054 |
| | C/A | 50 | 33 | 0.35 (0.15–0.84) | |
| | A/A | 13 | 10 | 0.54 (0.15–1.94) | |
| | C/A-A/A versus C/C | 63 | 43 | 0.39 (0.17–0.87) | 0.019 |
| **ADIPOQ rs266729** | | | | | |
| | C/C | 70 | 43 | Ref | 0.012 |
| | G/C | 26 | 43 | 2.55 (1.09–5.97) | |
| | G/G | 4 | 13 | 6.47 (1.2–34.83) | |
| | G/C-G/G versus C/C | 30 | 57 | 3.01(1.35–6.73) | 0.006 |

[a]Adjusted for West African genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth. [b]Adjusted for Native American genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth.

## 10. Genotypes and hormone receptor status

TABLE V summarizes the results of the significant association (p<.05) between ER/PR positivity and individual SNPS. We found inverse associations for the CC genotype of GCR rs12656106 and ER/PR positivity (OR, 0.47; 95% CI 0.17–1.35). For nH Black cases, GCR (rs10482616$_{GA+AA}$), ADIPOQ (rs1501299 $_{CA+AA}$) and BcL-2 (rs2279115$_{AA}$) genotypes were associated with ER or PR receptor positivity. None of the SNPS were significant at alpha<.05 among Hispanic cases. Overall, none of the associations between those SNPs and breast cancer characteristics remained statistically significant after adjustment for multiple comparisons.

**TABLE V**
LIST OF SNPS WITH SIGNIFICANT ASSOCIATION WITH HORMONE-RECEPTOR-POSITIVITY (p<.05)

| SNP | Genotype | HR positive (%) | HR Negative (%) | OR | p-value |
|---|---|---|---|---|---|
| **nH White cases [(a)]** | | | | | |
| **GCR rs12656106** | | | | | |
| | G/G | 40 | 35 | Ref | 0.025 |
| | C/G | 24 | 48 | 2.24(0.76–6.63) | |
| | C/C | 36 | 17 | 0.47 (0.17–1.35) | |
| | C/C versus G/G-C/G | 36 | 17 | 0.32(0.12–0.83) | 0.023 |
| **nH Black cases [(b)]** | | | | | |
| **GCR rs10482616** | | | | | |
| | G/G | 80 | 64 | Ref | 0.142 |
| | G/A | 17 | 31 | 2.05 (0.94–4.49) | |
| | A/A | 3 | 5 | 1.93(0.39–9.67) | |
| | G/A-A/A versus G/G | 20 | 36 | 2.03 (0.98–4.21) | 0.04828 |
| **ADIPOQ rs1501299** | | | | | |
| | C/C | 56 | 41 | Ref | 0.07604 |
| | C/A | 38 | 47 | 1.82 (0.95–3.47) | |
| | A/A | 7 | 12 | 2.78 (0.85–9.1) | |
| | C/A-A/A versus C/C | 44 | 59 | 1.96 (1.06–3.63) | 0.03116 |
| **BcL-2 rs2279115** | | | | | |
| | C/C | 53 | 57 | Ref | 0.02856 |
| | C/A | 42 | 27 | 0.6 (0.31–1.16) | |
| | A/A | 5 | 16 | 2.84 (0.78–10.28) | |
| | A/A versus C/C-C/A | 5 | 16 | 3.48 (0.99–12.24) | 0.02787 |

[a]Adjusted for European genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth. [b]Adjusted for West African genetic ancestry, health insurance, income, education, nulliparity, and age at first and last birth.

## D. **Discussion**

Breast cancer is a complex disease which is triggered by a multitude of factors including genetic variations. Activation of the glucocorticoid-mediated pathway plays an important role in several cellular processes, and disruption of GCR activity could play a role in breast cancer progression and aggression. We examined the association between genetic variants and breast cancer characteristics among self-reported racial/ethnic groups (nH White, nH Black, and Hispanic) separately.

To the best of our knowledge, this is the first study to examine the relationship between GCR and GCR-related gene polymorphisms and breast cancer characteristics. We used a sample of breast cancer patients from an urban, multiracial cohort to assess the association between breast cancer characteristics and genetic variants of functionally important SNPs in: GCR, the cochaperone FKBP5; apoptosis and oxidative stress related genes: SGK1, MnSOD, CAT, and BcL-2; and inflammation and adiposity related genes: IL-6, ADIPOQ, and LEPR. We found that several SNPs were associated with stage at diagnosis, grade at diagnosis, and hormone receptor status prior to adjustment for multiple comparisons. Those associations were observed among self-reported racial/ethnic groups even after correction for population stratification (TABLE VI).

The GCRs rs6191, rs33388 and rs41423247 were associated with higher grade at diagnosis only among White and Black cases, and not among Hispanic cases, but the variant associated with the phenotype was different among the different racial/ethnic groups. The minor allele G of rs6191 was associated with increased risk of high grade among nH White cases, while the minor allele T was associated with higher grade among nH Black cases. The minor allele T of rs33388 was associated with increased risk of high grade among nH White cases, while the minor allele A was associated with higher grade among nH Black cases. The rs41423247 variant in GCR is associated with hypersensitivity to glucocorticoids (van Rossum et al., 2006).

Similarly, rs9324924 was associated with higher grade at diagnosis among nH Black and Hispanic cases. But the minor G allele in nH Black and the GT and TT genotypes among Hispanics were associated with higher grade. The rs10482616$_{GA+AA}$ was associated with ER or PR receptor positivity among nH Black cases. None of these SNPs have been previously studied in breast cancer.

We observed an inverse relationship between stage at diagnosis and 3 FKPB5 SNPs (rs3800373, rs9296158, rs9470080) among nH Black cases. A cochaperone, FKBP5 belongs to the immunophilin family. Immunophilins are a large, functionally diverse group of proteins that are defined by their ability to bind immunosuppressive ligands. The FKBP5 expression is highly inducible by glucocorticoids and functions as a negative transcriptional regulator of GCR (Vermeer et al., 2003). In addition, overexpression of FKBP5 impairs nuclear localization of GCR (Binder, 2009). The rs3800373, rs9296158, and rs9470080 FKPB5 SNPs have been associated with a higher FKBP5 expression and a stronger induction of FKBP5 mRNA by cortisol (Binder et al., 2004). Romano et al. have observed a low/negative protein expression of FKBP5 among 10 breast cancer samples (Romano et al., 2010). If these associations are real and not a result of type 1 error, it is possible that these FKBP5 polymorphisms might be reducing GCR activation by inhibiting nuclear translocation.

We identified associations with two ADIPOQ SNPs (rs1501299 and rs266729) with stage at diagnosis among Hispanic cases. The ADIPOQ rs1501299$_{CA+AA}$ genotypes were protective against later stage (OR 0.39, 95% CI 0.17–0.87), while the ADIPOQ rs266729$_{GC+GG}$ genotypes were associated with later stage (OR 3.01, 95% CI 1.35–6.73). The ADIPOQ (rs1501299 $_{CA+AA}$) was associated with ER or PR receptor positivity among nH Black cases. These two SNPs have been previously associated with circulating levels of ADIPOQ and breast cancer. Kaklamani et

al. have previously shown that the rs1501299 was associated with increased breast cancer risk among African American women (Kaklamani et al., 2008). The G allele at rs266729 is associated with lower adiponectin levels and obesity (Siitonen et al., 2011).

Among the nH White cases, higher grade at diagnosis was associated with *IL-6* $rs1800797_{AG+AA}$ genotype. The cytokine IL-6 is an inflammatory cytokine, where high serum levels of IL-6 have been shown to correlate with poor outcome in breast cancer patients (Bachelot et al., 2003). Several IL6 SNPs have been associated with breast cancer risk and prognosis (DeMichele et al., 2009 ).

The BcL-2 gene encodes anti-apoptotic protein that are critical regulators of programmed cell death. Higher levels of BcL-2 expression in breast tumors have been shown to be an independent prognostic factor for improved survival from breast cancer (Dawson et al., 2010). In addition, BcL-2 ($rs2279115_{AA}$) was associated with ER or PR receptor positivity. Bachman et al. found that higher expression of BcL-2 was associated with the A-allele and survival analysis revealed a significant association of the AA genotype with improved survival (Bachmann et al., 2007).

We were able to examine the associations between breast cancer characteristics and GCR and GCR-related genes by using a sociodemographically diverse sample that captured the three major racial/ethnic groups from a population-based study. Several SNPs in the GCR, FKBP5, ADIPOQ, IL-6 and Bcl-2 genes were associated with breast cancer stage, grade, or hormone receptor status.

It is possible that those SNPs are not the causal SNPs nor are the real causal SNPs located in the vicinity. Given the large number of SNPs analyzed, genetic variants in tested genes were not associated with breast cancer characteristics after multiple comparison corrections. It is

possible that there is truly no association between breast cancer characteristics (stage, grade, ER/PR status) and our tested genes. In addition, the relatively small sample size and potential misclassification of ER/PR status, grade, and stage might tend to alter observed associations in unpredictable ways, by either attenuating or biasing associations away from the null. The statistical power, which is the probability of rejecting the null hypothesis while the alternative hypothesis is true, in this study was limited. We performed a post hoc power analysis. The results vary for each SNP investigated but using the rs33388 as an example with an MAF of 40%, an assumed population risk of 0.13 for breast cancer, a recessive genetic model, an OR of 2 and significance set at 5%, we had 62% power. Using the same assumptions we would need a sample at least double the size of the current study to have up to 80% power to detect the same effect. Future studies with larger sample size are needed to confirm our results, and if confirmed, further investigations would be required to identify the molecular pathogenesis. However, several SNPs were significant prior to multiple comparison adjustment. The adjustment may have contributed to false negative associations, thus the role of genetic variants of GCR or GCR-associated genes cannot be ruled out.

In conclusion, to the best of our knowledge, this is the first study to examine the relationship between GCR and GCR-associated gene polymorphisms and breast cancer characteristics. None of the associations from the present analysis remained statistically significant after adjusting for multiple comparisons, but several SNPs in the GCR, FKBP5, ADIPOQ, IL-6, and BcL-2 genes had interesting results.

# TABLE VI
SUMMARY OF SIGNIFICANT ASSOCIATIONS BETWEEN GENETIC VARIANTS AND BREAST CANCER

| | Breast cancer characteristics | | | | | |
|---|---|---|---|---|---|---|
| | Grade | | Stage | | ER/PR | |
| **Self-reported race/ethnicity** | **High grade** | **Low Grade** | **Late Stage** | **Early Stage** | **ER or PR Positive** | **ER and PR Negative** |
| **nH white** | **IL-6** rs1800797<br>**GCR** rs33388, rs6191 | -<br>**GCR** rs41423247 | | | | **GCR** rs12656106 |
| | 3 significant SNPs/49 comparisons=6%[a] | | | | 1 significant SNP/49 comparisons=2%[a] | |
| **nH Black** | **GCR** rs10052957, rs258813, rs2918418, rs33388, rs41423247, rs6188, rs619, rs9324924 | **GCR** rs10482616, rs10482672, rs7701443<br>**FKBP5** rs9296158 | **GCR** rs10482614 **FKBP5** rs3777747 | **FKBP5** rs3800373, rs9296158, 9470080 | **GCR** rs10482616, **ADIPOQ** rs1501299<br>**BCL2** rs2279115 | |
| | 8 significant SNPs/52 comparisons=15%[a] | | 5 significant SNPs/52 comparisons=10%[a] | | 3 significant SNPs/52 comparisons=6%[a] | |
| **Hispanic** | **GCR** rs9324924 | | **ADIPOQ** rs266729 | **ADIPOQ** rs1501299 | | |
| | 1 significant SNPs/49 comparisons=2%[a] | | 2 significant SNPs/49 comparisons=4%[a] | | | |

[a]Percentage of significant results compared to the number of comparisons: (number of significant results ($p<.05$)/total number of comparisons)*100

## II. SPECIFIC AIM 2

### A. Level and Subcellular Localization of the Glucocorticoid Receptor

Glucocorticoid, one of the primary mediators of stress, acts via its cytoplasmic receptor, the GCR, to regulate a myriad of physiological processes. Variations in GCR expression have been previously reported in malignant tissues. The main hypothesis is that alterations in the level or subcellular localization of GCR might contribute to breast cancer development or progression. **Method**: Cases from an urban, multiracial cohort were used to build tissue microarrays for the IHC study. We used digital image analysis to measure GCR protein expression and examined its association with breast cancer characteristics. **Results**: We observed nuclear and cytoplasmic GCR staining in normal ductal and lobular myoepithelial cells, whereas luminal cells were negative. The GCR staining significantly decreased in breast cancer tissue. We found that low nuclear GCR expression was associated with basal cell marker cytokeratin 5/6 positivity ($p<.001$). However, GCR expression was not associated with other breast cancer characteristics. Multivariate analyses showed that the basal cell marker was the strongest predictor for nuclear GCR positivity even after adjustment for self-reported race, stage, grade, age, histological category, and family history of early breast cancer. **Conclusion:** In this study, we show that GCR expression is reduced in breast cancer tissue and correlated with the basal cell marker, CK5/6 expression.

### B. Background

Breast cancer, one of the leading causes of death among women, is a complex disease where genetic, epigenetic, and environmental factors have been implicated in initiation and progression. Psychosocial stress has been hypothesized to play a role in the etiology of breast

cancer and prior prospective and retrospective epidemiological studies have been carried out to examine this relationship with conflicting findings. Although the majority of studies reported no association (Roberts et al., 1996; Lillberg et al., 2001; Kroenke et al., 2004; Michael et al., 2009; Surtees et al., 2010), some studies found a positive association (Helgesson et al., 2003; Lillberg et al., 2003), and surprisingly, a few reported a negative relationship between psychosocial stress and breast cancer risk (Kroenke et al., 2004; Nielsen et al., 2005). Furthermore, systematic reviews and meta-analyses of these studies (McKenna et al., 1999; Duijts et al., 2003; Nielsen and Gronbaek, 2006; Ribeiro et al., 2009; Antonova et al., 2011) were equivocal. A limitation of the literature had been the lack of epidemiological studies attempting to link psychosocial factors to biologically plausible intermediates. Although further downstream signals converting psychosocial stress into cellular dysregulation and finally into breast cancer is still unknown, several animal and in vitro studies have implicated glucocorticoid hormones in this process (Strange et al., 2000; Stark et al., 2002; Antonova and Mueller, 2008; Hermes and McClintock, 2008; Amirian et al., 2010; Nyante et al., 2011). Glucocorticoids play an important role in several cellular processes, including apoptosis, inflammation, mammary development, and tumorigenesis (Reichardt et al., 2001; Vilasco et al., 2011). Exposure to stress activates the HPA axis, instigating physiological responses leading to allostatic or adaptive responses including secretion of glucocorticoids from the hypothalamus; glucocorticoid exerts a negative feedback loop to discontinue the HPA response (McEwen, 1998; Pacak and Palkovits, 2001). However, chronic stimulation of the allostatic responses could lead to loss of this feedback loop resulting in greater allostatic load (the wear and tear cost of adaptation) that may have cumulative long-term adverse effects in the body leading to disease (McEwen, 1998; McEwen and Seeman, 1999; Pacak and Palkovits, 2001). During allostasis, glucocorticoids have protective effects on the

organism by regulating immune function, promoting memory of dangerous events, increasing blood pressure and heart rate to meet the physical demands of a fight or flight response, and making fuel available for sustaining increased physical activity. But prolonged stress-response conditions have been shown to predispose for immune dysfunction and cancer, including breast cancer (Holden et al., 1998; Strange et al., 2000; Vanitallie, 2002).

Glucocorticoid signaling is mediated through the functional isoform, GCRα, a member of nuclear receptor subfamily 3, group c, receptors. It resides predominantly in the cytoplasm in an inactive form in association with a chaperone complex that serves to stabilize the unbound receptor (Pariante and Miller, 2001). Upon binding to glucocorticoid, the chaperone complex dissociates, allowing GCR phosphorylation and translocation to the nucleus where it binds to GREs in target genes and regulates gene transcription in a cell-specific manner (Wiench et al., 2011). Thus, the expression and cellular location of GCR is important for appropriate glucocorticoid signal transduction (Oakley and Cidlowski, 2013).

The GCR is present in all tissue types and is predominantly expressed in myoepithelial cells in normal breast tissue and in all stages of breast cancer; however, the relationship between breast cancer progression and GCR expression and subcellular localization appears inconsistent. A wide range of GCR levels (0 to 90% positive cells) in the cytoplasmic and or nuclear compartments has been previously reported in breast cancer tissue (Lien et al., 2006; Conde et al., 2008; Belova et al., 2009; Buxant et al., 2010). We have undertaken this study to reexamine GCR expression because of these divergent results. We examined 287 invasive breast cancer samples constructed in a tissue microarray using multispectral digital imaging technology to investigate GCR expression and subcellular localization in a series of breast cancer cases with defined clinical and histological characteristics. The main hypothesis was that alterations in the

level of expression or subcellular localization of GCR are associated with breast cancer subtype or aggressiveness.

## C. Materials and Methods

### 1. Study population and biological samples

Patients and samples for this study are from the BCCC study, a population-based cross-sectional study of breast cancer cases with primary invasive breast cancer diagnosed between October 1, 2005 and February 29, 2008 from Chicago conducted by the UIC Center for Population Health and Health Disparities (NCI grant5 P50 CA 106743). The parent study protocol was approved by the UIC Institutional Review Board (IRB#2010-0519). Details of this study have been previously published (Rauscher et al., 2010). We obtained paraffin-embedded surgical samples of the tumor prior to initiation of any radiation, chemotherapy or hormone therapy from the diagnosing hospitals from breast cancer cases. The available clinical information included age at diagnosis, tumor stage at diagnosis, histologic grade, estrogen and progesterone receptor positivity and HER2/neu test results.

### 2. Construction of the tissue microarray

Three tissue microarrays were constructed from the BCCC breast cancer cases subcohort. Tumor tissue sufficient for IHC analyses was available for analyses for 287 women (29% of total BCCC cohort). We included 26 normal breast tissues from unaffected women obtained by reduction mastectomy procedures and five fibroadenomas from UIC Medical Center in the tissue microarray. Eligible unaffected women were nH White, nH Black, or Hispanic women aged 30–79. All samples used for this study were clinical samples that had been fixed in 10% formalin and embedded in paraffin. For each breast cancer case, a representative area of invasive breast cancer was identified by a trained study pathologist on hematoxylin- and eosin

stained sections and marked on the corresponding paraffin blocks for the creation of tissue microarrays (TMAs). Triplicate tissue cores (0.6 mm) were removed from the "donor" blocks and inserted into "recipient" paraffin blocks in a 1 mm spacing pattern using a Tissue Microarrayer (Beecher Instruments, Silver Spring, Maryland ).

3. **Immunohistochemical staining**

Serial sections from the TMAs were cut, deparaffinized, rehydrated, and subjected to the appropriate antigen retrieval method. Sections were then incubated with the appropriate primary and secondary antibody and visualized with 3,3-diaminobenzidine (DAB) and hematoxylin (counterstain). Immunohistochemical staining, performed by the UIC Research Histology and Tissue Imaging Core Facility, was optimized by testing different sources and dilutions of the primary antibody, and different methods of antigen retrieval. Additional staining was conducted for ER, PR, HER2/neu, CK5/6, and epidermal growth factor receptor (EGFR) in the surgical pathology laboratory at the University of Illinois Hospital using clinically validated antibodies and standard IHC staining procedures. Positive and negative controls were included in each assay series. A list of antibodies for IHC staining is summarized in Table VII.

**TABLE VII**
LIST OF ANTIBODIES FOR IMMUNOHISTOCHEMICAL STAINING

| Antigen | Manufacturer | Host | Clone # | Dilution | Retrieval Method |
|---|---|---|---|---|---|
| **GCR** | Lecia/Novocastra | Mouse | 4H2 | 1:25 | HIER |
| **IHC subtyping antibodies panel:** | | | | | |
| **ER** | Ventana | Rabbit | SP1 | Predilute | CC1 Mild |
| **PR** | Ventana | Rabbit | 1E2 | Predilute | CC1 Mild |
| **HER2** | Ventana | Mouse | 4B5 | Predilute | CC1 Mild |
| **CK 5/6** | DAKO | Mouse | D5 & 16B4 | 1:50 | HIER |
| **EGFR** | Ventana | Mouse | 3C6 | Predilute | CC1 Mild |

Abbreviations: HIER: Heat-induced epitope retrieval; CC1: cell conditioning solution 1.

4. **Manual and digital scoring**

For digital analysis, the Vectra® ( PerkinElmer, Waltham, Massachusetts ) multispectral image analysis system was used. The IHC-stained TMA slides were scanned at 20x with a Vectra slide scanner. The inForm® 2.0 software package was used to segment tissue compartments (epithelium versus stromal) and subcellular compartments (nucleus versus cytoplasm). The outcome of tissue segmentation for each core image was assessed by a trained pathologist. Then the target signals were quantitated within the selected tissue and subcellular compartment(s) of interest as optical density (OD) per unit of cytoplasmic or nuclear area. Separately, manual scoring was performed by a trained pathologist without knowledge of case outcomes. The GCR expression was evaluated based on the percentage of positive tumor cells and staining intensity. The H-score is a product of the percentage of cells (0–100%) in each intensity category (0, 1+, 2+, and 3+). The final score is on a continuous scale between 0 and 300. An average H-score of the triplicate cores was used during analysis. We recorded separate scores for nuclear and cytoplasmic staining.

5. **Molecular breast tissue subtyping**

We classified each breast cancer case by molecular subtype as determined by expression of ER, PR, HER2, CK5/6, and EGFR (Engstrom et al., 2013; Howland et al., 2013). Samples were scored as positive for ER or PR when 10% or more of tumor cell nuclei showed positive staining for the ER or PR, respectively. For HER2 and EGFR, American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guideline recommendations for HER2 testing in breast cancer were used with a membrane-staining score ranging from 0 to 3+ (Wolff et al., 2014). Briefly, a score of zero has no staining; 1+ has 10% of cells or less with faint, barely perceptible, incomplete cell membrane staining; 2+ has at least 10% of cells with complete, weak-to-moderate cell membrane staining; and 3+ has at least 10% of cells with circumferential, complete, and intense membrane staining. The HER2 or EGFR was considered positive when the score was 3+. The CK5/6 was scored as 0 (negative), R (rare—single cells stain), 1+ (5%–30% cells stain), 2+ (31%–60% cells stain) and 3+ (more than 60% of cells stain) (Dabbs et al., 2006). Any staining (1+ to 3+) was considered to be a positive result for CK5/6. From these results, breast cancers were classified as Luminal A ($ER^+$ or $PR^+/HER2^-$), Luminal B ($ER^+$ or $PR^+/HER2^+$), HER2 enriched ($ER^-/PR^-/HER2^+$), and triple negative ($ER^-/PR^-/HER2^-$). The triple negative cases were further subdivided into Basal-like phenotype ($CK5/6^+$ and/or $EGFR^+$) or non-basal-like ($CK5/6^-$ and $EGFR^-$). The IHC labeling was scored by a single investigator after a consensus was reached about cutoff levels with an experienced pathologist behind a multiheaded microscope.

6. **Statistical analysis**

We performed $\chi^2$ test for dichotomous variables and a one-way ANOVA model for continuous variables. Race/ethnicity was defined through self-identification and categorized

as nH White, nH Black, and Hispanic. The primary response variable was GCR expression, evaluated as continuous H-scores (scale: 0–300). We estimated Spearman coefficients and conducted linear regression models to assess correlations. Bland-Altman plots were then used to assess the extent of agreement between manual and digital scores. The GCR expression was dichotomized at the median to assess associations with our outcome variables. We used stage at diagnosis, hormone receptor status, and histologic grade (each abstracted from patient medical records) as markers of breast cancer progression or aggressiveness. Stage at diagnosis was categorized using the American Joint Committee on Cancer (AJCC) categories (0–4), with later stage at diagnosis defined as stage >=2 versus <=1. Histological grade was determined through the Nottingham grading system and defined as low, intermediate, and high. Higher grade was defined as grade intermediate and high versus low. The ER/PR status was defined as positive if the tumor contained either ER and/or PR receptors, and negative in the absence of both receptor types. Molecular subtypes were categorized as Luminal A, Luminal B, HER2+, and triple negative. Body mass index (BMI) was calculated as measured weight (kg) divided by measured height (m) squared and categorized according to Centers for Disease Control and Prevention (CDC) guidelines. We also fit logistic regression models to estimate the ORs and 95% CI. All reported p-values are two-sided and a p-value <.05 was considered statistically significant. Statistical analyses were conducted using Stata version 11 (College Station, Texas). The “baplot” command was used to plot the Bland-Altman graph and the aaplot was used to plot pairwise comparisons in STATA.

## D. Results

### 1. Characteristics of the cohort in the tissue microarray study

We measured GCR protein expression in breast cancer tissue from 287 cases. Descriptive statistics of this subset are summarized in TABLE VIII. We had valid results from 267 breast cancer cases with data from at least one of the triplicate cores available. The mean age at diagnosis was 56 years (SD±11), and cases consisted of 103 nH Black, 84 nH White, and 80 Hispanic patients; 79% were overweight or obese while 83% were postmenopausal. The valid samples included breast cancers of various subtypes and tumor progression stages. The majority of the cases were of the ductal histological type, luminal A molecular subtype, ER and/or PR positive.

### 2. Glucocorticoid receptor expression and subcellular localization

We analyzed the protein expression of GCR in both nuclear and cytoplasmic compartments using TMAs containing our BCCC breast cancer subcohort (267 cases) in addition to 26 samples of normal tissue from reduction mammoplasty and five samples of fibroadenoma. Representative images of nuclear GCR staining intensity in normal, fibroadenoma, and cancerous breast tissue along with digital imaging annotation are shown in Figure 4. In normal breast tissue, GCR was expressed predominantly in the nuclei of the myoepithelial cell layer that surrounds normal ducts and lobules. The luminal layer in normal breast tissue was negative for GCR. Among the fibroadenoma samples, GCR staining was not limited to the myoepithelial layer, as nuclear and cytoplasmic staining of luminal epithelial cells was also detected.

In breast cancer tissue, there was diffuse GCR staining throughout the cancer foci which is more likely to be attributed to the loss of normal glandular architecture and outlining myoepithelial cells.

# TABLE VIII
DISTRIBUTION OF DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF CASES

| BCCC Cohort Characteristics | N | % cases |
|---|---|---|
| **Self-Reported Race/Ethnicity** | 267 | |
| nH Blacks | | 39 |
| nH Whites | | 32 |
| Hispanics | | 30 |
| **Age at Diagnosis** | 224 | |
| Less than 50years | | 31 |
| Equal or greater than 50 years | | 69 |
| **Any Early (<50y) Family History Breast Cancer** | 223 | |
| No | | 94 |
| Yes | | 6 |
| **Menopausal** | 223 | |
| No | | 17 |
| Yes | | 83 |
| **CDC BMI Categories** | 265 | |
| Normal weight (18.5–24.9) | | 21 |
| Overweight (25.0–29.9) | | 37 |
| Obese (≥30.0) | | 42 |
| **Stage at Diagnosis** | 264 | |
| 0,1 (early stage) | | 42 |
| 2,3,4 (late stage) | | 58 |
| **Histologic Grade** | 261 | |
| Low/intermediate | | 62 |
| High | | 38 |
| **ER/PR Status** | 233 | |
| ER and/or PR Positive | | 77 |
| Double negative | | 23 |
| **Histological Subtypes** | 253 | |
| Ductal carcinoma | | 76 |
| Lobular carcinoma | | 12 |
| Mixed ductal/ lobular carcinoma | | 8 |
| Other | | 6 |
| **Molecular Subtypes** | 254 | |
| Luminal A | | 69 |
| Luminal B | | 6 |
| HER2 enriched | | 8 |
| Triple Negative | 44 | 17 |
| Basal-like | | 55 |
| Non-Basal-like | | 45 |

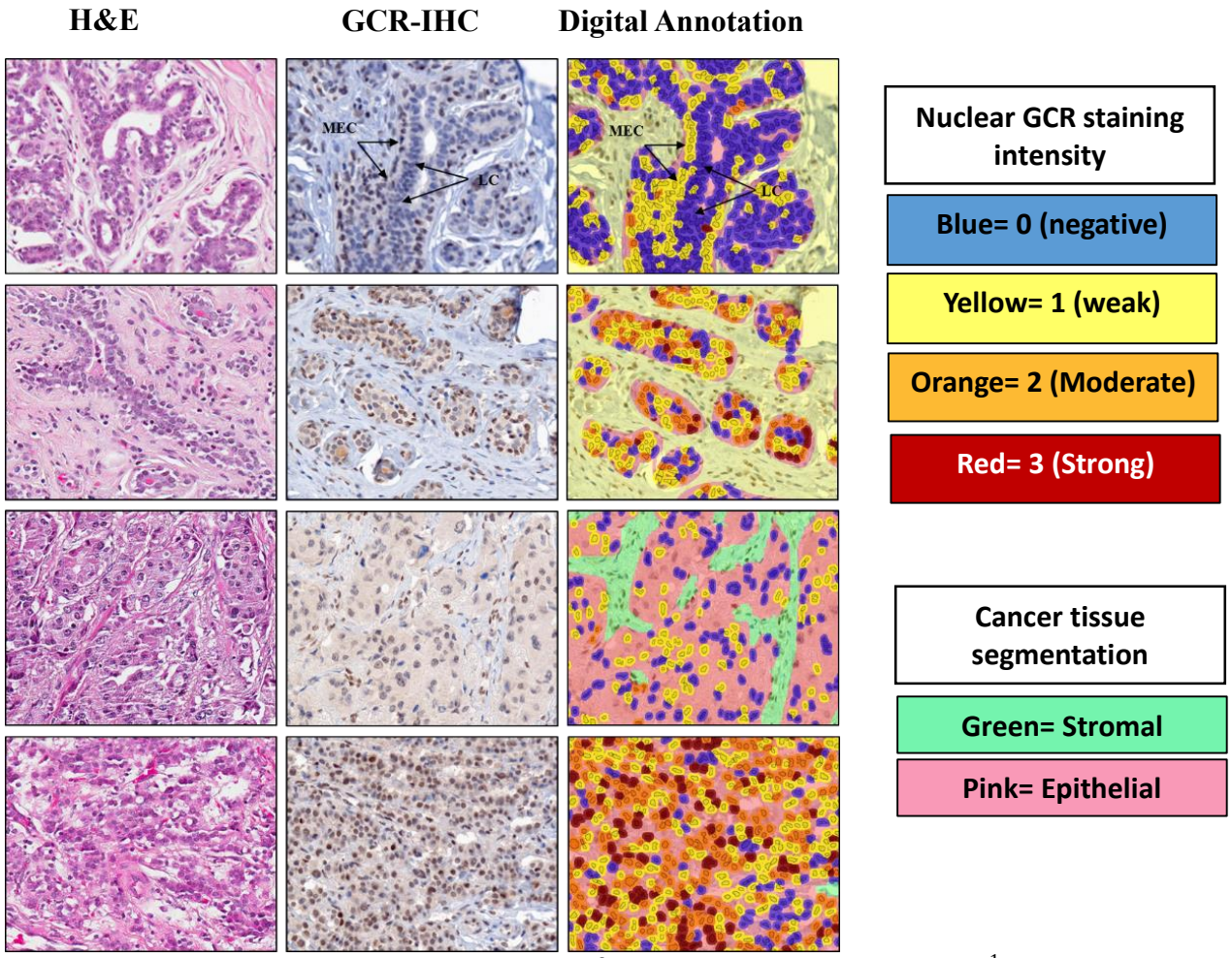


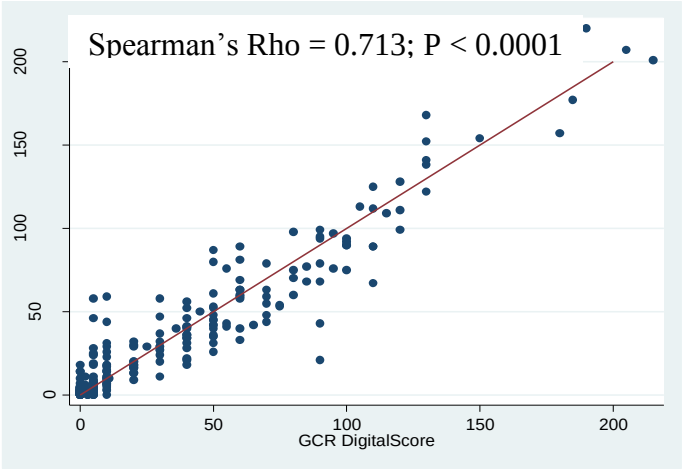

Figure 4. Immunohistochemical staining and digital output images for GCR in representative cases[1].

[1] For each case, H&E and the corresponding anti-GCR IHC image and digital analysis output are shown. (A) normal breast tissue, (B) fibroadenoma, (C) ductal carcinoma and (D) lobular carcinoma. *LC, luminal cells; MEC, myoepithelial cells.*

3. **Manual versus digital scoring**

As GCR is predominately a nuclear receptor, we used nuclear expression of GCR to compare manual versus digital scoring (Figure 5). There was a strong correlation between manual and automated scoring (R=.71; P<.0001). Bland-Altman plot and the Pitman's test of variance (p=.135) indicated that scoring using Vectra multispectral digital analysis system was similar to a pathologist visual scoring for GCR in breast cancer TMAs.

(A)




(B)

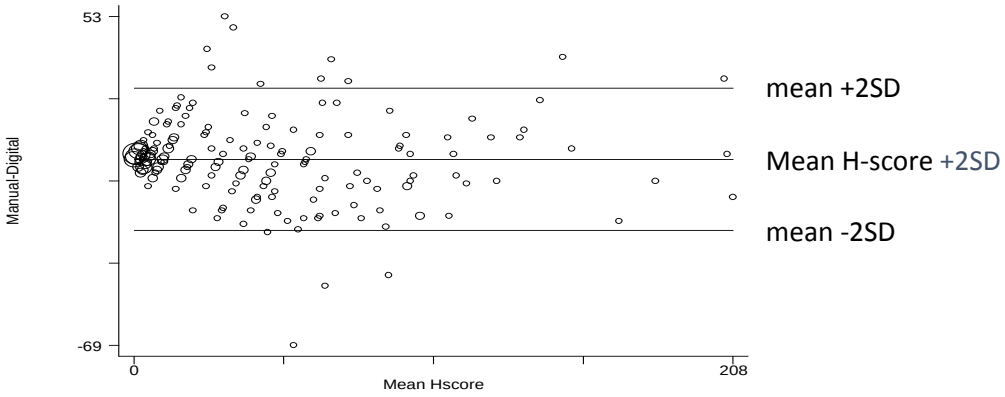



Figure 5. Manual versus digital scoring.[1]

[1] A) A plot of manual versus digital score for nuclear GCR showing a good correlation between the two measures. (B) Bland-Altman plot comparing manual score versus digital score of nuclear GCR expression.

**TABLE IX**
SUMMARY OF NUCLEAR AND CYTOPLASMIC GLUCOCORTICOID RECEPTOR EXPRESSION

| Breast tissue | N | Mean Nuclear H-Score | Nuclear GCR Staining (%>50) [a] | Mean Cytoplasmic H-Score |
|---|---|---|---|---|
| **Reduction Mammoplasty** | 26 | 83 | 77 | 20 |
| **Fibroadenoma** | 5 | 91 | 80 | 5 |
| **Breast Cancer** | 253 | 29 | 19 | 3 |
| | | p=.0053 | p<.0001 | p<.0001 |
| **Histological Breast Cancer Subtypes** | | | | |
| Ductal Carcinoma | 191 | 27 | 17 | 3 |
| Lobular Carcinoma | 29 | 36 | 28 | 4 |
| Mixed & Other | 33 | 32 | 21 | 2 |
| | | p=.13[b] | p=.43[c] | p=.91[b] |
| **Molecular Breast Cancer Subtypes** | | | | |
| Luminal A | 175 | 27 | 17 | 3 |
| Luminal B | 14 | 41 | 29 | 7 |
| Triple Negative | 45 | 26 | 20 | 8 |
| HER2 | 21 | 14 | 5 | 0.05 |
| | | p=.14[b] | p=.29[c] | p=.32[b] |

[(a)] Percentage positivity: A tissue was considered positive for nuclear GCR when the sample had an H-score >50
[(b)] F-test p-value.
[(c)] Chi-square p-value.

Expression of GCR was detected in both the cytoplasm and nuclear compartments of normal and breast cancer tissue. Despite the low expression of GCR in the cytoplasm relative to the nuclear compartment, there was a strong correlation between nuclear and cytoplasmic H-scores. (Spearman's Rho=.80; p<.00001) and a pairwise comparison showed similar results (Figure 6).

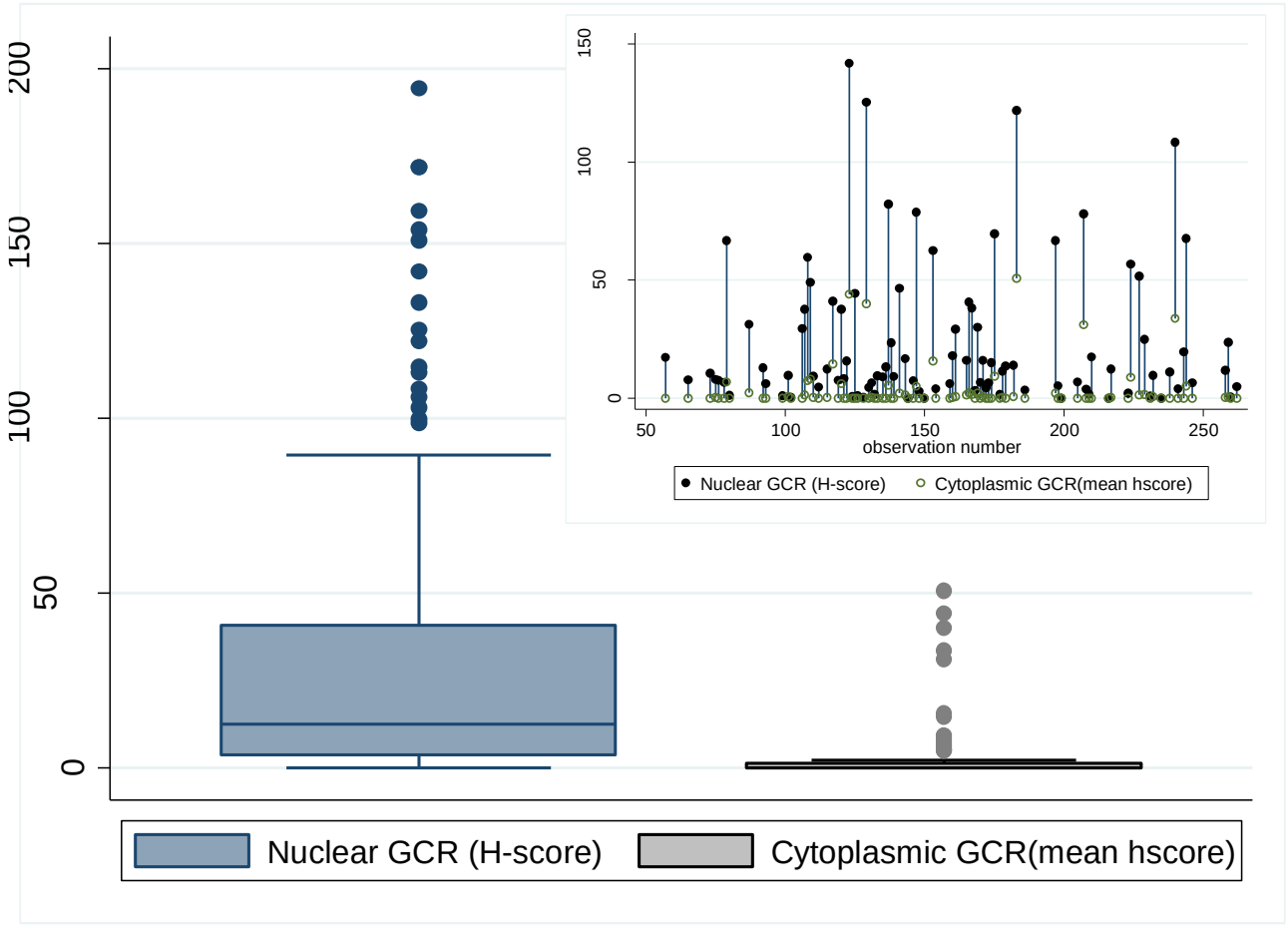



Figure 6. Summary of the nuclear and cytoplasmic GCR staining[1].

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[1](Inset) A plot of pairwise manual versus digital score for nuclear GCR showing a good correlation between the two measures.

The GCR staining (mean H-score) decreased in cancer tissue compared with normal tissue and fibroadenoma. When we dichotomized nuclear H-scores by their median value among breast cancer cases into low and high categories, only 19% had positive nuclear staining as opposed to 77% and 80% in normal breast tissue and fibroadenoma, respectively. In breast cancer tissue, cytoplasmic staining (mean H-score=3) was weaker than nuclear staining (mean H-score=29), as 57% of the breast cancer TMA cores had H-score=0 for cytoplasmic GCR. We did not observe a statistically significant difference in GCR staining among breast cancer subtypes. However, lobular carcinoma had the highest level of nuclear GCR expression (mean H-score: 36% versus 29% in ductal carcinoma) and percentage of nuclear positive cases (28% versus 17% in ductal). We also found that the majority of cases with basal-like subtype ($ER^-/PR^-/HER2^-/CK56^+/EGFR^+$) had low GCR expression.

4. **Correlation between nuclear glucocorticoid receptor expression and patient characteristics**

Positive nuclear GCR expression was weakly associated with any early family history of breast cancer (p=.069) but was not associated with self-reported race, BMI, nulliparity, menopausal status, stage or grade at diagnosis, or subtypes of breast cancer (TABLE X).

# TABLE X

## ASSOCIATION BETWEEN NUCLEAR GCR EXPRESSION AND DEMOGRAPHIC AND BREAST CANCER CHARACTERISTICS

| | All Cases | High GCR | P-value [a] |
|---|---|---|---|
| **Race/Ethnicity** | **n** | **%** | 0.549 |
| nH Blacks | 103 | 22 | |
| nH Whites | 84 | 18 | |
| Hispanics | 80 | 16 | |
| **Age at Diagnosis** | | | 0.599 |
| Less than 50 years | 70 | 17 | |
| Greater or Equal to 50 years | 154 | 20 | |
| **Nulliparous** | | | 0.865 |
| Yes | 44 | 18 | |
| No | 223 | 19 | |
| **Menopause Status** | | | 0.693 |
| Yes | 186 | 22 | |
| No | 37 | 19 | |
| **Any Early (<50y) Family History Breast Cancer** | | | 0.069 |
| Yes | 13 | 0 | |
| No | 210 | 21 | |
| **Stage at Diagnosis** | | | 0.133 |
| Early Stage (0,1) | 110 | 24 | |
| Late Stage (2,3,4) | 154 | 16 | |
| **Histological Grade** | | | 0.307 |
| Low/Intermediate | 161 | 21 | |
| High | 100 | 16 | |
| **Histology Subtype** | | | 0.394 |
| Ductal Carcinoma | 191 | 17 | |
| Lobular Carcinoma | 29 | 28 | |
| Mixed and Other | 33 | 21 | |
| **Positive for HER2** | | | 0.21 |
| Negative | 129 | 22 | |
| Positive | 27 | 11 | |
| **IHC Variables:** | | | |
| **IHC Molecular Subtypes** | | | 0.285 |
| Luminal A | 175 | 17 | |
| Luminal B | 14 | 29 | |
| Triple Negative | 45 | 20 | |
| HER2 Enriched | 21 | 5 | |
| **CK5/6** | | | 0.001 |
| Negative | 163 | 12 | |
| Positive | 93 | 28 | |

[(a)] Chi-square p-value.

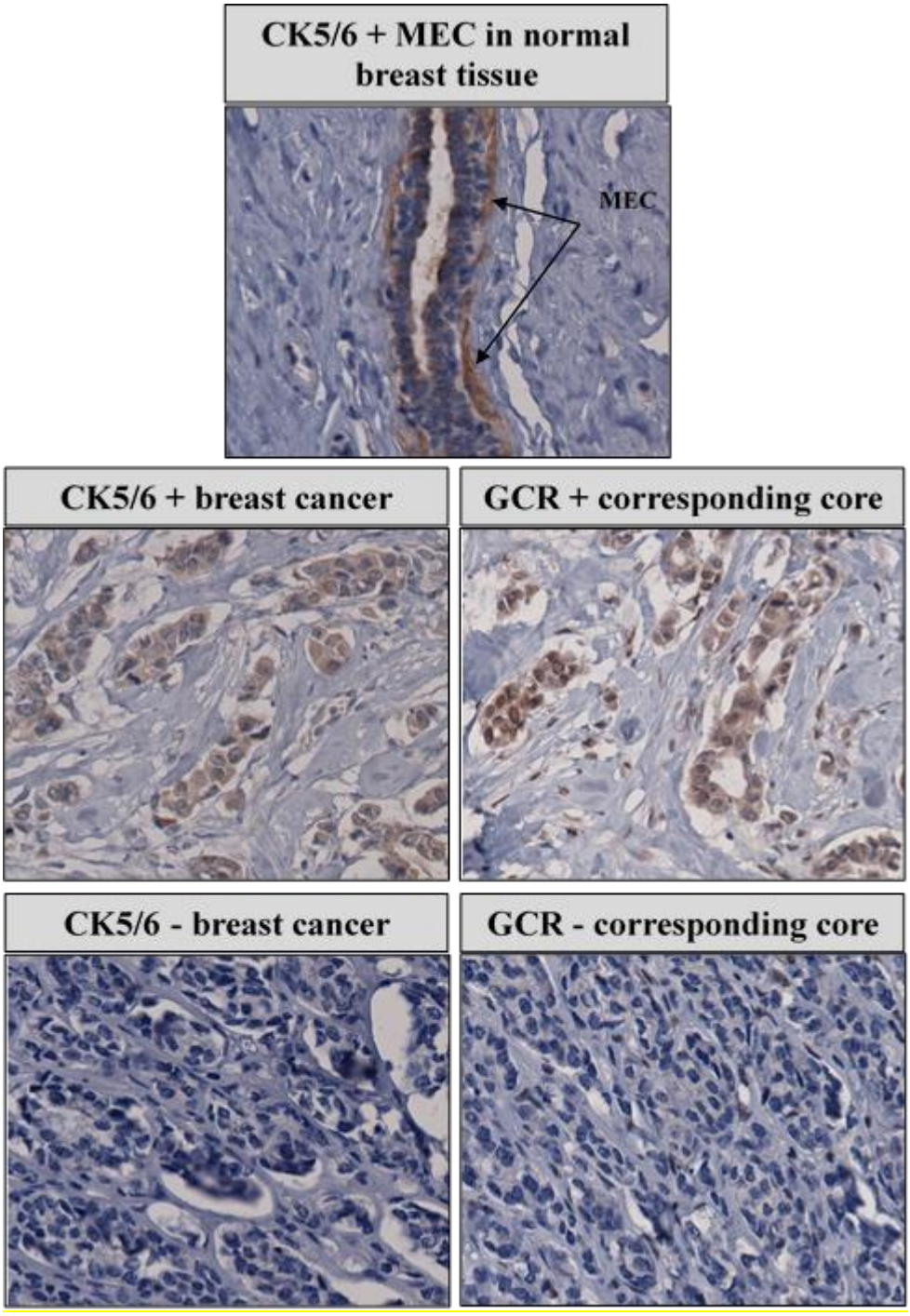



Figure 7. Correlation between CK5/6 and GCR expression in representative cases.

5. **Correlation between nuclear glucocorticoid receptor and cytokeratin 5/6 expression**

In our IHC study, nuclear GCR staining strongly correlated with cytoplasmic CK 5/6 expression, a marker of the basal nature of the tumor (TABLE XI).

There was a statistically significant difference in the mean H-score of nuclear GCR among CK5/6 positive (mean=36) and CK5/6 negative (mean=19) samples. Multivariate analysis with logistic regression adjusting for race; age at diagnosis; and stage, grade, and histological category revealed that high GCR expression was associated with CK5/6 expression (OR, 3.3; 95% CI, 1.6–6.9) TABLE XI. The CK5/6 was not associated with race/ethnicity, age at diagnosis, hormone receptor status, stage and grade at diagnosis, or subtypes of breast cancer.

**TABLE XI**
THE CORRELATION BETWEEN GCR POSITIVITY AND CK 5/6 EXPRESSION IN BREAST CANCER CASES

| GCR Positivity | Odds Ratio | 95% Confidence Interval | |
|---|---|---|---|
| **CK 5/6 Positivity** | 3.7 | 1.3 | 10.3 |
| **Self-Reported Race (nH White as Reference)** | | | |
| nH Black | 0.9 | 0.3 | 2.7 |
| Hispanic | 0.3 | 0.1 | 1.1 |
| **Greater than 50y at Diagnosis** | 2.0 | 0.6 | 6.5 |
| **High Grade at Diagnosis** | 0.8 | 0.3 | 2.6 |
| **Late Stage at Diagnosis** | 1.2 | 0.5 | 3.3 |
| **HER2 Positivity** | 1 | 0.2 | 5.2 |
| **Histological Subtypes (Ductal as Reference)** | | | |
| Lobular | 1.7 | 0.4 | 6.3 |
| Mixed & Other | 1.7 | 0.4 | 7.8 |

## E. **Discussion**

Glucocorticoid signaling via GCR regulates a myriad of physiological processes including those involved in mammary development and differentiation. We found that GCR was expressed in 100% of our normal and fibroadenoma samples and was mainly localized in the myoepithelial cells. There was a marked reduction in nuclear GCR expression in breast cancer tissue compared to normal or benign breast tissue lesions that might be due to disruption of the myoepithelial cell layer and basement membrane during tumor invasion (Khamis et al., 2012). Our findings could reflect either that GCR is involved in a biologic pathway leading to breast cancer or is a marker of other causal mechanisms associated with breast cancer development. It has been shown to promote both cell survival and cell death depending on the cell type. Based on our findings, we propose that GCR has tumor suppressor role in breast cancer. The down-regulation of the nuclear GCR was also observed in prostate cancer, another hormone-sensitive

tumor (Yemelyanov et al., 2007). It would be important to compare GCR levels from adjacent histologically normal areas, in situ and invasive components from the same patient to examine expression changes in the course of breast tumorigenesis.

Several studies from different countries across a range of ethnic groups have detected both cytoplasmic and nuclear GCR expression using either monoclonal (Conde et al., 2008; Belova et al., 2009; Buxant et al., 2010) or polyclonal antibody against GCR (Lien et al., 2006). Our results are in agreement with the pattern of GCR expression in these reports in that nuclear GCR expression decreased in breast cancer tissue. However, we did not observe a decrease in nuclear GCR expression and an increase of cytoplasmic GCR with tumor progression (Conde et al., 2008). We found that cytoplasmic GCR positively correlated with nuclear GCR expression. Unlike one of these studies (Belova et al., 2009), we did not find any correlation between GCR expression and age at diagnosis or histological and molecular subtypes of breast cancer.

We observed a strong correlation between GCR and the myoepithelial marker, CK5/6. The CK5/6 are found in the cells of the basal layer of normal breast ducts (Gusterson et al., 2005). Expression of CK5/6 has been associated with poor breast cancer prognosis (Abd El-Rehim et al., 2004). However, neither GCR nor CK5/6 were associated with race/ethnicity, age at diagnosis, hormone receptor status, stage and grade at diagnosis, or subtypes of breast cancer.

Strength of this TMA cohort is that it is a population-based study of breast cancer patients and therefore may be generalizable to an urban population. Another strength was the availability of detailed demographic and clinical data. The cross-sectional nature of this study prohibited the ability to assess temporal aspects of these associations. There were also limitations in the TMA and IHC staining technique used in this and other studies. Tissue stained might not

be representative of the tumor due to tumor heterogeneity, and the staining result can be affected by cross-reaction with other markers or other isoforms.

In conclusion, we observed a decrease in nuclear GCR expression between normal or benign breast lesions and tumor tissue, and a positive association in breast cancers between GCR expression and the basal cell marker, CK5/6. It will be important to understand the molecular mechanisms that underlie the decrease of GCR expression in breast cancer and its association with CK5/6.

# III. SPECIFIC AIM 3

**Background**: We have previously shown that the expression of the GCR was reduced in invasive breast carcinoma. The GCR regulates apoptosis via a wide array of target genes such as SGK1 and Bcl-2. Apoptosis is both a physiological and pathological process of cell death. The purpose of this study was to understand the relation between the reduction of GCR and the expression of SGK1 and Bcl-2 in primary breast cancer tissue. **Method**: Paraffin-embedded tumor tissue from an urban, multiracial cohort were used to quantify the level of SGK1 and Bcl-2. We also examined their association with breast cancer characteristics. **Results**: Higher SGK1 observed in breast cancer tissue compared with normal tissue. In addition, SGK1 expression was present in higher proportion in $ER^-/PR^-$ than $ER^+$ and/or $PR^+$ cases (66% versus 50% p=.039). But SGK1 expression did not vary among breast cancer subtypes. Expression of Bcl-2 was reduced in breast cancer compared with normal tissue, and was present at a lower proportion in $ER^-/PR^-$ than $ER^+$ and/or $PR^+$ cases (7% versus 74% p<.0001). The relationship between lower Blc-2 expression and hormone receptor status was present even after adjustment for several demographic and clinical features. There was an inverse correlation between SGK1 and GCR expression that was not statistically significant and no association between Bcl-2 and GCR. **Conclusion:** We observed a high SGK1 and a low Bcl-2 expression in breast cancer tissue. However, GCR expression did not significantly correlate with either SGK1 or Bcl-2 expression in our sample.

## A. The Level of Glucocorticoid Receptor-Targets in the Invasive Component of Breast Tumors

Breast cancer, one of the leading causes of death among women, is a complex disease where genetic, epigenetic and environmental factors have been implicated in initiation and progression. We had previously demonstrated that GCR expression was reduced in breast tumor tissue (Aim 2). Glucocorticoid plays an important role in several cellular processes, including apoptosis, inflammation, mammary development and tumorigenesis (Reichardt et al., 2001; Vilasco et al., 2011).

One of the hallmarks of cancer is the ability of cancer cells to evade apoptosis to grow and spread in the body (Hanahan and Weinberg, 2000). Apoptosis, or programmed cell death, occurs when cells undergo programmed cell death as a result of physiological and pathological signals involving a cascade of molecular events mediated by a family of proteins called caspases (Elmore, 2007). Glucocorticoids are widely used for their pro-apoptotic effect but they also have anti-apoptotic effect on mammary cells (Moran et al., 2000; Wu et al., 2004). The mechanism behind GCR regulation of apoptosis is not fully understood and seems to vary depending on cell type.

The GCR was shown to signal through two target genes involved in the apoptosis pathway: SGK1 (Tessier and Woodgett, 2006) and Bcl-2 (Almawi et al., 2004). The SGK1 reduces apoptosis in breast cancer cell line (Wu et al., 2004; Wu et al., 2006), and has been shown to be overexpressed in human breast cancers, promoting their malignant growth (Adeyinka et al., 2002; Sahoo et al., 2005). In vitro overexpression of SGK1 in breast cancer cell lines decreased cellular adhesiveness (Tangir et al., 2004). Furthermore, a variant of SGK1 is associated with increased glucocorticoid-dependent gene expression and has a marked allele frequency difference between populations of African and European ancestry (Luca et al., 2009).

The Bcl-2, a member of the Bcl-2 family of proteins with important roles in apoptosis, has anti-apoptotic characteristics (Siddiqui et al., 2015) and promotes cell survival by inhibiting adapters needed for the activation and cleavage of caspases leading to cell death. However, high Bcl-2 expression in breast tumor tissue specimens was associated with favorable prognosis (Callagy et al., 2008). The main hypothesis is that downregulation of GCR might alter SGK1 and Bcl-2 levels, which may result in decreased apoptosis that might contribute to the progression or aggression of breast cancer. We used TMA technology to investigate a series of breast cancer cases with defined clinical characteristics.

## B. Materials and Methods

### 1. Study population and biological samples

Patients and samples for this study are from the BCCC study, a population-based cross-sectional study of breast cancer cases with primary invasive breast cancer diagnosed between October 1, 2005 and February 29, 2008 from Chicago conducted by the UIC Center for Population Health and Health Disparities (NCI grant5 P50 CA 106743). The parent study protocol was approved by the UIC Institutional Review Board (IRB#2010-0519). Details of this study have been previously published (Rauscher et al., 2010). We obtained paraffin-embedded surgical samples of the tumor prior to initiation of any radiation, chemotherapy, or hormone therapy from breast cancer cases from the diagnosing hospitals. The available clinical information included age at diagnosis, tumor stage at diagnosis, histologic grade, estrogen and progesterone receptor positivity, and HER2/neu test results.

### 2. Construction of the tissue microarray

Three TMAs were constructed from the BCCC breast cancer cases subcohort. Tumor tissue sufficient for IHC was available for analyses for 287 women (29% of total BCCC

cohort). We included 26 normal breast tissues from unaffected women obtained by reduction mastectomy procedures and five fibroadenomas from UIC Medical Center in the TMA. Eligible unaffected women were nH White, nH Black, or Hispanic women aged 30–79. All samples used for this study were clinical samples that had been fixed in 10% formalin and embedded in paraffin. For each breast cancer case, a representative area of invasive breast cancer was identified by a trained study pathologist on hematoxylin- and eosin-stained sections and marked on the corresponding paraffin blocks for the creation of TMAs. Triplicate tissue cores (0.6 mm) were removed from the "donor" blocks and inserted into "recipient" paraffin blocks in a 1 mm spacing pattern using a Tissue Microarrayer (Beecher Instruments, Silver Spring, Maryland ).

3. **Immunohistochemical staining**

Serial sections from the TMAs were cut, deparaffinized, rehydrated, and subjected to the appropriate antigen retrieval method. Sections were then incubated with the appropriate primary and secondary antibody and visualized with 3,3-diaminobenzidine (DAB) and hematoxylin (counterstain). Immunohistochemical staining, performed by the UIC Research Histology and Tissue Imaging Core Facility, was optimized by testing different sources and dilutions of the primary antibody, and different methods of antigen retrieval. Additional staining was conducted for ER, PR, HER2/neu, CK5/6, and EGFR in the surgical pathology laboratory at the University of Illinois Hospital using clinically validated antibodies and standard IHC staining procedures. Positive and negative controls were included in each assay series. A list of antibodies for immunohistochemical staining is summarized in TABLE XII.

## TABLE XII

LIST OF ANTIBODIES FOR IMMUNOHISTOCHEMICAL STAINING

| Antigen | Manufacturer | Host | Clone # | Dilution | Retrieval method |
|---|---|---|---|---|---|
| **GCR** | Lecia/Novocastra | Mouse | 4H2 | 1:25 | HIER |
| **SGK1** | Novus/Biologicals | Rabbit | NB100-92054 | 1:50 | CC1 Mild |
| **Bcl-2** | Cell Marque | Mouse | 124 | Predilute | CC1 Mild |
| **IHC subtyping antibodies panel:** | | | | | |
| **ER** | Ventana | Rabbit | SP1 | Predilute | CC1 Mild |
| **PR** | Ventana | Rabbit | 1.00E+02 | Predilute | CC1 Mild |
| **HER2** | Ventana | Mouse | 4B5 | Predilute | CC1 Mild |
| **CK5/6** | DAKO | Mouse | D5 & 16B4 | 1:50 | HIER |
| **EGFR** | Ventana | Mouse | 3C6 | Predilute | CC1 Mild |

Abbreviations: HCF: UIC Histology Core Facility; MC: Medical Center at Chicago Clinical Reference Surgical Pathology laboratory; HIER: Heat-induced epitope retrieval; CC1: cell conditioning solution 1.

### 4. **Immunohistochemical scoring**

Manual scoring was performed by a trained pathologist without knowledge of case outcomes. The SGK1 and GCR expression was evaluated based on the percentage of positive tumor cells and staining intensity. The H score takes into account the percentage of cells (0–100%) in each intensity category (0, 1+, 2+, and 3+) and computes a final score, on a continuous scale between 0 and 300. An average H score of the triplicate cores was used during analysis. For Bcl-2 scoring a semiquantitative scale was used, which grades the tumors from 0 to 3 depending on the number of tumor cells stained and the intensity of the reaction, where 0=totally negative, 1=<20% of the cells show a reliable staining, 2=20%–80% show strong staining, and 3=all cells are strongly positive.

5. **Molecular breast tissue subtyping**

We classified each breast cancer by molecular subtype determined by expression of ER, PR, and HER2. Samples were scored as positive for ER or PR when 10% or more of tumor cell nuclei showed positive staining for the ER or PR, respectively. For HER2, ASCO/CAP guideline recommendations for HER2-testing in breast cancer were used with a membrane-staining score ranging from 0 to 3+ (Wolff et al., 2014). Briefly, a score of zero has no staining; 1+ has 10% of cells or less with faint, barely perceptible, incomplete cell membrane staining; 2+ has at least 10% of cells with complete, weak-to-moderate cell membrane staining; and 3+ has at least 10% of cells with circumferential, complete, and intense membrane staining. The HER2 was considered positive when the score was 3+. From these results, breast cancers were classified as Luminal A ($ER^+$ or $PR^+/HER2^-$), Luminal B ($ER^+$ or $PR^+/HER2^+$), HER2 enriched ($ER^-/PR^-/HER2^+$), and triple negative ($ER^-/PR^-/HER2^-$) (Engstrom et al., 2013). The IHC labeling was scored by a single investigator after a consensus was reached about cut off levels with an experienced pathologist behind a multiheaded microscope.

6. **Statistical analysis**

Race/ethnicity was defined through separate self-identification and categorized as nH White, nH Black, and Hispanic. The primary response variables were IHC scores for GCR, SGK1, and Bcl-2. The scores were dichotomized at the median to assess association with our outcome variables: stage at diagnosis, hormone receptor status, and histologic grade (each abstracted from patient medical records). Stage at diagnosis was categorized using the AJCC categories (0–4) with later stage at diagnosis defined as stage $\geq 2$ versus $\leq 1$. Histological grade was determined through the Nottingham grading system and defined as low, intermediate, and high. Higher grade was defined as grade intermediate and high versus low. The ER/PR status

was defined as positive if the tumor contained ER and/or progesterone (PR) receptors, and negative in the absence of both receptor types. Molecular subtypes were categorized as Luminal A, Luminal B, $HER2^{+}$, and triple negative. To compare clinical and histopathological characteristics, we performed $\chi^2$ test for dichotomous variables and a one-way ANOVA model for continuous variables. We also fit logistic regression models to estimate the ORs and 95% CIs. All reported p-values were two-sided and a p-value <.05 was considered statistically significant. Statistical analyses were conducted using Stata version 11 (College Station, Texas ).

## C. **Results**

### 1. **Characteristics of the BCCC cohort in the tissue microarray study**

We performed an IHC analysis using SGK1 and Bcl-2 antibodies on a TMA set composed of 287 of breast tissue samples. Descriptive statistics of this subset are summarized in TABLE XIII. We had valid results from 272 breast cancer cases in triplicates with a mean age at diagnosis of 56 (SD±11) years. Among them were 108 nH Black, 81 nH White, and 83 Hispanic breast cancer cases. The valid samples included breast cancers of various subtypes and tumor progression stages. The majority of the cases were of the ductal type (76%); 68% were of the luminal A molecular subtype; 59% were diagnosed at a late stage (1, 2, or 3); 61% were at low/intermediate grade at diagnosis; and 74% were positive for either ER or PR. Immunohistochemical subtyping has shown that 18% of our cases had a triple negative phenotype.

# TABLE XIII

DISTRIBUTION OF THE DEMOGRAPHIC AND TUMOR-RELATED FACTORS OF CASES

| BCCC Cohort Characteristics | % Cases |
|---|---|
| **Self-Reported Race/Ethnicity (n=267)** | |
| nH Blacks | 39 |
| nH Whites | 32 |
| Hispanics | 30 |
| **Stage at Diagnosis (n=264)** | |
| 0,1 (early stage) | 42 |
| 2,3,4 (late stage) | 58 |
| **Histologic Grade (n=261)** | |
| Low/intermediate | 62 |
| High | 38 |
| **ER/PR status (n=233)** | |
| ER and/or PR Positive | 25 |
| Double Negative | 76 |
| **Histological Subtypes (n=253)** | |
| Ductal Carcinoma | 76 |
| Lobular Carcinoma | 12 |
| Mixed Ductal/ Lobular Carcinoma | 8 |
| Other | 6 |
| **Molecular Subtypes (n=254)** | |
| Luminal A | 69 |
| Luminal B | 6 |
| Triple Negative | 17 |
| HER2 enriched | 8 |

2. **Description of serine/threonine-protein kinase expression**

We observed a diffuse cytoplasmic staining of both the myoepithelial and luminal layers in normal breast tissue and fibroadenomas (Figure 9).

The SGK1 lacks the exclusive myoepithelial staining pattern of GCR. We detected cytoplasmic staining in breast cancer tissue in all the histological and molecular subtypes, but the mean H-score and percent positivity were lower in normal breast tissues compared with tumor and fibroadenoma samples—this difference was statistically significant. The SGK1 expression did not vary much among histological or molecular subtypes of breast cancer. The highest mean H-score was among subtypes associated with poor prognosis and low survival such as hormone-receptor-negative (66 mean H-score), triple negative (56 mean H-score), HER2+ (56 mean H-score), and mixed/other types (52 mean H-score) breast cancer subtypes. We observed the same upregulation of cytoplasmic SGK1 in fibroadenoma and all subtypes of breast cancer tissue compared with normal tissue when we categorized SGK1 H-score according to the median H-score (low $<30$ and high $\geq 30$). With this categorization, only 25% of the normal tissues were strongly positive for SGK1 compared with 50% among fibroadenoma and 53% among breast cancer tissue. We also found high nuclear GCR staining among samples with high cytoplasmic SGK1 staining, but this association was not statistically significant (TABLE XIV).

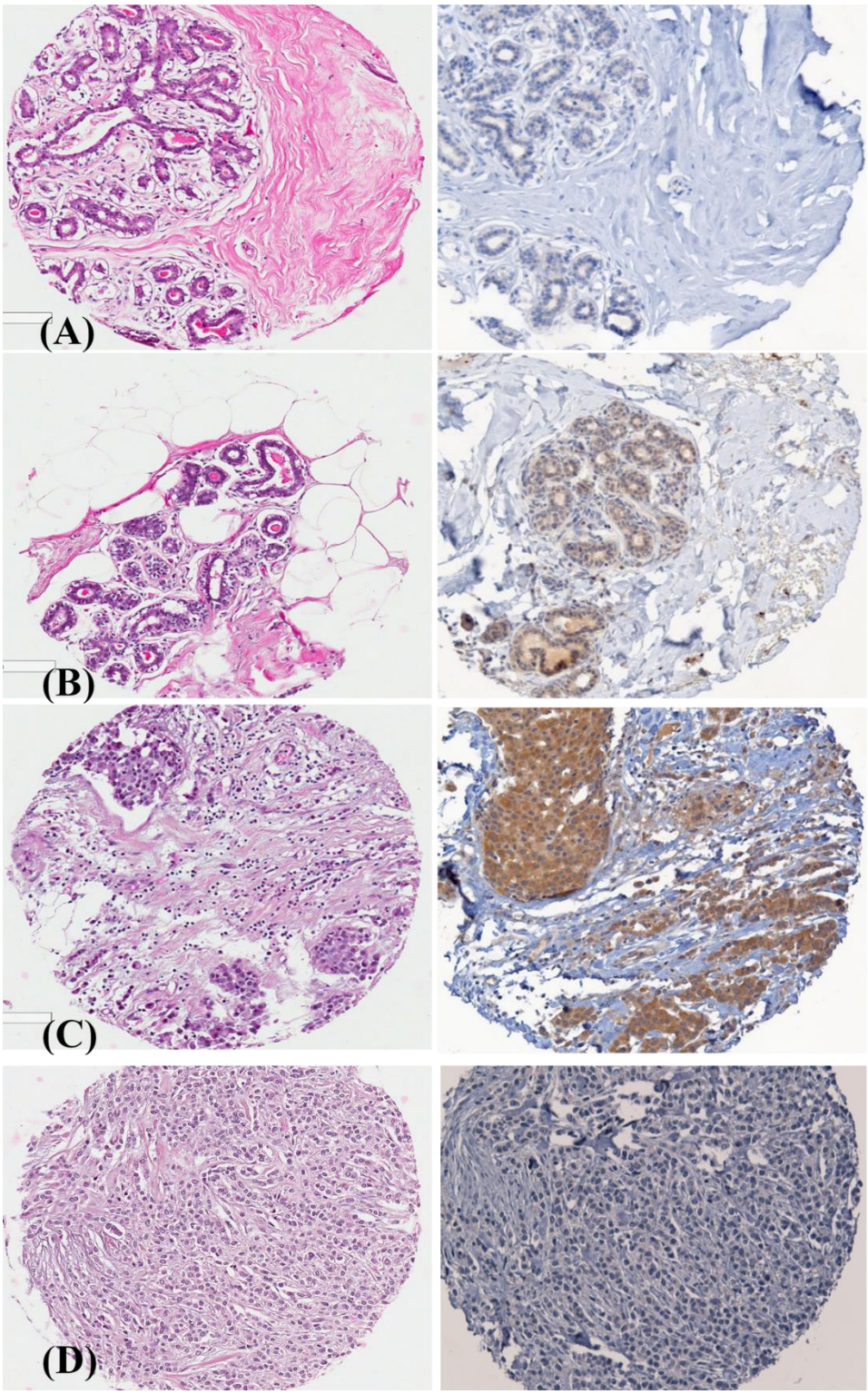



Figure 8. Immunohistochemical staining for SGK1 in representative cases of (A) normal breast tissue, (B) benign breast lesion (fibroadenoma), (C) invasive ductal carcinoma and (D) invasive lobular carcinoma.

3. **Description of B-cell lymphoma 2expression in normal and cancer breast**

Figure 9 shows representative case showing Bcl-2 staining in breast cancer, fibroadenoma, and normal breast tissue. The Bcl-2 staining was invariably cytosolic. We observed a strong cytoplasmic staining of both the myoepithelial and luminal layers in normal breast tissue and fibroadenomas. We also detected cytoplasmic staining in breast cancer tissue in all the histological and molecular subtypes. There was a statistically significant difference in the mean score and percent positivity between normal and fibroadenoma samples compared with tumor breast tissue.

# TABLE XIV

## SUMMARY OF SGK1 AND BCL2 EXPRESSION IN BREAST TISSUE

| | | SGK1 | | Bcl-2 | |
|---|---|---|---|---|---|
| **Breast tissue** | **N** | **Mean H-Score** | **Positivity (% >30) [a]** | **Mean Score** | **Positivity (% >2) [b]** |
| **Reduction Mammoplasty** | 24 | 20 | 25 | 1.97 | 91 |
| **Fibroadenoma** | 6 | 30 | 50 | 2 | 100 |
| **Breast Cancer** | 258 | 46 | 53 | 1.46 | 59 |
| | | p=.0154[c] | p=.017[d] | p=.004[c] | <p=.000[d] |
| **Histological Breast Cancer Subtypes** | | | | | |
| Ductal Carcinoma | 195 | 47 | 54 | 1.36 | 54 |
| Lobular Carcinoma | 28 | 29 | 39 | 1.69 | 79 |
| Mixed & Other | 35 | 52 | 57 | 1.8 | 67 |
| | | p=.1044[c] | p=.287[d] | p=.0383[c] | <p=.000[d] |
| **Molecular Breast Cancer Subtypes** | | | | | |
| Luminal A | 179 | 41 | 49 | 1.95 | 80 |
| Luminal B | 14 | 44 | 57 | 1.21 | 43 |
| Triple Negative | 48 | 56 | 60 | 0.4 | 11 |
| HER2 | 21 | 56 | 67 | 0.13 | 0 |
| | | p=.1227[c] | p=.25[d] | <p=.000[c] | <p=.000[d] |
| **Hormone Receptor Status** | | | | | |
| ER+ and/or PR+ | 177 | 42 | 50 | 1.8 | 74 |
| ER- and PR- | 61 | 57 | 66 | 0.29 | 6.9 |
| | | p=.0189[c] | p=.039[d] | <p=.000[c] | <p=.000[d] |
| **Glucocorticoid Receptor Status** | | | | | |
| Low (<50 H-Score) | 120 | 44 | 51 | 1.44 | 58 |
| High (≥50 H-Score) | 142 | 54 | 65 | 1.7 | 67 |
| | | p=.184[c] | p=.074[d] | p=.1227[c] | p=.294[d] |

[a] Percentage positivity for SGK1: A tissue was considered positive for cytoplasmic SGK1 when the sample had an hscore≥30
[b] Percentage positivity for Bcl2: A tissue was considered positive for Bcl2 when the sample had a score ≥2
[c] F-test p-value.
[d] Chi-square p-value

# TABLE XV

## SGK1 AND BCL-2 EXPRESSION AND DEMOGRAPHIC AND BREAST CANCER CHARACTERISTICS

| | All Cases | High SGK1 | P-value[a] | High Bcl-2 | P-value[a] |
|---|---|---|---|---|---|
| **Race/Ethnicity** | **n** | **%** | 0.5 | **%** | 0.049 |
| nH Blacks | 108 | 57 | | 53 | |
| nH Whites | 81 | 52 | | 71 | |
| Hispanics | 83 | 49 | | 58 | |
| **Age at Diagnosis** | | | 0.6 | | 0.5 |
| <50years | 72 | 56 | | 57 | |
| ≥50years | 157 | 52 | | 61 | |
| **Any Early (<50y) Family History Breast Cancer** | | | 0.4 | | 0.4 |
| No | 213 | 54 | | 61 | |
| Yes | 14 | 43 | | 50 | |
| **Nulliparous** | | | 0.7 | | 0.033 |
| No | 229 | 53 | | 57 | |
| Yes | 43 | 56 | | 74 | |
| **Menopause Status** | | | 0.3 | | 0.4 |
| No | 37 | 46 | | 54 | |
| Yes | 191 | 55 | | 62 | |
| **Stage at Diagnosis** | | | 0.99 | | <.0001 |
| 0,1 (early stage) | 111 | 53 | | 79 | |
| 2,3,4 (late stage) | 158 | 53 | | 47 | |
| **Histologic Grade** | | | 0.5 | | <.0001 |
| Low/Intermediate | 161 | 52 | | 75 | |
| High | 103 | 56 | | 33 | |
| **Positive for ER or PR** | | | 0.039 | | <.0001 |
| No | 61 | 66 | | 66 | |
| Yes | 177 | 50 | | 46 | |
| **HER2 status** | | | 0.009 | | <.0001 |
| Negative | 131 | 47 | | 25 | |
| Positive | 27 | 74 | | 76 | |
| **Nuclear GCR (H-Score) 0<50, 1=>50** | | | 0.07 | | 0.3 |
| Low (<50 H-Score) | 120 | 51 | | 58 | |
| High (≥50 H-Score) | 142 | 65 | | 67 | |

[a]Chi-square p-value

The Bcl-2 expression varied much among histological or molecular subtypes of breast cancer. The highest mean score was among subtypes associated with poor prognosis and low survival such as hormone-receptor-negative (0.29 mean score), triple negative (0.4 mean score) and HER2+ (0.13 mean score) breast cancer subtypes.

Figure 10 is an example of the reduction in Bcl-2 expression in HER2 and triple negative subtypes compared with Luminal A. We observed the same pattern of cytoplasmic Bcl-2 expression among our samples when we categorized Bcl-2 score according to the median score (low $<2$ and high $\geq 2$). We detected Bcl-2 staining in only 59% of the breast cancer tissues compared with 100% for fibroadenoma and 91% for normal tissue.

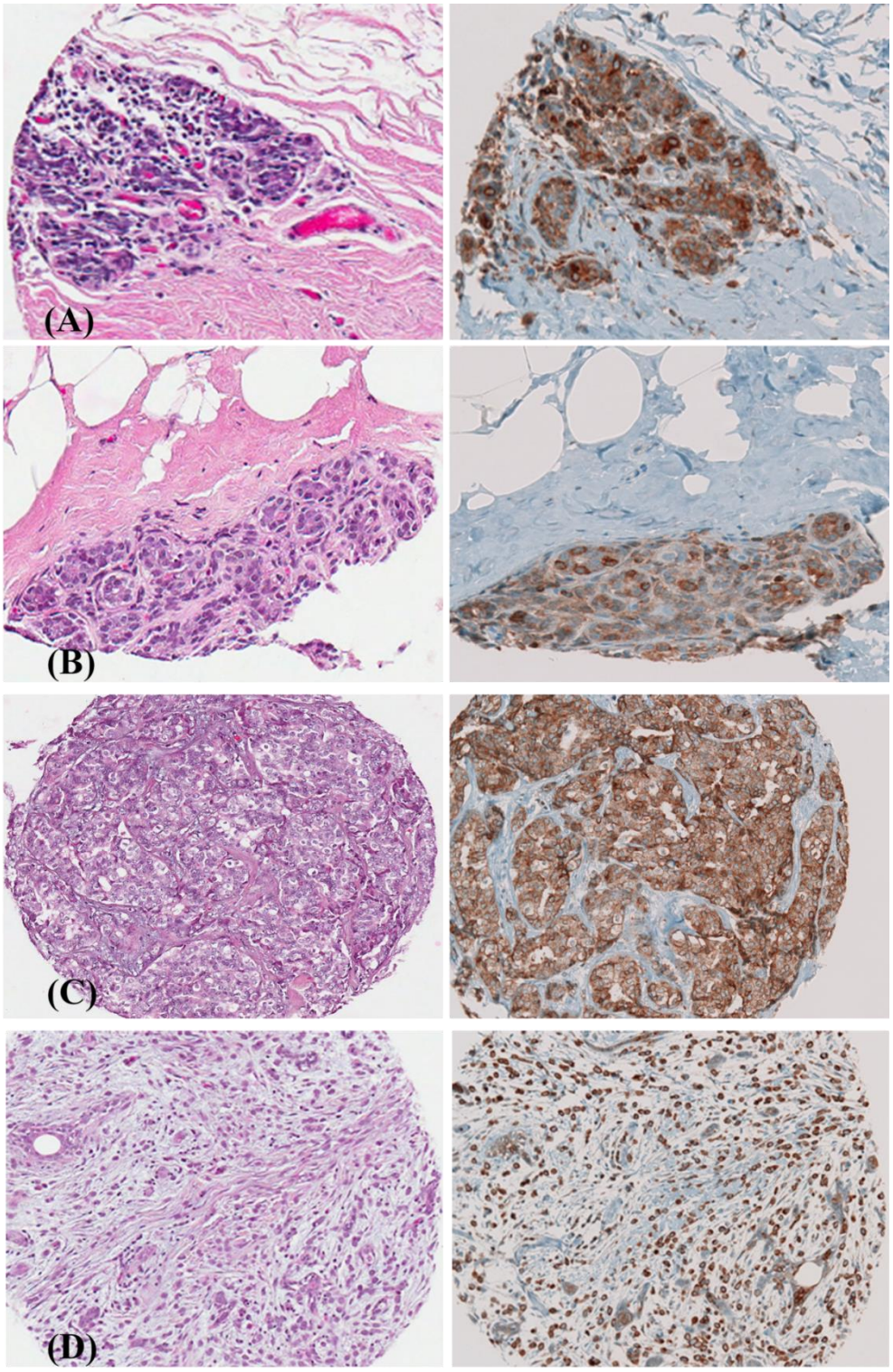



Figure 9. Immunohistochemical staining for Bcl-2 in representative cases of (A) normal breast tissue, (B) benign breast lesion (fibroadenoma), (C) invasive ductal carcinoma and (D) invasive lobular carcinoma.

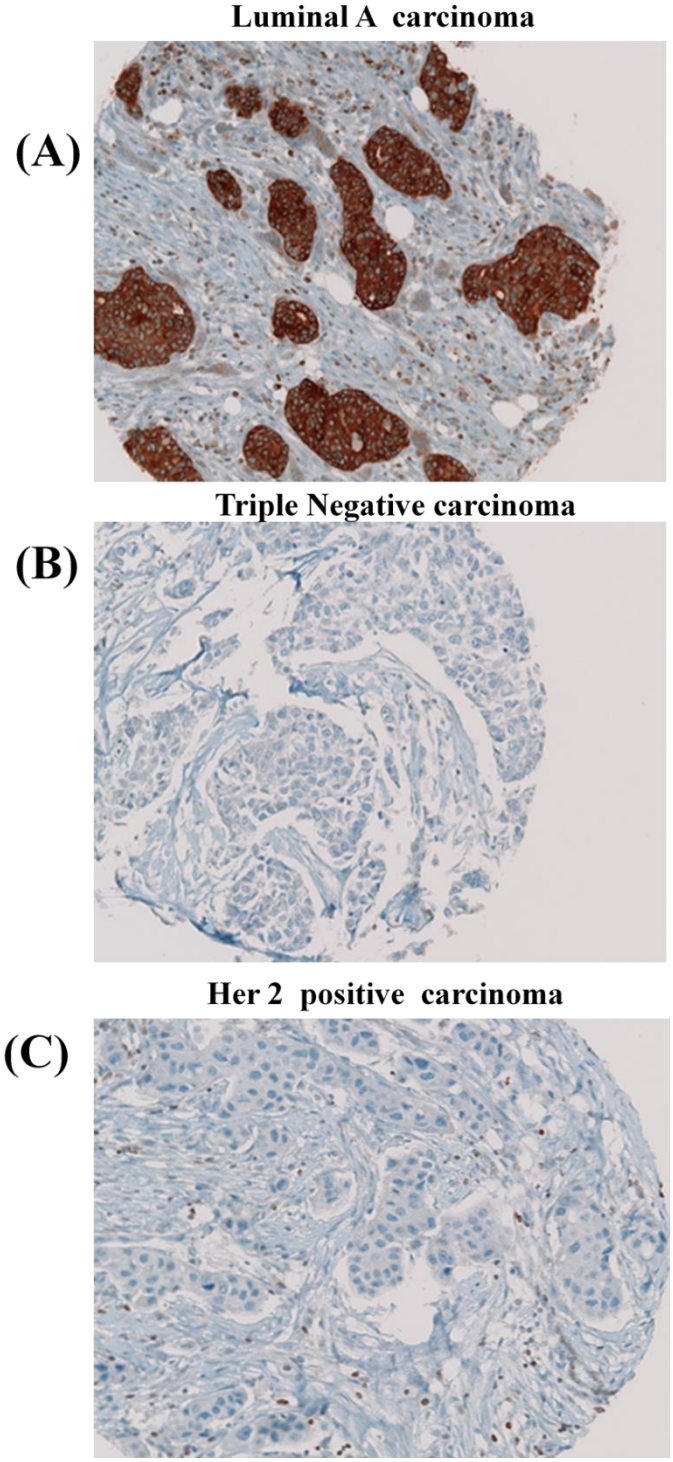



Figure 10. Immunohistochemical staining for Bcl-2 in representative cases of (A) luminal A, (B) triple negative, and (C) HER2.

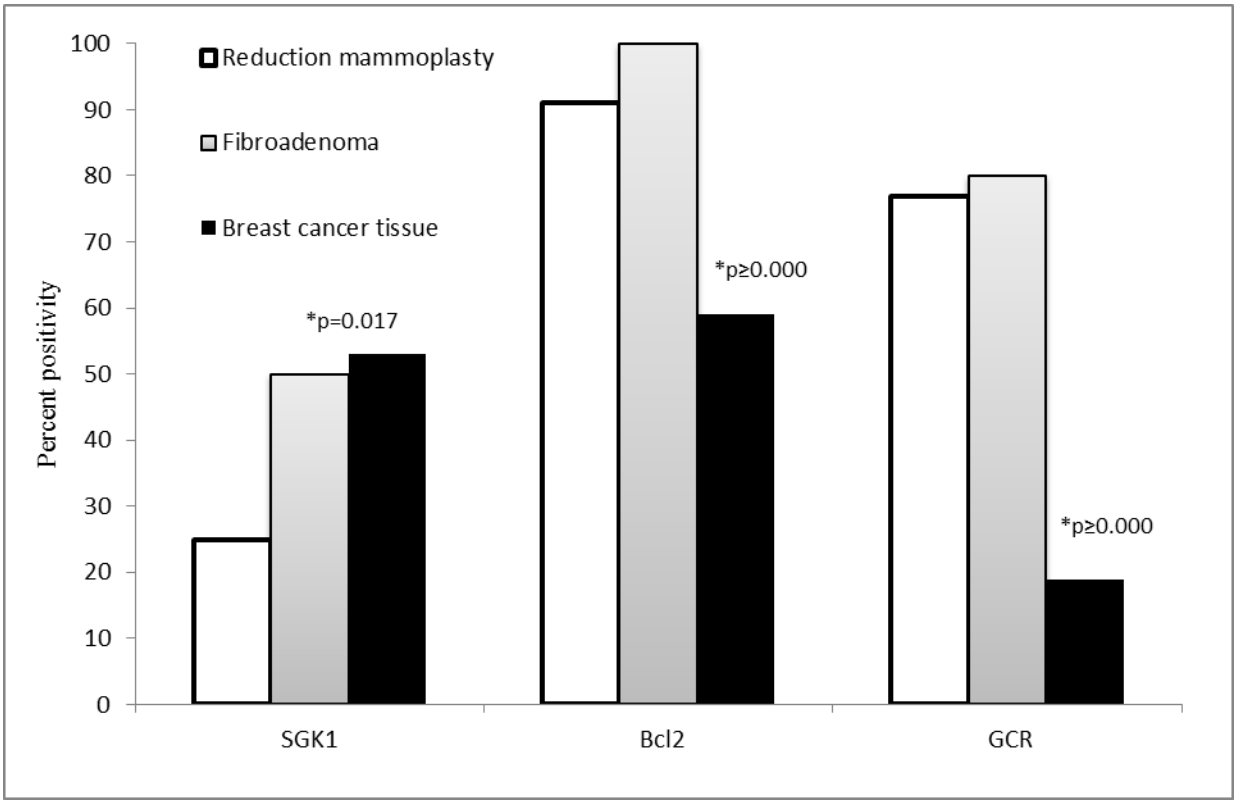
4. **Correlation between SGK1, Bcl-2, GCR expression and breast cancer characteristics**

Associations between SGK1 and Bcl-2 expression and breast cancer characteristics are summarized in TABLE XIV. High SGK1 expression was associated with $ER^-/PR^-$ status (p=.039), $HER2^+$ (0.009) from medical records and IHC $ER^-$ (p=.021), $PR^-$ (p=.04) status. Low nuclear GCR expression seems to be associated with high SGK1 expression but this relationship is not statistically significant (p=.074). However, SGK1 expression was not associated with self-reported race, stage, grade, and histological or molecular subtypes of breast cancer. High SGK1 was associated with lower odds of $ER^+$ and/or $PR^+$ status (OR 0.54, 95% CI 0.29–0.97). Adjusting for potential confounders such as age at diagnosis, self-reported race, stage, or grade at diagnosis, did not change the point estimate but the CI became wider and included one (OR 0.6, 95% CI 0.3–1.3). The Bcl-2 expression was associated with White racial/ethnic self-classification (p=.049), $ER^+/PR^+$ status (p<.0001), early stage, (p<.0001), and low grade (p<.0001), $HER2^+$ (p<.0001). The Bcl-2 expression was not associated with age at diagnosis, family history of breast cancer, or menopausal status. Unlike SGK1, GCR expression was not associated with Bcl-2.

## D. **Discussion**

We have previously shown that GCR is reduced in breast cancer tissue (Aim2). Here, we used the same series of breast cancer cases with defined clinical characteristics to measure SGK1 and Bcl-2 expression. Figure 11 summarizes the expression pattern of the GCR, SGK1, and Bcl-2 among cases from the BCCC subcohort. We observed a reduction in GCR and Bcl-2 and an increase in SGK1 protein expression in cancer compared with normal breast tissue.

The SGK1, a phosphatidylinositol 3-kinase-dependent serine/threonine kinase, is expressed in many tissues types and induced by several hormones such as glucocorticoids (Lang and Shumilina, 2013). It promotes cell survival signal and cell cycle progression, acting as an anti-apoptotic factor (Lang and Cohen, 2001). However, the role of SGK1 for tumor growth is conflicting, because its expression is upregulated in some tumors, such as breast cancer (Sahoo et al., 2005), multiple myelomas (Fagerli et al., 2011), and lung cancer, and downregulated in prostate cancer (Szmulewitz et al., 2012). It was mainly expressed in the cytoplasmic compartment of myoepithelial cells in normal breast tissue and benign breast lesions. As SGK1 is known to promote cell survival, it was not surprising to find that SGK1 expression is increased in breast cancer tissue compared with normal tissue. This strong cytoplasmic SGK1 expression was associated with negative ER/PR status but not with racial/ethnic category, age at diagnosis, stage or grade at diagnosis, or molecular subtypes of breast cancer. The GCR expression correlated with SGK1 expression in breast cancer tissue as the SGK1 protein is induced by glucocorticoids, but this relationship was not statistically significant.




* Chi-square p-value of breast cancer versus non-cancer tissue (Reduction mammoplasty and fibroadenoma)

Figure 11. Percentage positive staining for SGK1, Bcl-2, and GCR staining in breast tissue.

Our results are in agreement with the pattern of SGK expression in earlier reports. Expression of SGK1 in breast cancer has been previously examined in a small number of breast cancer cases. Sahoo et al. found that 19 of 40 tumors from 37 patients had positive SGK1 staining (≥10% positivity) with the majority showing exclusive cytoplasmic subcellular localization (Sahoo et al., 2005). Zhang et al. used a multitumor tissue microarray from the

Tissue Array Research Program (TARP-2), to find low or undetectable SGK1 in normal breast tissues (5/5) and high SGK1 in the majority of breast cancer tissue (29/38).

The Bcl-2 is a key anti-apoptotic gene that is associated with tumor growth and survival (Siddiqui et al., 2015). We observed a statistically significant decrease in Bcl-2 expression in breast cancer compared with normal breast tissue. We also observed an association between expression of the Bcl-2 and favorable clinical features of the tumors: lower grade, early stage, and hormone receptor expression that was not explained by any potential confounder such as age at diagnosis, self-reported race, or stage and grade at diagnosis. Expression of Bcl-2 has been consistently associated with a better prognosis for breast cancer in previous reports despite its anti-apoptotic properties (Callagy et al., 2006; Martinez-Arribas et al., 2007; Callagy et al., 2008).

The present study of 267 breast cancer tissue is the largest study to date to assess SGK1 expression in breast cancer and the first to test the correlation between two apoptotic proteins—SGK1 and Bcl-2—with GCR. A strength of this TMA cohort is the availability of demographic and clinical data on a diverse population of incident cancer cases, which means our findings will be more generalizable to an urban, ethnically diverse population of US breast cancer patients. We observed that SGK1 was mainly expressed in the cytoplasmic compartment that was associated ER/PR status in the univariate analysis. The association of high SGK1 expression with ER/PR negativity was robust and remained evident even after adjustment for multiple demographic and clinical factors including race, age, tumor stage, and grade at diagnosis. Of great interest in the context of our findings is the resistance of SGK1 knockout mice against cancer after chemical carcinogenesis (Nasir et al., 2009). Furthermore, SGK1 inhibitors decreased head and neck carcinoma (Berdel et al., 2014). We report an inverse relationship between SGK1 and GCR that

was not statistically significant. On the other hand, Bcl-2 expression was reduced in cancer when compared with normal breast tissue. Similar to previous reports, high Bcl-2 was associated with favorable prognosis such as hormone-receptor-positivity, early stage, and low grade and diagnosis. However, Bcl-2 expression was not associated with GCR expression among our cases. There are limitations to this study. This is a cross-sectional study and the direction of any associations could potentially suffer from reverse causality. There are also the limitations of TMA and IHC staining technique.

In conclusion, we observed an increase in SGK1 and a decrease in Bcl-2 expression in cancer compared with normal breast tissue. There is evidence of a correlation between GCR and SGK1 but not Bcl-2.

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## SCIENTIFIC PUBLICATIONS

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