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Hormone Receptor Activity and Chronic Disease Risk in Migrant Populations

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Hormone Receptor Activity and Chronic Disease Risk in Migrant Populations

by

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ABSTRACT

Hormone Receptor Activity and Chronic Disease Risk in Migrant Populations

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Non-communicable diseases (NCDs), including type 2 diabetes (T2D) and breast cancer, account for a vast majority of deaths worldwide. The incidence rates of these morbidities vary widely among different ethnic groups with racial minorities disproportionately affected by these chronic pathologies. Known risk factors among those afflicted with T2D and breast cancer include genetic markers, diet, obesity, and other lifestyle factors. However, these risk factors cannot fully explain the observed incidence rates across different ethnic populations. For migrant populations whose environment is often altered throughout their lifetime, environmental factors play a significant role in the development of chronic diseases. For this reason, approaches that are aimed at identifying non-genetic factors are necessary. This dissertation makes use of an exposome approach known as the receptorome. This methodology was used to investigate differences in hormone receptor activity and their association to breast cancer and T2D in migrant populations. The introduction in **Chapter 1** provides an overview of the exposome approach highlighting the role of steroid hormones and environmental mimics in the development of two chronic diseases that disproportionately affect migrant populations. **Chapter 2** investigates the association between estrogenic activity and nativity, genetic ancestry, and lifestyle factors among Mexican women. **Chapter 3** is an extension of the previous chapter, providing further evidence that ancestry and other breast cancer risk factors may be associated with mechanisms linked through the endocrine system. This study demonstrates the importance of endogenous and exogenous estrogens among different racial/ethnic groups and their potential role in breast cancer incidence rates. **Chapter 4** examines hormone receptor activity and the association to T2D and persistent organic pollutants in South Indian Asians and European Whites living in London. Lastly, **Chapter 5** summarizes all findings and elucidates a secondary technique, known as metabolomics, to aid in the identification of novel environmental risk factors affecting the incidence rates of NCDs in migrant groups.

DEDICATION

I dedicate this dissertation to all immigrant families, including my own, whose stories are often left unheard, unspoken, and forgotten in scientific research. May this work be a starting point for other investigators.

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Chapter 1. Introduction

Overview

In 2012, the World Health Organization (WHO) estimated that non-communicable diseases (NCDs) accounted for over 38 million deaths worldwide, with more than 70 percent of deaths occurring in low and middle income countries[1]. According to the WHO report, the top causes of deaths can be attributed to the following (in descending order): cardiovascular disease (CVD), cancers, respiratory disease and diabetes mellitus[1]. This dissertation will focus on two leading NCDs, specifically breast cancer and type 2 diabetes (T2D), in two migrant populations comprised of Hispanic/Latina women and South Indian Asians. Moreover, this dissertation elucidates the role of endocrine disrupting compounds (EDCs) as a potential source for the discrepancies in these disease rates.

While breast cancer is characterized by excessive cell proliferation and T2D is related to elevated blood sugar levels and insulin resistance, the incidence rate of both diseases is steadily increasing both across the Unites States (U.S.), and worldwide. About 1.7 million new cases of breast cancer are diagnosed annually worldwide[2]. Furthermore, breast cancer is the most common cancer in women and the second cause of cancer death worldwide (after lung cancer)[3]. In the U.S., it is estimated that the lifetime risk of being diagnosed with breast cancer is 1 in 8, or 12.4 percent[4,5]. Similarly, diabetes mellitus, with T2D being the most common form, has quadrupled over the past few decades from 108 million adults in 1980 to 422 million in 2014[6]. It is projected that diabetes mellitus will affect 1 in 10 adults in the U.S., with over 90 percent of cases attributed to T2D[7]. While both diseases disproportionately affect low and middle income countries[6], the incidence rates vary among different ethnic groups, partly due to the various risk factors associated with these diseases, some of which remain unknown.

The Exposome

The exposome is defined as the totality of exposures from conception onwards[8]. This paradigm includes an individual's internal as well as external environments[8,9]. Epidemiological studies benefit by using an exposomics approach by comparing different -omic (i.e. genomics, transcriptomics, adductomics, receptorome, metabolomics, etc.) platforms between populations to discover novel biomarkers. One branch of the exposome referred to as the receptorome, has been previously used in multiple studies to measure disruption in hormone levels via receptor signaling. Measurements are obtained through the use of luciferase gene reporter bioassays, which cover a broad range of receptors including the estrogen, androgen, and glucocorticoid receptors. Within the past decade, these assays have progressed from using environmental samples[10], like waste water, to human sample types, including blood[11]. This transition facilitated the measurement of the exposome since blood samples provide an inclusive representation of chemicals derived from internal and external processes that affect hormone signaling within a person's body. For instance, in a Singapore Chinese Health Study of postmenopausal women[12], it was suggested that factors other than endogenous estrone and estradiol may activate the estrogen receptor signaling pathways to increase breast cancer risk. In fact, estrogenic (E) activity correlates better with breast cancer risk than measurements of estradiol and its metabolites alone[13,14]. Consequently, this dissertation relies on the use of luciferase gene reporter bioassays to help understand the differences in breast cancer and T2D risk between two different racial/ethnic migrant groups.

Risk Factors

The risk of breast cancer and T2D are affected by both genetic and environmental risk factors. While advances in genetic research have been achieved, environmental risk factors are less understood and still undefined. For breast cancer, genetic risk factors are more pronounced for inherited mutations in the most widely studied human breast cancer genes—*BRCA1, BRCA2 and p53*. These genes account for approximately 5 to10 percent of female breast cancer cases[15,16]. Another well-known risk factor is age, with the mean age of cases being around 62 years, or about ten years after the onset of menopause[17]. A family history of diabetes mellitus and age (usually over the age of 45[18]) is also an important risk factor for T2D, though the age at onset of the disease has rapidly consumed younger generations, including children[7]. Nonetheless, other non-genetic factors play a more critical role, including sedentary lifestyle, obesity, poor nutrition and abnormal lipid levels. Some environmental exposures have been linked to breast cancer and T2D such as pollutants[19,20], smoking[21,22], hormone replacement therapy[23,24], *in utero* exposures to potent synthetic estrogens like diethylstilbestrol[25,26], alcohol consumption[27–29], sugar enriched diets[30,31], and postmenopausal obesity[32,33]. One common theme among many of these environmental factors is their relationship to the endocrine system and endocrine hormones.

Endocrine Hormones and Endocrine Disrupting Chemicals

Research on endocrine disrupting chemicals, or EDCs, is growing rapidly. EDCs are exogenous chemical compounds (or mixture of compounds) that interfere with the endocrine system and the action of its hormones[34]. These chemicals interfere with hormone synthesis and secretion, binding of nuclear receptors (NRs) and changes to homeostatic systems[35]. NRs regularly bind naturally occurring hormones circulating in the blood. These natural hormones- estrogens, androgens, and glucocorticoids- are variable in concentrations throughout a person's life due to age and gender differences. More importantly, variations can result as a result of various adverse health outcomes that have been linked to EDCs.

For example, breast cancer risk has been directly linked to hormone receptor disruptors in animal models[36,37] and in occupational exposure studies[38,39]. Environmental compounds, including metals (e.g. cadmium), natural food components (i.e. genistein), and industrial chemicals (i.e. bisphenol A (BPA), phthalates) can bind the receptor and either activate or suppress downstream signaling of ER target genes[40–43]. Though not as heavily studied, more research on T2D and metabolic ailments is being explored using sex hormones as means to explain the differences in disease onset[44,45]. Moreover, EDCs like dioxins, polychlorinated biphenyls, BPA and dichlorodiphenyltrichloroethane (DDT) have also been hypothesized to affect the clinical manifestation of metabolic diseases regardless of their persistence in the body[46–50].

It is imperative to determine how EDCs interact with endogenous hormones via NRs to fully understand how these chemicals impact disease development. More importantly, there is a need to effectively and efficiently develop new screening strategies. In this dissertation the receptorome serves as an excellent tool to capture a glimpse of endogenous and environmental exposures in two unique populations.

Migrant Populations

With the ever changing demographics across the globe, migrant health is an area of research that is relatively new and requires further investigation. There is an interest in assessing migrations impact on disease occurrence, which would benefit from applying exposomic tools,

such as the receptorome. While research on migrant health is very complex, it is also limited by many biological as well as social factors including one's country of origin, diet, new place of residence, sex differences, lifestyle, language barriers, and health systems. Incidence variations in several chronic illnesses—cardiovascular diseases, cancers and diabetes—are evident across migrant groups with one theory being that migrant populations typically carry a higher burden of different environmental exposures that they encountered in early life or prior to settling in their new place of residence[50–52]. Yet, other researchers propose that the increase in the incidence of disease rates occurs after relocating to a new environment[53–56]. Both outlooks highlight the importance of the timing of exposures and the diverse nature of migrant groups for which there is a knowledge gap that requires improvements and development of new strategies to address these ethnic health disparities.

To understand the impact of migration on hormone receptor activity and disease risk, this dissertation focuses on two migrant populations based within and outside the U.S. The first population derives from a case control study consisting of foreign-born and U.S. born Mexican women from the San Francisco Bay Area Breast Cancer Study (SFBCS). The parent study is comprised of approximately 5,000 women representing three different racial/ethnic groups: Hispanics/Latinas, Non-Latina Whites, and Non-Latina Blacks. The second migrant population includes both men and women residing in London as participants in the London Life Sciences Population (LOLIPOP) Study. While the parent study consists of over 30,000 participants, findings will be reported for a cross-sectional study of about 400 samples. These South Asian Indians are at a 2 to 3-fold risk of T2D as compared to European Whites. Using an exposome approach that considers early-life exposures, as well as genetic and non-genetic risk factors on both of these populations, would be ideal for examining differences in disease onset in immigrant populations.

Conclusion

NCDs, specifically cancer and diabetes, have been growing in burden which require public health efforts to prioritize the identification of more risk factors. These risk factors should include those of environmental origin given their complex origin and less understood mechanisms. Growing research on EDCs and their effect on migrant health should not only expand discovery of environmental risk factors, but it is of utmost importance to develop better regulatory policies to protect vulnerable migrant populations.

Glossary

BPA	bisphenol A
CVD	cardiovascular diseases
DDT	dichlorodiphenyltrichloroethane
EDC	endocrine disrupting chemical
LOLIPOP	London Life Sciences Population Study
NCD	noncommunicable disease
NR	nuclear receptor
SFBCS	San Francisco Bay Area Breast Cancer Study
T2D	type 2 diabetes
WHO	World Health Organization

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Chapter 2. Association of lifestyle and demographic factors with estrogenic and glucocorticogenic activity in Mexican American women

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Abstract

Breast cancer risk is higher in US-born than in foreign-born Hispanics/Latinas and also increases with greater length of US residency. It is only partially known what factors contribute to these patterns of risk. To gain new insights, we tested the association between lifestyle and demographic variables and breast cancer status, with measures of estrogenic (E) and glucocorticogenic (G) activity in Mexican American women. We used Chemical-Activated LUciferase gene eXpression assays to measure E and G activity in total plasma from 90 Mexican American women, without a history of breast cancer at the time of recruitment, from the San Francisco Bay Area Breast Cancer Study. We tested associations of nativity, lifestyle and sociodemographic factors with E and G activity using linear regression models. We did not find a statistically significant difference in E or G activity by nativity. However, in multivariable models, E activity was associated with Indigenous American ancestry (19% decrease in E activity per 10% increase in ancestry, $P = 0.014$) and with length of US residency (28% increase in E activity for every 10 years, $P = 0.035$). G activity was associated with breast cancer status (women who have developed breast cancer since recruitment into the study had 21% lower G activity than those who have not, $P = 0.054$) and alcohol intake (drinkers had 25% higher G activity than non-drinkers, $P = 0.015$). These associations suggest that previously reported breast cancer risk factors such as genetic ancestry and alcohol intake might in part be associated with breast cancer risk through mechanisms linked to the endocrine system.

Introduction

Breast cancer risk in US Latina women, although lower than that of non-Latina Whites[1], is higher in those born in the USA, and risk increases with younger age at migration[2]. Age-adjusted incidence rates for the period 1988–2004 showed 38% higher rates for US-born than for foreign-born Latinas[3]. It is only partially known what factors contribute to these patterns of increasing risk[2,3].

Previous studies have attempted to explain the changes in breast cancer incidence among Latina immigrants using measures of exposure obtained through questionnaires or through record linkage to census data to evaluate the effect of socioeconomic status (SES) or neighborhood effects[2,3]. However, by themselves, these results are limited because they are bound to provide information about exposures that have already been associated with breast cancer risk, or because they do not tell us much about the possible precursors and the biological effects underlying the associations. Furthermore, it is known that individuals with similar reported exposures are not equally susceptible to disease, due to inter-individual variation in the metabolism of endogenous and exogenous compounds[4,5].

Conducting analysis of endocrine disruptors by measuring elevated or reduced hormone activity in plasma is a novel way to understand the differences in breast cancer risk between Latina women born in the USA compared with foreign-born Latinas. Enzyme activation by exposure to hormone receptor binding compounds can lead to increased hormone catabolism and compromise hormone signaling[6]. Breast cancer risk has been directly linked to hormone receptor disruptors in animal models[7,8] and in occupational exposure studies[9,10]. There is also evidence linking endocrine disruptors to breast cancer risk through regulation of microRNAs' expression[11], as well as through their involvement in the formation of reactive electrophiles such as reactive oxygen species and subsequent DNA adduct formation[12].

Cell-based reporter bioassays have been commonly used to identify estrogenic (E) compounds present in the environment[13–16], but few studies have used them to test the association

between overall E activity in human blood and breast cancer risk[17–20], as was originally proposed by Brouwers *et al*.[20] An analysis conducted in samples collected prospectively from the Singapore Chinese Health Study tested the associations between levels of estrogens and estrogen receptor (ER)-mediated bioactivity and breast cancer risk among postmenopausal women and found results suggesting that factors other than estrone and estradiol may activate ER-mediated signaling pathways to increase breast cancer risk[19].

There is extensive evidence for the role of estrogens in breast cancer risk and prognosis. Selective estrogen receptor modulators, such as tamoxifen and raloxifene, as well as aromatase inhibitors, are cornerstones of breast cancer treatment and have been shown in randomized trials to prevent breast cancer, particularly ER-positive disease[21–24]. Epidemiologic studies have documented about a 2-fold higher risk of breast cancer in postmenopausal women in the top versus bottom 20–25% of plasma estradiol, estrone or estrone sulfate levels[25,26]. Estrogens affect breast tissue largely through binding ER, which in turn leads to expression of ER target genes[27]. However, multiple other compounds can also bind ER and either activate or suppress downstream signaling, including metals (e.g. cadmium), chemicals for industrial or household use (e.g. bisphenol A, parabens and phthalates), natural food components (e.g. isoflavones) and endogenous compounds (e.g. 27-hydroxycholesterol and estrogen metabolites)[27–32].

Exposure to endogenous and exogenous glucocorticoid receptor (GR) modulators is also likely to contribute to breast cancer development. Glucocorticoids are adrenocortical steroid hormones involved in several physiological and cellular processes, including cell differentiation, metabolism and programmed cell death by interacting with the GR[33]. Reduced expression of the GR gene was observed in a panel of human liver, lung, prostate, colon and breast cancers and found to play an important role in promoting accurate chromosome segregation during mitosis, which highlights its role as a tumor suppressor[34]. In addition, GR expression in breast cancer tissue has been associated with smaller tumor size and lower grade[35]. In addition, glucocorticogenic (G) activity might reflect cortisol levels, which, given their link to stress[36], could be particularly relevant to this population of immigrant women.

In the present study, we used cell-based assays[37,38] to measure overall E and G activity in plasma of 90 Mexican American women who participated as controls in the San Francisco Bay Area Breast Cancer Study (SFBCS), a population-based case–control study of women aged 35–79 years. The specific goal of our study was to test if nativity (ref. US-born) and other breast cancer risk factors were associated with E and G activity in total plasma.

Methods

Study samples

The San Francisco Bay Area Breast Cancer Study (SFBCS), described elsewhere[2,39], is a multiethnic population-based case–control study of breast cancer initiated in 1995, and with biospecimen collection added for cases diagnosed between 1 April 1997 and 30 April 2002 and matching controls. Briefly, participating women aged 35–79 years resided in the San Francisco Bay Area when diagnosed with a first primary histologically confirmed invasive breast cancer between April 1995 and April 2002. Controls identified by random-digit dialing were frequency matched to cases based on race/ethnicity and the expected 5 year age distribution of cases. Trained interviewers administered a structured questionnaire in English or Spanish at a home visit and took anthropometric measurements. Trained phlebotomists collected a fasting blood sample. Since for

some women, the blood was collected a few years after recruitment into the study, a phlebotomy questionnaire was administered at the time of blood draw to update some key variables.

For the present study, 90 women were selected from the set of 603 Latina controls with stored plasma if they had developed breast cancer since the time of blood collection or if they remained free of breast cancer and were of Mexican origin. Through linkage with the California Cancer Registry in 2013, 15 Latina women were identified who developed breast cancer after blood collection. The remaining 75 women were randomly selected within subsets according to age at migration to the USA if foreign-born (balancing the number of younger and older age at migration) and menopausal status (balancing the number of premenopausal and postmenopausal women within each demographic category). The final sample included 60 foreign-born women (8 cases and 52 controls) and 30 US-born women (7 cases and 23 controls) (Supplementary Figure S1). Overall, 33 women were premenopausal and 57 were postmenopausal. Since we included all Latina women who developed breast cancer after blood collection, some of them were not of Mexican origin. Of the 15 cases, one was from Colombia, one from Puerto Rico and two from Nicaragua. Given that 97% of the women included in the present analysis were of Mexican origin, we refer to them generally as Mexican American throughout the manuscript, despite the fact that three women had different national origins.

Measures

The questionnaire for the main study obtained data on demographic background (education in years, country of birth, and age at migration if not US born) and known or suspected breast cancer risk factors. For the present analysis, we selected specific risk factors that we hypothesized could be associated with E or G activity at the time of blood draw, such as use of menopausal hormone therapy (HT), alcohol intake, body mass index (BMI) or socioeconomic and sociocultural background. The phlebotomy questionnaire collected information on use of oral contraceptives (OCs), menopausal HT and alcohol (beer, wine, hard liquor) during the 6 months prior to the blood draw. OC and HT use at the time of blood draw was categorized as current, former, and never. For alcohol intake, grams per day were calculated. BMI was obtained by dividing measured weight (kg) by measured height (m) squared. Neighborhood level SES was estimated using a composite index including income, education, poverty, unemployment, occupation and housing and rental values, based on 2000 Census block-group data[40,41]. Individual proportion of Indigenous American genetic ancestry was available for 86 of the 90 samples and was included in the analyses as a proxy for unmeasured sociocultural and/or biological differences. Details about the procedure for ancestry estimation have been previously reported[42]. Briefly, we estimated global individual ancestry as the average locus-specific ancestry across 59211 loci for each individual. Locus-specific ancestry estimates obtained with the HAPMIX software[43] were available from a previous genome-wide genotyping effort described elsewhere[42] and were estimated based on a three-way admixture model (African, European and Indigenous American components).

Luciferase assay overview

Chemically Activated LUciferase gene eXpression, or CALUX, bioassays are highly sensitive and reliable high throughput screenings used to measure biologically relevant exposures in various media including sediment[13], house dust[14], drinking water[15,16] and human blood[19,20]. CALUX assays are used to identify receptor-mediated signaling pathways of gene expression by specific compounds such as estrogens and androgens[44,45]. We relied on these bioassays to

agnostically measure ER and GR agonist and antagonist activity profiles. Two breast cancer cell lines, T47D-Kbluc and MDA-Kb2, were stably transfected with a luciferase promoter gene construct to detect total E and G activity, respectively, for endogenous and exogenous compounds in human plasma. The process is initiated when compounds found in the plasma enter the cell and bind to the hormone receptor in the cytoplasm. If the compound that is bound to the receptor is an agonist, the agonist will cause the ligand bound receptor to translocate to the nucleus. The DNA binding domain of the receptor will then bind to its respective responsive element and transcription of the luciferase gene will take place. Upon cell lysis and substrate addition, promoter activity is measured by the amount of emitted light, referred to as relative light units (RLUs). Higher RLUs usually indicate agonists are binding the receptor and producing more luciferase protein. A decrease in RLUs can result if agonists are scarce or when an antagonist binds to the receptor and blocks nuclear translocation or inhibits transactivation, which leads to less production of the luciferase protein.

Cell culture and treatments for the ER bioassay

The methods used were similar to those previously described by Wilson *et al.*[46]. The transfected breast cancer cell line T47-Kbluc was used to measure total endogenous and exogenous estrogens, such as 17-beta estradiol (E2), ethynyl estradiol and diethylstilbestrol in human plasma for both premenopausal and postmenopausal women in our study. Cells were cultured in phenol red Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) until 1 week prior to plasma addition. Phenol red media can act as a weak estrogen[30] and interfere with the bioassay. In order to remove all external sources of estrogen mimics, cells were treated with 'stripped' phenol red free DMEM supplemented with 10% charcoal–dextran stripped FBS for 1 week. After 1 week in stripped medium, cells were seeded at a density of 27,000 cells per well and 200 µl final volume in white, 96-well microtiter plates (Thermo Scientific, Waltham, MA) and incubated at 37°C for 24 hours. The 8 µl of plasma used per sample was diluted in phenol red free medium and then added in quadruplicate directly onto the cells. This step was followed by a final incubation period of 24 hours at 37°C before cell lysis with 5× passive lysis buffer (Promega, Madison, WI). Luciferase gene expression was measured using a microplate luminometer (Berthold Technologies, Centro XS3 LB 960 Instrument). Reporter activity was measured per well by the fluorescence emitted from the chemiluminescent reaction when the enzyme is activated by the substrate. Readings for each well were expressed in RLUs. RLUs from quadruplicate wells were averaged to get one measure per individual. The intra-assay and inter-assay coefficients of variation (CVs) of this assay are 7–23%. The minimum detection limit for E2 is 1.0 pM.

Cell culture and treatments for the GR bioassay

Similar to the ER bioassay, the GR bioassay also used methods previously described by Wilson *et al.*[47]. However, the transfected breast cancer cell line MDA-Kb2 expresses two receptors. This cell line was used to measure androgens such as testosterone and dihydrotestosterone present in human plasma for premenopausal and postmenopausal women in our study. Because the androgen receptor and the GR have homologous DNA binding domains and act on the mouse mammary tumor virus promoter, this cell line also has the ability to measure glucocorticoids such as corticosterone and aldosterone using the GR. To distinguish between A and G activities, the use of a potent androgen receptor inhibitor, hydroxyflutamide (OHF), was needed. Cells were cultured in Leibovitz's-15 (L-15) medium with 10% FBS until 1 week before plasma addition. External sources of androgens and glucocorticoids were removed by treating the

cells for 1 week with 'stripped' medium composed of L-15 medium and 10% charcoal–dextran serum. The cells were seeded at 27,000 cells per well at 200 µl final volume in white, 96-well plates and incubated at 37°C for 24 hours. The 165 µl of plasma used per female sample was diluted in stripped medium and then added in quadruplicate directly onto cells, both in the presence and absence of 0.5 µM OHF. After 24 hours of incubation at 37°C, the cells were lysed and the microplate was read using the luminometer to obtain RLU readings. To get G activity measurements, RLUs from quadruplicate wells in the presence of OHF were averaged to get one measure per individual. The intra-assay and inter-assay CVs of this assay are 5–10%. The minimum detection limit for cortisol is 4.4nM.

Statistical analysis

Differences in means and proportions for all analyzed variables between US-born and foreign-born Mexican American women were assessed using two-sided *t*-tests and Fisher's exact tests, respectively. E and G activity measures were ln-transformed in order to approximate the normal distribution. We used linear regression models with the receptor activity as the outcome and demographic and lifestyle factors as predictors. We also included a batch or plate variable to account for experimental variation. To facilitate interpretation of regression results, we calculated the percent change in RLUs per unit change of predictor variables using the formula $[e^{\beta} - 1] * 100$. Analyses were conducted in R[48] or STATA[49].

The multivariable regression models included E or G activity (continuous, ln-transformed RLUs) as the outcome and height (continuous, in meters; m), BMI (continuous and ln-transformed, in kg/m^2), proportion of Indigenous American ancestry (continuous, 10% ancestry unit), level of education (less than high school versus high school or more), neighborhood SES (continuous score, based on first component of principal components analysis), age at blood draw (continuous, 10 years unit), alcohol intake at first interview (yes, no), nativity (US-born, foreign-born) and menopausal status (premenopausal, postmenopausal) as predictors. We also conducted two additional analyses, one stratifying by menopausal status, which included use of HT (never, former or current), and the other by nativity, in which we were able to assess the association between E or G activity and years of residence in the USA (continuous, 10 years unit). Models included all predictors (based on the *a priori* hypothesis that they could all influence E and G activity). We did not include use of OCs because only one woman in the study was taking them at the time of blood draw and was therefore excluded from the analysis. African genetic ancestry is relatively low in women of Mexican origin and, therefore, estimates obtained are less reliable than for the major components (Indigenous American and European). As a result, the present analyses only included Indigenous American ancestry (which is the complement of the European ancestry and therefore collinear). Seventeen women had discordant alcohol intake answers during the calendar year prior to selection into the parent study versus during the 6 months prior to the blood draw. All 17 reported to drink some alcohol in the first interview (ranging from half a drink per week to approximately two drinks a day) and no alcohol at blood draw. The E activity analysis included education level, but not SES as predictor, and the G activity analysis included SES but not education level. Since SES and education were highly correlated, for each model we kept the variable that had the largest effect on the adjusted R^2. The multivariable regression analyses excluded individuals with missing data on genetic ancestry (one case from Puerto Rico and three Mexican American cases) and education (three controls), a breastfeeding woman and a woman

currently using OCs. The final sample set included 11 breast cancer cases and 70 controls for E activity and 9 cases and 50 controls for G activity.

Ethical statement

All participants provided written informed consent and the study was approved by the Institutional Review Boards at the University of California San Francisco and the Cancer Prevention Institute of California.

Results

In the present study, we tested if first-generation Mexican immigrants had different E and/or G activity in plasma, as measured using a CALUX cell-based assay, compared with US-born women of Mexican origin, and if those levels were associated with other known breast cancer risk factors. Median E activity of transfected cells after addition of plasma was 2925 RLUs [interquartile range (IQR): 8226], and G was 178232 RLUs (IQR: 72207). Table 1 describes the levels of all considered variables by place of birth (US-born versus foreign-born). There were no differences in E or G activity, age at blood draw, height, BMI and menopausal status (the latter due to selection of similar number of postmenopausal and premenopausal women from the two migration groups during the study design). There was a statistically significant difference in the level of education and neighborhood SES, with US-born Latinas having higher levels for both variables, and suggestive differences in alcohol intake during the calendar year prior to selection into the study (higher intake among US-born women), and menopausal HT (higher use in US-born women). A higher proportion of breast cancer cases were US-born.

Estrogenic activity

We tested the association between E activity of transfected cells after addition of plasma from 86 Mexican American women and multiple anthropometric, lifestyle and demographic factors using univariate and multivariable regression models. In univariable analyses, we found a strong positive association with age at blood draw, where for every 10 years increase in age, E activity decreased 50% ($P = 1\times10^{-12}$) (Supplementary Figure S2). Mean E activity level among postmenopausal women was 79% lower than that of premenopausal women ($P < 1\times10^{-16}$) (Supplementary Figure S3). Variation in age at blood draw and menopausal status explained ~40% of the variation in E activity (adjusted $R^2 = 0.43$).

We did not find an association between E activity level and nativity (US-born versus foreign-born) in univariate or multivariable models.

The multivariable model suggested a negative association between E activity and proportion of Indigenous American genetic ancestry, where for every 10% increase in ancestry there was a 19% decrease in E activity ($P = 0.014$) (Table 2). In analyses stratified by menopausal status, we did not observe any significant heterogeneity for the described associations, though *P* values increased due to the reduced sample size (Supplementary Table S1). When we stratified the analyses by nativity (US-born, $N = 28$ versus foreign-born, $N = 57$), we observed an important change in the Indigenous American ancestry coefficient, with a strong association among the foreign-born Mexicans (23% change in E activity, $P = 0.009$), but no association among US-born individuals (5% change in E activity, $P = 0.770$) (Table 3). In the model that included foreign-born individuals, we observed a positive association between E activity and years of

residence in the USA (For every 10 years of US residence, there was a 28% increase in E activity, $P = 0.035$) (Table 3).

Glucocorticogenic activity

Glucocorticogenic (G) activity was only obtained for 60 of the 86 women due to lack of plasma availability for 26 women. We found no association between G activity, age at blood draw, menopausal status or nativity (Table 4). There was an inverse association with breast cancer status, suggesting that women who had developed breast cancer after recruitment into the study had 21% lower G activity than those who did not ($P = 0.054$). We also observed a positive association with alcohol intake during the year prior to the first interview (compared with non-drinkers, women who reported drinking at least some alcohol had a 25% higher G activity, $P = 0.015$) and a positive association with height (per every 10cm there was a 17% increase in G activity, $P = 0.037$) (Table IV). Stratified analyses did not suggest heterogeneity by menopausal status (Supplementary Table S2) or nativity (Supplementary Table S3) regarding these two variables. The association between G activity and alcohol intake was not observed when alcohol intake at blood draw instead of interview was included in the model (Supplementary Table S4). However, we found a statistically significant association between discordant status for the two alcohol variables and G activity (Supplementary Table S5).

Discussion

We presented the results of a semi-targeted analysis of E and G activity in plasma in Mexican American women from the San Francisco Bay Area. Our results suggest that E activity in plasma is associated with Indigenous American ancestry and length of US residence among foreign-born Mexican American women. We also observed an association between G activity and alcohol intake. Indigenous American ancestry has consistently been associated with breast cancer risk, with lower risk among women with high Indigenous American ancestry[50,51]. A genome-wide association study in Latinas reported the existence of a single nucleotide polymorphism, only present in Indigenous American populations, and associated with protection against breast cancer[52]. In addition to this protective variant, non-genetic factors are likely to contribute to the decreased breast cancer risk in highly Indigenous American women, given that genetic ancestry is correlated with sociodemographic, reproductive and lifestyle factors such as education, neighborhood SES, use of HT and alcohol intake[53].

Our results suggest that lower levels of E activity among women with high Indigenous American ancestry could partly contribute to the inverse association between Indigenous American ancestry and breast cancer risk. It is unclear from our results if the lower E activity is due to lower levels of endogenous estrogens or lower levels of endocrine disruptors, and further studies will need to be conducted to clarify these results. Targeted studies looking at the association between endogenous estrogens and breast cancer risk have already shown a positive relationship[25,26], and a previous study of E activity in Asian women strongly suggested increased activity among women who developed breast cancer, beyond the effect of endogenous estrogen levels[19].

Analyses did not include variables such as age at menarche, number of live births and breastfeeding, which are important breast cancer risk factors related to variation in estrogen levels. We focused on factors that were likely to be acting on hormone levels near the time of sample extraction. Given the average age of women in the study, remote events such as age at menarche or breastfeeding were not thought as likely to be reflected in estrogen levels. In order to confirm

our assumption, we ran a model that included these variables, which did not show any meaningful change in estimates compared with those in the model without those variables (data not shown). There were only four women in the study who reported using HT at the time of blood draw. In the model for postmenopausal women, it was clear that the difference in activity was between current users versus former or never users. To make sure that the current use of HT was not affecting the association between years of residence in the USA and E activity, we tested a model that included only the postmenopausal foreign-born women and information on hormone replacement therapy use, which confirmed that the association with years of residence in the USA was independent of HT (*P* value for years in the USA was slightly lower when including HT, $P = 0.026$).

The analysis of G activity, even though limited by a small sample size, also provided interesting results that warrant replication. Despite there being only nine breast cancer cases in the sample, we observed a negative association with G activity (the average G activity was lower among the women who developed breast cancer compared with those who did not). This is consistent with the observation that GR stimulation decreases the risk of relapse in breast tumors that are ER positive[54] due to cross talk between ER and GR[54,55]. We also observed an association between G activity and alcohol intake as reported during the first interview (which for some of the individuals was 4 years before the time of blood draw). Due to low levels of alcohol intake among Mexican American women, we decided to compare individuals who responded that they do not drink at all to those who responded that they drink some alcohol. Women who reported drinking at least some alcohol had higher G activity than those who responded that they never drank. Stress-induced glucocorticoid secretion triggers changes in gene expression through activation of the GR, which might alleviate immediate negative feelings associated with stress but lead to behavioral pathologies such as addiction, anxiety and depression[56]. Studies have shown that inactivation of GR decreases motivation to take alcohol or other drugs[57,58]. Our finding that G activity is higher among drinkers is consistent with these results and suggests the possibility that stress-induced activation of the GR might lead to increased levels of alcohol intake among some women in this overall low alcohol consumption group.

The study had some limitations. One limitation was that the data analyzed only included 11 women who had developed breast cancer. Ideally, we would have analyzed a larger number of cases to test if the associations observed between E or G activity and breast cancer risk factors mediated the association between those factors and breast cancer risk. Due to the small number of cases, we focused our analysis on the relationship between E and G activity and other factors that have been associated with breast cancer risk and were likely to be correlated with this activity. Another limitation was the relatively small overall sample size. However, we were able to discover some interesting associations that warrant replication and further investigation in future studies including a larger number of Latina women as well as women from other race/ethnicities. In addition, we lacked measures of endogenous estrogen, which would have allowed us to estimate what proportion of the variability in E activity might be due to differences in the level of estrogen-like compounds of exogenous origin versus differences in endogenous levels of estrogen. Finally, endogenous hormone levels as well as exposure to other receptor antagonists and agonists vary on a daily basis and therefore a measure of E and G activity obtained from a single plasma sample might not represent the average level of exposure for the individual. However, we believe that, at the population level, observed associations are informative and should be further explored, while absence of association cannot be taken as conclusive given that it is possible that for certain exposures the time at which the biospecimen was collected could be crucial.

Conclusions and Future Directions

In summary, we have investigated the levels of plasma estrogenic and glucocorticogenic activity in Mexican American women born in and outside the USA and tested their association with lifestyle, demographic and anthropometric breast cancer risk factors. Despite the null association with the main predictor (nativity), the cell-based measure of E and G activity reflected the expected differences by age at blood draw and menopausal status and also suggested previously unknown associations with genetic ancestry, years of US residence and alcohol intake. Future research will use cutting edge mass spectrometry-based technology to further identify the specific chemicals, and their precursors, that contribute to the observed associations and possibly to breast cancer risk. If modifiable, these agents could be targets of prevention programs, which would eventually reduce breast cancer incidence.

Glossary

BMI	body mass index
E	estrogenic
ER	estrogen receptor
FBS	fetal bovine serum
G	glucocorticogenic
GR	glucocorticoid receptor
HT	hormone therapy
IQR	interquartile range
OC	oral contraceptive
RLU	relative light unit
SES	socioeconomic status

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Table 1. Sample characteristics by migration status in Mexican American women (N=90).

	U.S.-born	Foreign-born	P value[a]
Estrogenic activity level (RLUs), median (IQR)	2,403. (9,833)	3,437 (7,000)	0.546
Glucocorticogenic activity level (RLUs), median (IQR)[b]	177,443 (73,473)	183,735 (68,784)	0.813
Age at blood draw (years), mean (sd)	54 (11)	53 (11)	0.624
% Indigenous American ancestry, mean (sd)	44 (12)	42 (14)	0.530
History of breast cancer, n (%)			
No	23 (77)	52 (87)	0.245
Yes	7 (23)	8 (13)	
Education, n (%)			
<High School	7 (23)	40 (67)	<0.001
High School+	21 (70)	15 (25)	
Unknown	2 (7)	5 (8)	
Neighborhood SES (statewide quintiles), n (%)			
1st quintile (lowest)	0	4 (7)	0.011
2nd quintile	2 (7)	19 (32)	
3rd quintile	10 (33)	16 (27)	
4th quintile	9 (30)	15 (25)	
5th quintile (highest)	7 (23)	4 (7)	
Unknown	2 (7)	2 (3)	
Menopausal status at blood draw[d], n (%)			
Premenopausal	11 (37)	21 (35)	1.000
Postmenopausal	19 (63)	38 (63)	
Unknown	0	1 (2)	
Height (cm), mean (sd)	157 (6)	156 (6)	0.632
Body Mass Index (kg/m2), mean (sd)	29 (6)	30 (6)	0.358
Oral Contraceptive use at blood draw[d], n (%)			
Never	8 (27)	26 (43)	0.157
Former	22 (73)	32 (53)	
Current	0 (0)	1 (2)	
Unknown	0	1 (2)	
Hormone therapy use at blood draw[c,d], n (%)			
Never	8 (42)	21 (55)	0.186
Former	8 (42)	16 (42)	
Current	3 (16)	1 (3)	
Alcohol intake during calendar year before selection into parent study[d], n (%)			
None	16 (53)	44 (73)	0.086
Some	12 (40)	14 (23)	
Unknown	2 (7)	2 (3)	
Alcohol intake during the 6 months prior to blood draw[d], n (%)			0.154
None	25 (83)	56 (93)	
Some	5 (17)	4 (7)	
Unknown	0	0	

SES: socioeconomic status; RLU: relative light unit; IQR: interquartile range; sd: standard deviation.

[a] We used t-test (for continuous variables) or Fisher's exact test (for categorical variables) to assess the significance of the difference in variable distribution between U.S.-born and foreign-born women.

[b] Seven U.S. born and 20 foreign-born women did not have information on glucocorticogenic activity.

[c] Only among postmenopausal women.

[d] The participants answered two questionnaires, one at first interview and one at blood draw. The one at interview asked about behavior within the year prior to interview. The one at blood draw asked about behavior within the 6 previous months.

Table 2. Association between estrogenic activity, lifestyle and demographic factors in Mexican American women (N=81).

	Univariate analysis			Multivariate analysis		
	Coef. (95%CI)*	% Change in RLUs**	P value	Coef. (95%CI)	% Change in RLUs**	P value
Nativity (ref. U.S.-born)	0.12 (-0.46, 0.71)	13	0.672	-0.07 (-0.57, 0.43)	-7	0.781
Age at blood draw (per 10 years)	-0.69 (-0.89, -0.50)	-50	<0.001	-0.40 (-0.69, -0.12)	-33	0.007
Indigenous American ancestry (per 10%)	-0.16 (-0.37, 0.06)	-15	0.156	-0.21 (-0.37, -0.04)	-19	0.014
Breast Cancer (ref. no)	-0.57 (-1.30, 0.16)	-43	0.122	0.13 (-0.50, 0.77)	14	0.680
Education (ref. <High School)	-0.02 (-0.60, 0.55)	-2	0.934	-0.15 (-0.63, 0.33)	-14	0.543
Postmenopausal (ref. premenopausal)	-1.55 (-2.00, -1.09)	-79	<0.001	-1.17 (-1.79, -0.55)	-69	<0.001
Height (per 10 cm)	0.21 (-0.23, 0.65)	23	0.349	-0.19 (-0.56, 0.18)	-17	0.315
BMI (ln kg/m2)	-0.04 (-0.19, 0.11)	-4	0.591	0.00 (-0.12, 0.11)	0	0.968
Alcohol intake (ref. none)* **	0.10 (-0.52, 0.71)	11	0.759	0.04 (-0.44, 0.51)	4	0.877

*The coefficients and 95%CI are based on the ln-transformed RLUs.

** Percent change in RLUs (untransformed) per unit change in predictor was estimated using the formula $[e^{\beta}-1]*100$.

***Alcohol intake during calendar year before selection into parent study.

Table 3. Association between estrogenic activity, lifestyle and demographic factors by place of birth (N=81).

	U.S.-born women (N=28)			Foreign-born women (N=53)		
	Coef. (95% CI)*	% Change in RLUs**	P value	Coef. (95% CI)	% Change in RLUs**	P value
Age at blood draw (per 10 years)	-0.59 (-1.19, 0.03)	-45	0.060	-0.77 (-1.25, -0.28)	-54	0.003
Indigenous American ancestry (per 10%)	0.05 (-0.33, 0.44)	5	0.770	-0.26 (-0.46, -0.07)	-23	0.009
Breast Cancer (ref. No)	0.62 (-0.58, 1.83)	86	0.291	-0.19 (-1.05, 0.67)	-17	0.659
Education (ref. <=High School)	-0.58 (-1.54, 0.41)	-44	0.238	-0.07 (-0.69, 0.55)	-7	0.824
Years in the U.S. (per 10 years)				0.25 (0.02, 0.49)	28	0.035
Postmenopausal (ref. premenopausal)	-1.27 (-2.51, 0.02)	-72	0.047	-0.75 (-1.54, 0.03)	-53	0.060
Height (per 10 cm)	-0.14 (-1.01, 0.73)	-13	0.735	-0.25 (-0.69, 0.19)	-22	0.254
BMI (ln kg/m2)	-0.00 (-0.22, 0.22)	0	0.980	-0.08 (-0.24, 0.09)	-8	0.359
Alcohol intake (ref. None)* **	0.29 (-0.60, 1.19)	34	0.504	-0.30 (-0.95, 0.34)	-26	0.346

*The coefficients and 95%CI are based on the ln-transformed RLUs.

** Percent change in RLUs (untransformed) per unit change in predictor was estimated using the formula $[e^{\beta}-1]*100$.

***Alcohol intake during calendar year before selection into parent study.

Table 4. Association between glucocorticogenic activity, lifestyle and demographic factors (N=59).

	Univariate analysis			Multivariate analysis		
	Coef. (95%CI)*	% Change in RLUs**	P value	Coef. (95%CI)	% Change in RLUs**	P value
Nativity (ref. U.S.-born)	-0.4 (-0.20, 0.12)	-33	0.628	-0.05 (-0.22, 0.13)	-5	0.589
Age at blood draw (per 10 years)	0.04 (-0.03, 0.11)	4	0.238	0.03 (-0.06, 0.13)	3	0.468
Indigenous American ancestry (per 10%)	-0.03 (-0.09, 0.03)	-3	0.305	-0.03 (-0.09, 0.03)	-3	0.338
Breast Cancer (ref. No)	-0.09 (-0.29, 0.11)	-9	0.359	-0.23 (-0.45, 0.00)	-21	0.054
Neighborhood SES (continuous score)	-0.07 (-0.17, 0.04)	-7	0.217	-0.09 (-0.20, 0.02)	-9	0.106
Postmenopausal (ref. premenopausal)	0.15 (-0.01, 0.31)	16	0.073	0.16 (-0.06, 0.37)	17	0.157
Height (per 10 cm)	0.11 (-0.04, 0.26)	12	0.149	0.16 (0.01, 0.32)	17	0.037
BMI (ln kg/m2)	-0.01 (-0.05, 0.03)	-1	0.604	0.01 (-0.04, 0.05)	1	0.781
Alcohol intake (ref. None)* **	0.16 (-0.00, 0.33)	17	0.057	0.22 (0.05, 0.40)	25	0.015

*The coefficients and 95%CI are based on the ln-transformed RLUs.

** Percent change in RLUs (untransformed) per unit change in predictor was estimated using the formula $[e^{\beta}-1]*100$.

***Alcohol intake during calendar year before selection into parent study.

Supplementary Materials

Supplementary Figure 1 (Figure S1) — Schematic of sample selection process.

Supplementary Figure 2 (Figure S2) — Graph displays inverse association between age at blood draw and estrogenic activity in Mexican American women.

Supplementary Figure 3 (Figure S3) — Histogram showing the distribution of estrogenic activity in premenopausal and postmenopausal women.

Supplementary Table 1 (Table S1) — Association between estrogenic activity, lifestyle and demographic factors in Mexican American women by menopausal status.

Supplementary Table 2 (Table S2) — Association between glucocorticogenic activity, lifestyle and demographic factors in Mexican American women by menopausal status.

Supplementary Table 3 (Table S3) — Association between glucocorticogenic activity, lifestyle and demographic factors by place of birth.

Supplementary Table 4 (Table S4) — Association between glucocorticogenic activity, lifestyle and demographic factors, including alcohol intake variable at interview and at blood draw.

Supplementary Table 5 (Table S5) — Association between glucocorticogenic activity, lifestyle and demographic factors, including alcohol intake variable that identifies women who reported discordant values at first interview and blood draw.

Figure S1: Schematic of sample selection process. White rectangles represent stage of sample selection into the study. Gray rectangles represent subcategories from which samples were selected into the study (pre: premenopausal; post: postmenopausal).

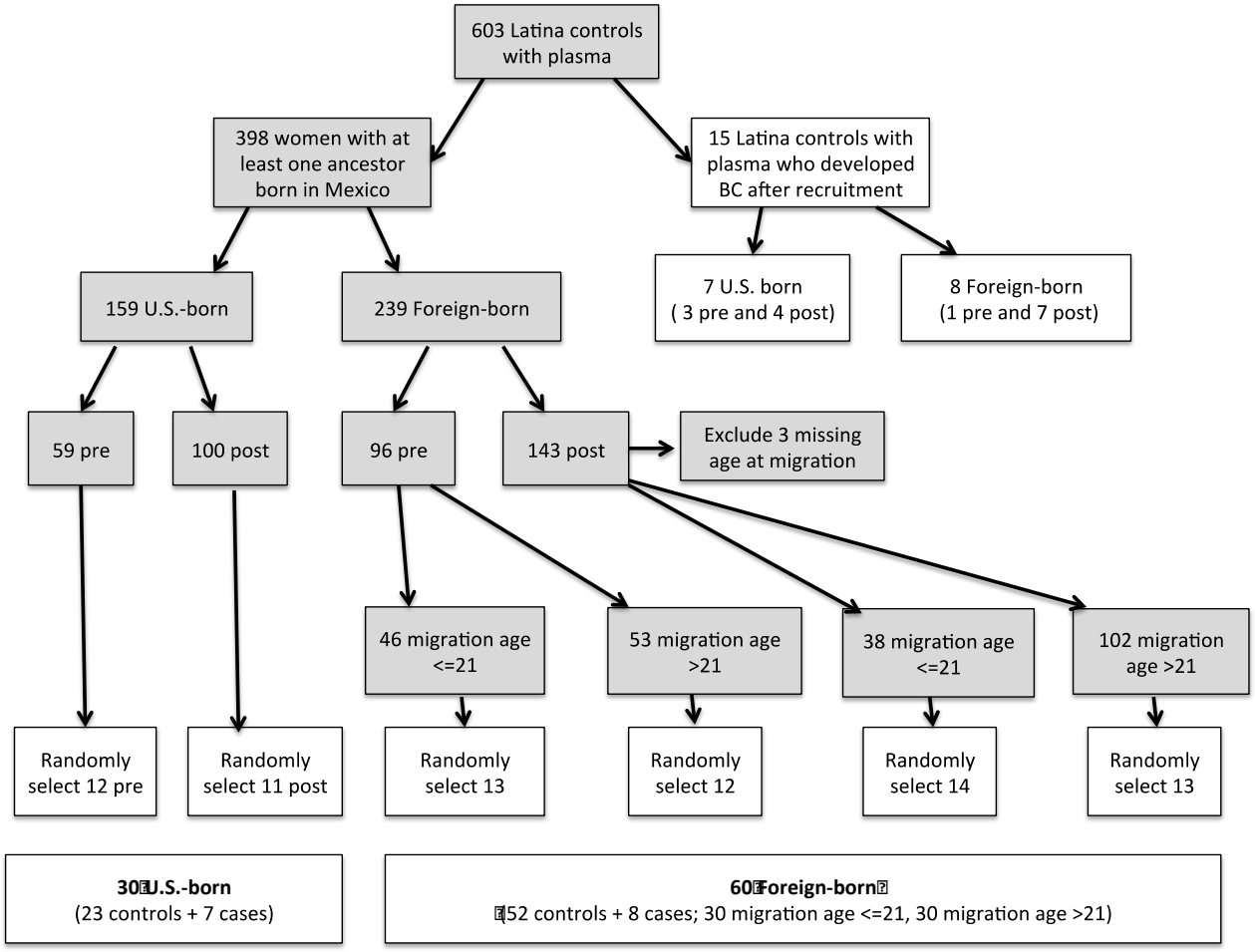

Figure S2: Inverse association between age at blood draw (in years) and estrogenic activity [measured as the natural logarithm of average relative light units (RLUs) of cell-based estrogen receptor (ER) assay] in Mexican American women (Univariate analysis linear regression coefficient of -0.70, 95%CI: -0.89, -0.50, $P=1x10^{-12}$).

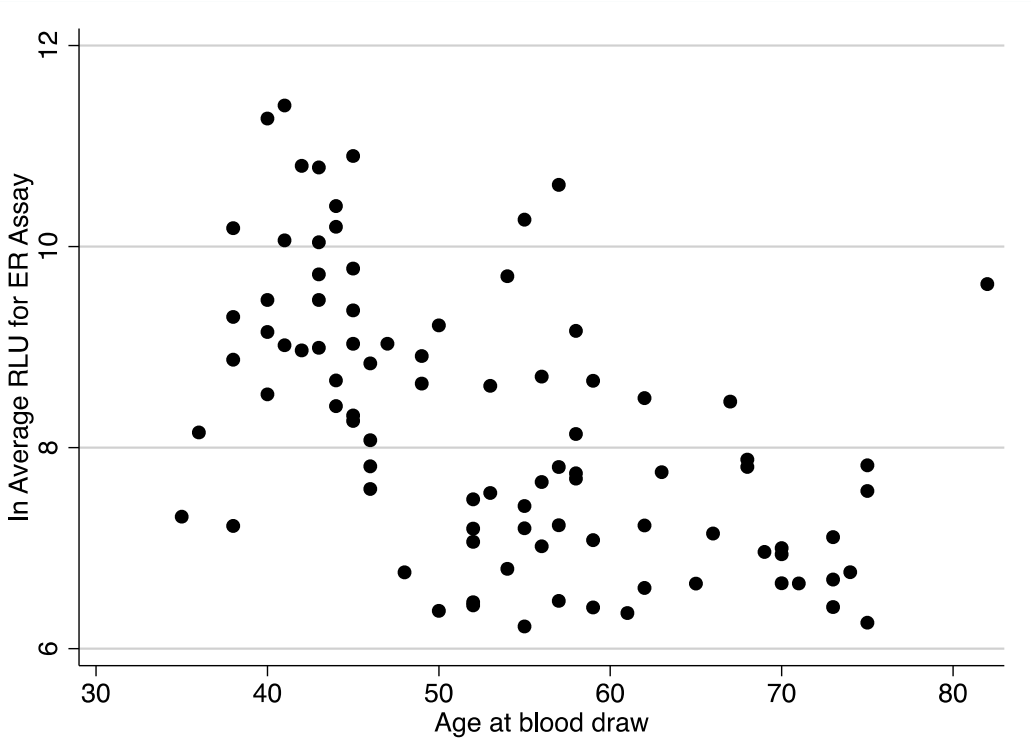

Figure S3: Histogram showing the distribution of estrogenic activity (ln-transformed ER RLUs) in premenopausal (red line) and postmenopausal (black line) women (Univariate analysis linear regression coefficient -1.90, premenopausal as reference, 95%CI: -2.31, -1.49, $P<1x10^{-16}$).

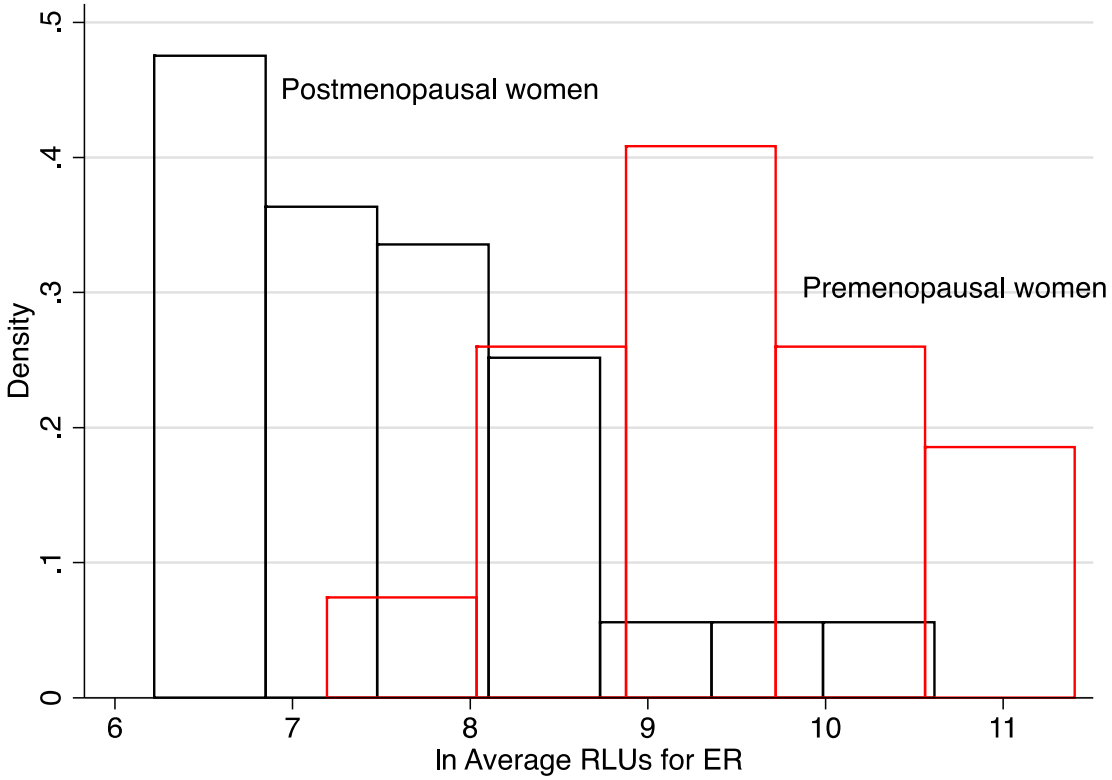

Table S1. Association between estrogenic activity, lifestyle and demographic factors in Mexican American women by menopausal status (N=81).

	Premenopausal women N=29 Coef. (95%CI)	**P value**	**Postmenopausal women N=52 Coef. (95%CI)**	**P value**
Nativity (ref. U.S.-born)	-0.43 (-1.27, 0.41)	0.297	0.47 (-0.12, 1.05)	0.116
Age at blood draw (per 10 years)	-0.34 (-1.35, 0.67)	0.487	-0.27 (-0.57, 0.03)	0.077
Indigenous American ancestry (per 10%)	-0.13 (-0.39, 0.12)	0.286	-0.24 (-0.44, -0.05)	0.014
Breast Cancer (ref. No)	0.66 (-1.10, 2.41)	0.443	0.06 (-0.56, 0.68)	0.847
Education (ref. <High School)	-0.50 (-1.21, 0.22)	0.161	0.04 (-0.53, 0.60)	0.893
Height (per 10 cm)	-0.33 (-0.90, 0.24)	0.244	-0.10 (-0.56, 0.37)	0.679
BMI (ln kg/m2)	-0.07 (-0.25, 0.13)	0.477	0.09 (-0.05, 0.24)	0.211
Hormone therapy use (ref. Never)				
Former			-0.29 (-0.81, 0.23)	0.269
Current			2.03 (1.10, 2.96)	<0.001
Alcohol intake (ref. None)*	0.18 (-0.59, 0.96)	0.625	0.28 (-0.32, 0.88)	0.354

* Alcohol intake during calendar year before selection into parent study.

Table S2: Association between glucocorticogenic activity, lifestyle and demographic factors in Mexican American women by menopausal status (N=59).

	Premenopausal women N=19	**P value**	**Postmenopausal women N=40**	**P value**
	Coef. (95%CI)		**Coef. (95%CI)**	
Nativity (ref. U.S.-born)	-0.11 (-0.65-0.43)	0.653	-0.12 (-0.36-0.11)	0.292
Age at blood draw (per 10 years)	0.12 (-0.39-0.63)	0.606	0.03 (-0.08-0.15)	0.583
Indigenous American ancestry (per 10%)	0.00 (-0.17-0.18)	0.990	-0.04 (-0.12-0.04)	0.327
Breast Cancer (ref. No)	-0.20 (-1.03-0.63)	0.595	-0.26 (-0.54-0.02)	0.069
Neighborhood SES (continuous score)	-0.16 (-0.47-0.16)	0.290	-0.10 (-0.26-0.07)	0.235
Height (per 10 cm)	-0.03 (-0.42-0.37)	0.887	0.19 (-0.03-0.41)	0.087
BMI (ln kg/m2)	0.02 (-0.06-0.09)	0.667	-0.01 (-0.08-0.06)	0.853
Hormone therapy use (ref. Never)				
Former			-0.01 (-0.23-0.22)	0.961
Current			-0.02 (-0.75-0.71)	0.954
Alcohol intake (ref. None)*	0.08 (-0.41-0.58)	0.714	0.26 (0.01-0.51)	0.042

* Alcohol intake during calendar year before selection into parent study.

Table S3. Association between glucocorticogenic activity, lifestyle and demographic factors by place of birth (N=59).

	U.S.-born women N=21 Coef. (95%CI)	P value	Foreign-born women N=38 Coef. (95%CI)	P value
Age at blood draw (per 10 years)	0.20 (-0.02-0.41)	0.069	-0.01 (-0.18-0.16)	0.891
Indigenous American ancestry (per 10%)	-0.01 (-0.16-0.14)	0.888	-0.06 (-0.14-0.01)	0.096
Breast Cancer (ref. No)	-0.44 (-0.91-0.03)	0.066	-0.28 (-0.61-0.05)	0.095
Neighborhood SES (continuous score)	-0.05 (-0.26-0.16)	0.615	-0.11 (-0.27-0.04)	0.147
Years in the US (per 10 years)			-0.03 (-0.12-0.06)	0.540
Postmenopausal (ref. premenopausal)	0.10 (-0.30-0.51)	0.591	0.14 (-0.17-0.45)	0.371
Height (per 10 cm)	0.27 (-0.04-0.59)	0.085	0.11 (-0.10-0.31)	0.300
BMI (ln kg/m2)	0.01 (-0.06-0.07)	0.847	-0.01 (-0.08-0.05)	0.646
Alcohol intake (ref. None)*	0.31 (0.00-0.61)	0.047	0.28 (0.00-0.55)	0.048

* Alcohol intake during calendar year before selection into parent study.

Table S4. Association between glucocorticogenic activity, lifestyle and demographic factors, including alcohol intake variable at interview and at blood draw (N=59).

	Alcohol at interview		**Alcohol at blood draw**	
	Coef. (95%CI)	P value	Coef. (95%CI)	P value
Nativity (ref. U.S.-born)	-0.05 (-0.22, 0.13)	0.589	-0.10 (-0.29, 0.08)	0.252
Age at blood draw (per 10 years)	0.03 (-0.06, 0.13)	0.468	0.01 (-0.09, 0.11)	0.778
Indigenous American ancestry (per 10%)	-0.03 (-0.09, 0.03)	0.338	-0.03 (-0.10, 0.03)	0.281
Breast Cancer (ref. No)	-0.23 (-0.45, 0.00)	0.054	-0.14 (-0.38, 0.11)	0.262
Neighborhood SES (continuous score)	-0.09 (-0.20, 0.02)	0.106	-0.08 (-0.20, 0.03)	0.163
Postmenopausal (ref. premenopausal)	0.16 (-0.06, 0.37)	0.157	0.18 (-0.05, 0.41)	0.116
Height (per 10 cm)	0.16 (0.01, 0.32)	0.037	0.15 (-0.01, 0.31)	0.073
BMI (ln kg/m2)	0.01 (-0.04, 0.05)	0.781	-0.01 (-0.05, 0.03)	0.677
Alcohol intake (ref. None)*	0.22 (0.05, 0.40)	0.015	-0.04 (-0.34, 0.20)	0.592

* Alcohol intake during calendar year before selection into parent study.

Table S5. Association between glucocorticogenic activity, lifestyle and demographic factors, including alcohol intake variable that identifies women who reported discordant values at first interview and blood draw (N=59).

	Coef. (95%CI)	P value
Nativity (ref. U.S.-born)		
Age at blood draw (per 10 years)	0.02 (-0.07, 0.12)	0.594
Indigenous American ancestry (per 10%)	-0.03 (-0.09, 0.03)	0.254
Breast Cancer (ref. no)	-0.19 (-0.42, 0.03)	0.094
Neighborhood SES (continuous score)	-0.09 (-0.20, 0.02)	0.094
Postmenopausal (ref. premenopausal)	0.15 (-0.06, 0.37)	0.154
Height (per 10 cm)	0.18 (0.02, 0.33)	0.024
BMI (ln kg/m2)	0.00 (-0.04, 0.04)	0.880
Alcohol intake (ref. none)		
Concordant drinker	0.05 (-0.21, 0.31)	0.693
Discordant	0.29 (0.10, 0.47)	0.004

Chapter 3. Estrogenic activity, race/ethnicity, and Indigenous American ancestry among San Francisco Bay Area women

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Abstract

Estrogens play a significant role in breast cancer development and are not only produced endogenously, but are also mimicked by estrogen-like compounds from environmental exposures. We evaluated associations between estrogenic (E) activity, demographic factors and breast cancer risk factors in Non-Latina Black (NLB), Non-Latina White (NLW), and Latina women. We examined the association between E activity and Indigenous American (IA) ancestry in Latina women. Total E activity was measured with a bioassay in plasma samples of 503 women who served as controls in the San Francisco Bay Area Breast Cancer Study. In the univariate model that included all women with race/ethnicity as the independent predictor, Latinas had 13% lower E activity ($p = 0.239$) and NLBs had 35% higher activity ($p = 0.04$) compared to NLWs. In the multivariable model that adjusted for demographic factors, Latinas continued to show lower E activity levels (26%, $p = 0.026$), but the difference between NLBs and NLWs was no longer statistically significant ($p = 0.431$). An inverse association was observed between E activity and IA ancestry among Latina women (50% lower in 0% vs. 100% European ancestry, $p = 0.027$) consistent with our previously reported association between IA ancestry and breast cancer risk. These findings suggest that endogenous estrogens and exogenous estrogen-like compounds that act on the estrogen receptor and modulate E activity may partially explain racial/ethnic differences in breast cancer risk.

Introduction

Epidemiological studies have consistently reported that endogenous sex hormones play a critical role in the etiology of numerous diseases including breast cancer[1–4]. Interestingly, among U.S. racial/ethnic groups the reported variation in endogenous hormone levels is consistent with observed racial/ethnic differences in the incidence of breast cancer[5–7]. For example, in the Women's Health Initiative Dietary Modification Trial, African American women had significantly higher concentrations of endogenous reproductive hormones compared to non-Latina White women[8], whereas higher levels of urinary concentrations of estrogens were strongly associated with breast cancer risk in Asian women in the Shanghai Women's Study cohort[9]. Moreover, in the Multiethnic Cohort study, postmenopausal Native Hawaiian and African American women tended to have higher levels of endogenous hormones when compared to non-Latina Whites (NLWs)[10], while foreign-born Hispanic/Latina women (referred to as Latinas hereafter) had lower hormone levels which correlates with their lower incidence of breast cancer[5].

Changes in lifestyle and demographic factors can influence endogenous estrogen levels through changes in adiposity. Across racial/ethnic groups, multiple studies have consistently identified a positive association between body mass index (BMI) and estrogen levels[11,12]. In Latina women, adaptation of a Western lifestyle, along with greater physical inactivity have served as potential explanations for changes in BMI[13]. Estrogen plays a significant role in breast cancer and is not only produced endogenously, but is also mimicked by exogenous sources including xenoestrogens and phytoestrogens[14–16] like bisphenol A (BPA), diethylstilbestrol (DES), atrazine, and soy products. It is because of this dual role that endogenous and exogenous sources could contribute to differences in breast cancer incidence rates in different racial/ethnic populations across the U.S.[17].Various methods are available to quantify estrogen receptor (ER) function and activation. Luciferase assays have been widely used and have proven useful in predicting breast

cancer risk[18,19]. Generally, due to the fluctuations of estrogens throughout the menstrual cycle, estrogen measures have been more consistent for postmenopausal than premenopausal women[20]. In a previous pilot study among foreign-born and U.S.-born Mexican women who participated as controls in a population-based case-control study of breast cancer, we found that plasma estrogenic (E) activity was associated with genetic ancestry and years of U.S. residence[18], suggesting the possibility of a hormone related pathway for the observed racial/ethnic differences in breast cancer risk. In the present study, we examined total E activity accounting for both endogenous and exogenous sources of estrogenic compounds in plasma of 503 women, the majority of them postmenopausal, from three racial/ethnic groups. We hypothesize that differences in breast cancer risk in different racial/ethnic groups could be partly due to variations in endogenous estrogens and exogenous estrogen-like exposures. We evaluated the association between E activity, demographic and lifestyle factors in non-Latina Black (NLB), NLW, and Latina women, accounting for endogenous estrogen levels. We also used a larger sample of Latina women to validate the previously observed association between E activity and Indigenous American (IA) ancestry[18]. The approach we used provides an efficient way to assess endogenous and exogenous exposures and provides insights on a mechanistic connection that explains the differences in breast cancer incidence rates in different racial/ethnic populations living in the US.

Materials and Methods

Study samples/ control selection

Participants were selected from the control group of the San Francisco Bay Area Breast Cancer Study (SFBCS), a population-based case-control study in Latina, NLB, and NLW women[13]. Controls living in San Francisco, San Mateo, Alameda, Contra Costa or Santa Clara counties were identified through random-digit dialing and frequency matched to breast cancer cases diagnosed from 1995–2002 in the same counties on race/ethnicity and 5-year age group. Blood samples were collected for a subset of study participants (cases diagnosed from 1997–2002 and their matched controls). Professional trained interviewers administered a questionnaire on breast cancer risk factors in English or Spanish at a home visit and took anthropometric measurements. Blood samples were collected shortly after the interview and in some instances a few years after the interview as part of an ancillary study. A short questionnaire was administered at time of blood draw to obtain updates on key variables (current menstrual status and weight; pregnancy, breast-feeding, smoking, alcohol consumption, and medication use in the previous 6 months).In the present study, we measured E activity in plasma samples of 503 women (329 Latinas, 100 NLBs, and 74 NLWs) from the control group of SFBCS. Eight percent of the participants were premenopausal and equally distributed among the different racial/ethnic groups. To prevent exposure misclassification of exogenous estrogen use, we excluded women who were using hormone therapy at the time of blood draw. Participants who had one or more missing covariates were excluded from the analyses (11 women in analyses without adjustment for endogenous estrogens; 15 women in analyses with adjustment for endogenous estrogens). All study participants provided written informed consent. The study was approved by the Institutional Review Boards at the University of California, San Francisco and the Cancer Prevention Institute of California.

Endogenous hormone measurements

Plasma hormone measurements were performed at the Reproductive Endocrine Research laboratory at the University of Southern California under the direction of one of the contributing authors (FZS). Methods are described elsewhere[5]. Briefly, plasma concentrations of estrone and bioavailable estradiol were obtained by a radioimmunoassay method after organic solvent extraction and Celite column partition chromatography. Reported endogenous estrogen levels are the sum of estrone and bioavailable estradiol in free and albumin bound form. The assay sensitivities for estrone and estradiol are 4 pg/ml and 2 pg/ml, respectively, and the inter-assay coefficient of variation (CV) for each assay is less than 12%.

Estrogenic activity measurement

We utilized a receptor-mediated Chemical-Activated Luciferase gene eXpression (CALUX) assay for the assessment of total E activity profiles in human plasma, which captures levels of both endogenous and exogenous estrogenic compounds. Methods followed procedures as previously described[18,21]. Briefly, endogenous and exogenous estrogens and estrogen-like compounds (e.g., 17-beta estradiol (E2), phytoestrogens, 4-nonylphenol) in human plasma were measured using a transfected human breast cancer cell line with luciferase reporter, T47D-Kbluc (ATCC, Manassas, VA). Cells were cultured in phenol red DMEM with 10% FBS until one week prior to cell treatment with plasma. Cells were placed in phenol red free DMEM media with 10% charcoal dextran serum to ensure removal of all external sources of estrogens for one week prior to plasma addition. After one week in phenol red free medium, cells between passage 10 and 16 were seeded at a density of 27,000 cells per well and a final volume of 200 uL per well in 96-well microtiter plates and incubated at 37 degrees Celsius. After a 24 hour incubation period, 8uL of plasma per sample was diluted in phenol red free medium and added in quadruplicates directly onto the cells. A second incubation period of 24 hours at 37 degrees Celsius followed plasma addition. Lastly, cells were lysed using 1X passive lysis buffer (Promega, Madison, WI, USA) and results were obtained using a microplate luminometer (Berthold Technologies, Oak Ridge, TN, USA). Reporter activity was measured by the fluorescence emitted per well. Results were expressed in relative light units (RLUs) with higher RLU values reflecting greater E activity in plasma. Four RLU readings per sample were averaged to express results as one measure. The measurement was converted to picomolar (pM) equivalents based on the standard curve of (17β)-estra-1,3,5(10)-triene-3,17-diol, or 17β-estradiol (Tocris Bioscience, Bristol, UK) on each plate expanding a range of 0.0 pM to 25.0 pM. Samples from each racial/ethnic group were included within each batch. Lab personnel were blinded to the race/ethnicity of the samples. The minimum limit of detection for estradiol is 1.0 pM and the intra-assay (technical) and inter-assay (biological) CVs of this assay are between 7–23%, with higher values often corresponding to premenopausal women.

Statistical methods

All hormone values were natural log (ln) transformed to approximate normal distribution. Differences in means or proportions for all analyzed variables between racial/ethnic groups were assessed using two-sided t-tests and Fisher's exact tests, respectively. Linear regression was used to analyze the relationship between ln-transformed and plate adjusted E activity (dependent variable) and race/ethnicity (main predictor). Plate adjusted values were obtained by first estimating average plate effects using linear regression and then subtracting the average plate

effect from each individual value. Percent change in RLUs per unit change of predictor variables was calculated using the formula $[e^{\beta}-1]*100$. Statistical significance was set at $p<0.05$. All statistical analyses were performed using the program Stata[22].The present study assessed differences in E activity by race/ethnicity and tested associations between E activity and various factors that were hypothesized to affect E activity levels (i.e., height, BMI, age, alcohol intake, and neighborhood SES). Multivariable regression models included several independent variables such as race/ethnicity (Latina, NLW, NLB), age at blood draw (categorical, <55, 55 to 65 and ≥ 66 years), height (continuous, in centimeters), body mass index (BMI) at blood draw (categorical, <25, 25–29.9, and ≥30 kg/m^2), neighborhood socioeconomic status (SES) (categorical, 1 = low SES, 5 = high SES) geocoded to a Census 2000 block group, alcohol intake during the calendar year before selection into the parent study (categorical, grams per day), and E activity (continuous, log-transformed). Selection of these variables was based on factors we previously hypothesized could affect E activity at time of blood draw[18]. BMI was calculated as self-reported weight (kg) at blood draw / height squared (m) measured at interview. For a subset of Latina women (n = 276), information was available on IA ancestry. Proportion of IA ancestry was used as a continuous variable with values ranging from 0 to 100%. The multivariable model for the ancestry analysis included IA ancestry, age at blood draw, height, BMI, neighborhood SES, alcohol intake, and nativity (foreign-born vs. U.S.-born). Analyses were also adjusted for endogenous estrogen measurements.

Data on reproductive variables were available from the interview (i.e., age at menarche, age at first full term pregnancy, number of full term pregnancies). We ran multivariable models adjusting for reproductive variables, but as we had previously hypothesized, they did not have an effect on E activity at blood draw, which for most women occurred many years after the individual's last pregnancy. Therefore, final models do not adjust for reproductive variables. Additionally, because the average age at blood draw for all three racial/ethnic groups was over the age of 60 years, which is about 10 years after the average age of menopause in U.S. women, final models did not adjust for age at menopause.

Results

Descriptive characteristics by racial/ethnic groups are presented in Table 1. After adjusting for plate, ER RLUs were highest for NLB women (mean of 8844 RLUs), followed by NLWs (mean of 8155 RLUs), and lowest for Latina women (mean RLUs of 6226). NLW women were older at blood draw (66.8 yrs, sd. 10.5 yrs) when compared to NLB (61.7 yrs, sd. 9.8 yrs) and Latina (61.5 yrs, sd. 9.5 yrs) women. Age at menarche was similar for the three groups. NLB women had the lowest age at first full term pregnancy (21 yrs, sd. 5 yrs). However, Latina women had a greater number of full term pregnancies (3.8) compared to the NLW (2.4) and NLB women (2.8). Latina women were on average shorter than NLBs and NLWs. A large percentage (66%) of the Latina women in this study were foreign-born, while over 90% of the NLW and NLB women were U.S.-born. About 87% and 84% of the Latina and NLB women, respectively, were categorized as overweight or obese. A greater proportion of NLW women reported consuming some alcohol (54%), while 76% and 71% of NLB and Latina women, respectively, reported no alcohol intake during the year before the interview. Lastly, women in this study were predominantly postmenopausal either naturally or due to surgery. Although a statistically significant greater proportion (27%) of NLB women had a history of hysterectomy and/or

oophorectomy compared to NLW and Latina women, menopausal status did not differ between the three racial/ethnic groups.

In the univariate model (Table 2), Latinas had 13% lower E activity ($p = 0.239$) and NLBs had 35% higher activity ($p = 0.04$) compared to NLWs. After adjusting for endogenous estrogens, the trend was consistent with 11% higher E activity in NLB women and 21% lower activity in Latina women; however, the association was statistically significant only for Latina women ($p = 0.019$). In the multivariable model, Latinas showed lower E activity before (26%, $p = 0.026$) and after (25%, $p = 0.01$) adjusting for endogenous estrogens. Although E activity in NLBs continued to remain higher than that of NLWs, the difference between the two groups was no longer statistically significant before (13%, $p = 0.431$) or after (5%, $p = 0.673$) adjustment for endogenous estrogens. We observed a decrease in E activity with increasing age, which was no longer statistically significant after adjustment for endogenous estrogens. In unadjusted models, E activity was 67% higher for obese women ($p<0.001$) and 40% higher for overweight women ($p = 0.006$) when compared to those with normal BMI ($p<0.001$), but these associations were no longer apparent after adjusting for endogenous estrogen levels. Overall, no statistically significant associations were observed with neighborhood SES before or after adjustment for endogenous estrogens. Compared to women who reported no alcohol consumption, those who consumed <10gms of alcohol had lower E activity before (21%, $p = 0.017$) and after (17%, $p = 0.024$) adjusting for endogenous estrogens.

Restricting the analysis to Latina women with information on genetic ancestry ($N = 276$), we found that in the univariate model higher IA ancestry was associated with lower E activity (Table 3). This finding was statistically significant prior to adjusting for endogenous estrogens (52%, $p = 0.031$) and marginally significant after adjustment (39%, $p = 0.088$). In the multivariable analysis, increasing age was associated with lower E activity. As with the full sample, the significant associations with BMI were no longer statistically significant after adjusting for endogenous estrogens. No statically significant associations were observed for height, neighborhood SES or nativity.

Discussion

Our study shows that there are significant differences in E activity in women across three racial/ethnic groups, partly due to differences in BMI (in the case of NLWs vs. NLBs) and potential exposure to exogenous estrogen-like compounds (NLWs vs. Latinas). Results also suggest that higher IA ancestry in Latina women is associated with lower levels of E activity, which is consistent with the observation that Latina women with high IA ancestry have lower risk of developing breast cancer.

There was an association between BMI and E activity in all racial/ethnic groups. In NLB women, higher E activity was mostly attributed to BMI. After inclusion of the BMI variable in the multivariable model, the level of E activity among NLBs was no longer statistically different from NLWs. The attenuation of the positive association between E activity and BMI after adjusting for endogenous estrogen levels in the regression model is consistent with previous observations[23–25]. In overweight and obese postmenopausal women, the adipose tissue serves as the primary source of estrogen synthesis and leads to elevated estrogen measures. This is likely to be one of multiple mechanisms underlying the association between BMI and breast cancer risk in postmenopausal women[11]. Interestingly, weight loss interventions[26] in postmenopausal women have been

associated with lower circulating estradiol, suggesting a reversal of peripheral production of estrogens via reduction of adipose tissue. Since the association between E activity and BMI is present in all racial/ethnic groups, a reduction of E activity through weight management could lead to breast cancer risk reduction in all women. The lower E activity among Latinas, especially among Latina women with high IA ancestry, was not fully explained by the relationship between BMI and endogenous estrogen levels and should be further investigated.

Another lifestyle factor that showed association with E activity was alcohol intake. Alcohol intake is associated with risk of several cancers including breast cancer. However, studies looking at the effects of alcohol consumption on circulating levels of endogenous estrogen have produced inconsistent results. Some studies reported that increased alcohol consumption is associated with higher circulating estrogens[27], while others found no association[28]. Our results are not consistent with previous findings, given that our data show an inverse association between E activity and alcohol consumption.

More studies that include samples from diverse populations are needed to identify the effects of alcohol consumption on endogenous estrogen levels. The association between IA ancestry and E activity among Latinas even after adjust for endogenous estrogens suggests that in this group, the E activity could be related to environmental exposures such as exogenous chemicals or dietary constituents. Soy-based and vegetable-derived foods containing several phytoestrogens are common in the diet of Latino populations[29]. Because phytoestrogens have a similar chemical structure to estradiol, they may compete with estrogens for binding to ER. Dietary lignan consumption has been found to be associated with reduced risk of postmenopausal breast cancer specifically in ER and progesterone receptor-positive cases, suggesting that these compounds are acting through an ER related mechanism[30]. However, this hypothesis needs to be further investigated. Another explanation for the lower E activity among Latina women with high IA ancestry after adjusting for endogenous estrogen levels would be a biological difference in the metabolism of estrogen and estrogen-like compounds, resulting in a fewer number of molecules that could adequately bind to the ER[31]. Some studies have demonstrated that estrogen metabolism and hydroxylation of parent estrogen compounds at different positions around the steroid ring, mainly 2-hydroxylation, is associated with reduced risk of postmenopausal breast cancer[32].

An important strength of the present study is the diverse study population and the larger sample set of Latina women included in the analysis of IA ancestry (n = 276) compared to our previous pilot study that included only 90 Mexican women[18]. Using this larger sample size, we have validated our previous findings of a negative association between E activity and IA ancestry. The association between endogenous estrogen levels and IA ancestry was statistically significant and could partly explain why Latina women with higher IA ancestry have a lower incidence of breast cancer. Furthermore, we were able to assess E activity using a commonly used bioassay and correlate measures with endogenous estrogen levels. The Pearson correlation coefficient between the two methods was high (0.62, $p<0.0001$). Although the methods were not perfectly correlated, our results highlight the potential role of exogenous estrogenic compounds in the activation of the ER and the concomitant effects on the endocrine system. More importantly, given that most breast cancer grows in the presence of estrogens, understanding what factors (of endogenous and exogenous origin) might stimulate or block the estrogen receptor among women of different racial/ethnic backgrounds is of great relevance.

Our study also has some limitations. Although we were able to assess total E activity in plasma samples and account for endogenous estrogens, this approach does not allow us to

determine the specific estrogen-like compounds that are acting on the ER. However, we report total E activity, which encompasses both endogenous and exogenous estrogens and is a comprehensive representation of estrogenic exposure. Additionally, our results are based on blood samples from a single time point, which may not represent the fluctuations of estrogens over time. Yet, the endogenous estrogen levels in postmenopausal women are relatively stable over time[33] and fluctuations in total estrogens will be mostly due to exogenous estrogens that are captured by the bioassay. Our study only included control women; assessing associations with breast cancer risk using a case-control design is not feasible because measuring E activity in plasma from breast cancer cases would not reflect E activity before diagnosis. However, the association between levels of circulating estrogens and breast cancer risk is well established[31], highlighting the significance of the associations reported in the present study for breast cancer risk. Further replication of the association between race/ethnicity, BMI, genetic ancestry, E activity, endogenous hormones and breast cancer risk in a prospective cohort with information about possible sources of exogenous estrogens (i.e., diet) and genetic data will provide the ideal setting to investigate the role that estrogen-related factors play in explaining differences in breast cancer incidence between racial/ethnic groups. Such knowledge could lead to race/ethnicity/ancestry-specific interventions focused on lowering E activity to reduce the risk of developing breast cancer in all racial/ethnic groups.

Conclusions

Given the central role of the activation of the ER in the etiology of breast cancer, a better understanding of the receptor's interaction with the receptor ligands, whether endogenous or exogenous, is imperative. Additionally, the discovery of specific compounds that are modulating the receptor and are present at different levels in different populations, would lead to changes in exposure to these compounds and ultimately, to changes in breast cancer risk.

Glossary

BMI	body mass index
CALUX	Chemical Activated Luciferase gene eXpression
CV	coefficient of variation
E	estrogenic
ER	estrogen receptor
FBS	fetal bovine serum
IA	Indigenous American
IQR	interquartile range
NLB	Non-Latina Black
NLW	Non-Latina White
RLU	relative light unit
SES	socioeconomic status
SFBCS	San Francisco Bay Area Breast Cancer Study

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Table 1. Participant characteristics by racial/ethnic group (N=503).

Continuous variables	Latinas			NLWs			NLBs			P value
	N	Mean	Sd.	N	Mean	Sd.	N	Mean	Sd.	
Estrogenic Activity in RLUs	329	6226	12899	74	8155	13307	100	8844	11260	0.0157
Median (IQR)		2452 (1582- 4478)			2248 (1722-4691)			3335 (1883-10124)		
Endogenous estrogen level[a], pg/mL	327	58.9	49.4	74	52.1	32.1	100	64	38.2	0.0613
Median (IQR)		46.7 (34.5-64.5)			40.9 (34.5-60.4)			53.6 (36.5-81.3)		
Age at blood draw (years)	329	61.5	9.5	74	66.8	10.5	100	61.7	9.8	0.0001
Age at menarche, yrs	326	12.9	1.9	74	12.8	1.5	99	12.7	1.7	0.6396
Age at first full term pregnancy, yrs	306	23	5.2	58	24.2	4.8	88	20.8	4.9	0.0001
Number of full term pregnancies	329	3.8	2.7	74	2.4	1.8	100	2.8	2.2	<0.0001
Height (cm)	324	155.5	6.9	73	161.1	7.3	100	163.3	6.2	<0.0001
Individual African Ancestry proportion (%)	285	8	7							
Individual Indigenous American ancestry proportion (%)	285	41	15							

Categorical variables	Latinas		NLWs		NLBs		P value
	N	%	N	%	N	%	
Nativity							
U.S.-born	112	34	68	92	97	97	<0.0001
Foreign-born	217	66	6	8	3	3	
Family history of breast cancer[b]							
No	290	88	62	84	88	88	0.559
Yes	39	12	12	16	12	12	
Alcohol intake (gms per day)[c]							
None	235	71	34	46	76	76	<0.0001
<10	82	25	20	27	15	15	
≥10	12	4	20	27	9	9	
BMI (kg/m^2)							
<25	42	13	22	30	16	16	<0.0001
25-29.9	122	38	30	41	24	24	
≥30	160	49	21	29	60	60	
Age at blood draw (years)							
<55	94	29	14	19	31	31	0.001
55-65	117	36	13	18	31	31	
≥66	118	36	47	64	38	38	
Menopausal status at blood draw							
Premenopausal	28	9	8	11	6	7	0.571
Postmenopausal	274	91	63	89	86	93	
History of oophorectomy and/or hysterectomy	46	14	10	14	27	27	0.01
Neighborhood socioeconomic status (SES)							
1 (low SES)	17	5	3	4	23	23	<0.0001
2	77	24	6	8	30	30	
3	91	28	12	16	25	25	
4	80	25	21	29	11	11	
5 (high SES)	61	19	31	42	10	10	

NLW, Non-Latina White; NLB, Non-Latina Black; RLUs, relative light units reported by bioassay (untransformed)

[a] Sum of endogenous estrogens including estrone and bioavailable estradiol (free and albumin bound); [b] First degree relatives; [c] Alcohol intake during calendar year before selection into parent study

Table 2. Association of estrogenic activity with lifestyle and demographic factors in all women (N=488) using univariate and multivariable analysis.

Univariate	**Coefficient[a] (95% CI)**	**% Change in RLUs***	**P value**	**Adjusted by endogenous estrogens[b]**	**% Change in RLUs***	**P value**
Race/ethnicity[#]						
NLW	ref			ref		
NLB	0.30 (0.01, 0.58)	35	0.04	0.10 (-0.13, 0.32)	11	0.398
Latina	-0.14 (-0.38, 0.10)	-13	0.239	-0.23 (-0.42, -0.04)	-21	0.019
Multivariable						
Race/ethnicity						
NLW	ref			ref		
NLB	0.12 (-0.18, 0.43)	13	0.431	0.05 (-0.20, 0.31)	5	0.673
Latina	-0.30 (-0.57, -0.04)	-26	0.026	-0.29 (-0.50, -0.07)	-25	0.01
Age at blood draw (years)						
<55	ref			ref		
55-65	-0.32 (-0.53, -0.11)	-27	0.003	-0.01 (-0.19, 0.16)	-1	0.884
>65	-0.41 (-0.62, -0.21)	-34	<0.001	-0.06 (-0.24, 0.11)	-6	0.491
Height, cm	0.01 (-0.01, 0.02)	1	0.33	0.00 (-0.01, 0.01)	0	0.684
BMI (kg/m^2)						
<25	ref			ref		
25-29.9	0.34 (0.10, 0.59)	40	0.006	0.12 (-0.10, 0.32)	13	0.272
≥30	0.51 (0.27, 0.75)	67	<0.001	0.13 (0.08, 0.33)	14	0.225
Neighborhood SES						
1 (low)	ref			ref		
2	0.27 (-0.06, 0.60)	31	0.114	0.12 (-0.15, 0.39)	13	0.39
3	0.10 (-0.23, 0.43)	11	0.565	0.01 (-0.26, 0.28)	1	0.915
4	0.31 (-0.03, 0.65)	36	0.078	0.20 (-0.08, 0.48)	22	0.163
5 (high)	0.25 (-0.10, 0.60)	28	0.157	0.10 (-0.18, 0.39)	11	0.475
Alcohol intake (gms/day)[c]						
None	ref			ref		
<10	-0.24 (-0.44, -0.04)	-21	0.017	-0.19 (-0.35, -0.02)	-17	0.024
≥10	-0.25 (-0.57, 0.07)	-22	0.122	-0.20 (-0.46, 0.06)	-18	0.129

CI, confidence interval

[#] ANOVA p-value = 0.002

* Luciferase reporter assay results expressed in relative light units (RLUs). Percent change in RLUs (untransformed) per unit change in predictor was estimated using the formula $[e^{\beta} - 1] * 100$.

[a] The coefficients and 95% CI are based on the ln-transformed RLUs

[b] Sum of endogenous estrogens including estrone and bioavailable estradiol (free and albumin bound)

[c] Alcohol intake during calendar year before selection into parent study

Table 3. Association between estrogenic activity and Indigenous American ancestry in Latina women (N=276) using univariate and multivariable analyses.

Univariate	**Coefficient (95% CI) [a]**	**% Change in RLUs***	**P value**	**Coefficient (95% CI) [a]**	**% Change in RLUs***	**P value**
Indigenous American ancestry	-0.74 (-1.41, -0.07)	-52	0.031	-0.49 (-0.06, 0.07)	-39	0.088
Multivariable		**Model A [b]**		**Model A + endogenous estrogens**		
Indigenous American ancestry	-1.10 (-1.84, -0.35)	-67	0.004	0.71 (-1.34, -0.081	-51	0.027
Age at blood draw (years)						
<55	ref			ref		
55-65	-0.28 (-0.55, -0.01)	-24	0.041	-0.01 (-0.24, 0.22)	-1	0.918
>65	-0.37 (-0.64, -0.10)	-31	0.008	-0.05 (-0.28, 0.19)	-5	0.681
Height, cm	-0.00 (-0.02, 0.01)	0	0.58	-0.01 (-0.02, 0.01)	-1	0.431
BMI (kg/m^2)						
<25	ref			ref		
25-29.9	0.49 (0.15, 0.84)	63	0.005	0.25 (-0.05, 0.54)	28	0.107
≥30	0.47 (0.13, 0.82)	60	0.006	0.17 (-0.12, 0.47)	19	0.251
Neighborhood SES						
1 (low)	ref			ref		
2	0.17 (-0.32, 0.67)	19	0.491	0.01 (-0.41, 0.43)	1	0.956
3	-0.02 (-0.50, 0.47)	-2	0.948	-0.03 (-0.44, 0.38)	-3	0.887
4	0.23 (-0.27, 0.73)	26	0.364	0.12 (-0.30,0.54)	13	0.589
5 (high)	0.18 (-0.33, 0.70)	20	0.49	0.09 (-0.35, 0.52)	9	0.697
Alcohol intake (gms/day)[c]						
None	ref					
<10	-0.35 (-0.59, -0.10)	-30	0.007	-0.24 (-0.45, -0.03)	-21	0.026
≥10	-0.16 (-0.69, 0.38)	-15	0.561	-0.15 (-0.60, 0.30)	-14	0.511
Foreign born						
Yes	-0.02 (-0.26, 0.22)	-2	0.899	-0.03 (-0.23, 0.17)	-3	0.779

CI, confidence interval

* Luciferase reporter assay results expressed in relative light units (RLUs). Percent change in RLUs (untransformed) per

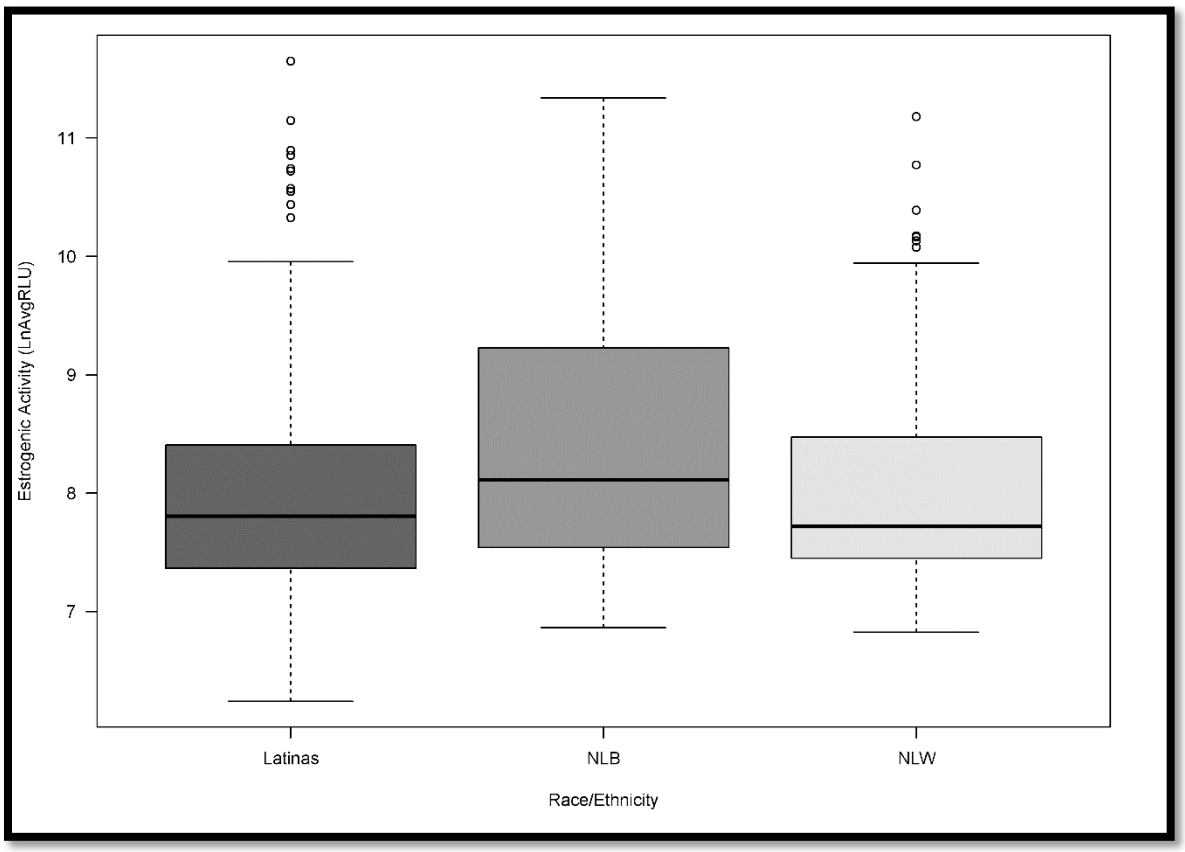
[a] The coefficients and 95% CI are based on the ln-transformed RLUs

[b] Unadjusted for endogenous estrogen levels

[c] Alcohol intake during calendar year before selection into parent study

Supplementary Material

Supplementary Figure 1 (S1 Figure) – Unadjusted estrogenic activity in all 503 women.



Chapter 4. Association of persistent organic pollutants and hormone receptor activity in South Indian Asians living in London

Abstract

Type 2 diabetes (T2D) is a metabolic disorder with a global impact affecting over 2.7 million individuals in the United Kingdom (U.K.) alone. Modifiable risk factors including unhealthy diet, being overweight or obese, physical inactivity, and smoking are unable to completely explain the rise in T2D incidence. Furthermore, Indian Asians living in the U.K. have a 2 to 3-fold higher risk of developing T2D than European Whites, indicating increased susceptibility to this disease. Endocrine disrupting chemicals (EDCs), such as persistent organic pollutants (POPs), may contribute to the onset of T2D and increased susceptibility among Indian Asians. Our group previously reported higher POP levels among South Indian Asians with T2D living in London. These same POPs have also been shown to bind cellular endocrine receptors that influence T2D risk. Here, we evaluated the association between POPs and hormone receptor activity. Cell-based reporter bioassays were used to measure plasma estrogenic (E), androgenic (A), and glucocorticogenic (G) activity levels in a sample set of men and women (n=375) from the London Life Sciences Population (LOLIPOP) Study. As expected, E activity differed by sex and menopausal status. No statistically significant associations were observed between POPs and hormone receptor activity. Our findings do not provide strong evidence that the higher body burden of POPs in South Indian Asian immigrants alter hormone receptor activity. Therefore, POPs may influence T2D risk through alternative mechanisms.

Introduction

Type 2 diabetes (T2D) is a chronic illness that affects nearly 3 million individuals in the United Kingdom (U.K.), with a disproportionate proportion of those affected being of Indian Asian descent. Compared to European Whites, Indian Asians have a 2 to 3-fold higher risk of developing T2D [1,2]. Both heritable [3,4] and inheritable traits[5–7] such as diet and lifestyle factors, adiposity, and lipid levels can explain some of the elevated risk in this population. However, these traditional genetic markers and non-heritable traits only explain a small portion of their increased risk, leaving data gaps in understanding T2D etiology and susceptibility in this population. The current hypothesis is that the metabolic phenotype of Indian Asians, which consists of higher levels of adiponectin, greater body fat, and differences in fat storage, contributes to increased susceptibility to T2D [8,9].

Multiple studies have shown that environmental pollutants are a possible risk factor for T2D[10–12]. It is not surprising that endocrine disrupting chemicals (EDCs) are found in higher concentrations in Indian Asians given their country's long history of chemical production and use of persistent organic pollutants (POPs), specifically the world's higher producer of dichlorodiphenyltrichloroethane (DDT)[13]. POPs, in parent form or metabolites, aggregate mainly in adipose tissue and are known to influence glucose regulation and lipid metabolism[14–17]. Moreover, there is substantial evidence in human and animal models that POPs alter several endocrine hormones such as estrogens, androgens and insulin[18–21].

One of the main targets of several EDCs is nuclear receptors[22–24]. Nuclear receptors, such as the estrogen, androgen and glucocorticoid receptors (ER, AR, and GR), are expressed on all T2D related organs including adipose tissue, pancreatic cells, blood cells and skeletal muscle. More importantly, these receptors are important for regulating energy expenditure (ER), systemic homeostasis (AR) and metabolic homeostasis (GR)[25–27]. *In vivo* and *in vitro* studies have

demonstrated that POPs and other environmental pollutants can bind these receptors[22] and disrupt hormone levels. Altered receptor activity and hormone levels have also been linked to the onset of various metabolic disorders associated with coronary heart disease, including obesity, T2D, and metabolic syndrome[1,12,28].

It is critical to identify a potential mechanistic connection between EDCs and T2D to better understand the causes of this disease. Luciferase assays have been used extensively as a measure of endocrine disruption in environmental and epidemiological studies to agnostically measure the total effect of all receptor ligands deriving from endogenous and exogenous sources[29–33]. Therefore, a similar approach can be applied to assess whether EDCs contribute to differences in T2D susceptibility among Indian Asians.

In a previous study[34], we reported that South Indian Asian immigrants, of Tamil and Sri Lankan Telegu descent, have a higher body burden of organochlorine (OC) pesticides, namely DDT and hexachlorocyclohexanes (HCHs), than European Whites. Furthermore, when compared to European Whites, the levels of OC pesticides were 3-9 fold higher and 9-30 fold higher in Tamils and Telegus, respectively. Two of the POPs measured, p,p'-DDE and β-HCH, were also strongly associated with diabetes case status in the South Indian Asians. Currently, no established mechanism exists to explain the association between OC pesticides and T2D, but as previously mentioned, POPs have been shown to perturb endocrine function by binding to hormone receptors. Therefore, we hypothesize that POPs present in the blood of South Indian Asians increase T2D risk by altering hormone receptor activity. To determine the relationship between POPs and hormone receptor activity in this population, we utilize cell-based reporter bioassays to measure plasma estrogenic (E), androgenic (A) and glucocorticogenic (G) activity in 375 individuals of South Indian Asian and European descent. This study will evaluate whether hormone receptor activity links OC pesticides to the elevated risk of T2D among South Indian Asians.

Materials and Methods

Study population

The London Life Sciences Population (LOLIPOP) Study was established to better understand the causes of heart disease and other major medical problems, primarily among participants of Indian Asian ancestry who have a higher risk of heart disease. Since its inception in 2002, this prospective cohort study has followed over 30,000 Indian Asians and European Whites living in West London[34,35]. Participants have been followed annually with a detailed health assessment (health physical, electrocardiography, blood pressure, lipid profiles and physical measurements) and collection of blood and urine samples. A comprehensive survey with questions pertaining to demographic information, drinking and exercise habits, occupation, family history and time of residence in London was also collected at time of visit.

Our cross sectional study makes use of 375 plasma samples collected in 2012 from both men and women of South Indian Asian and European descent. The Indian Asians represent two groups of native or foreign-born first and second-generation immigrants of Telegu or Sri Lankan Tamil descent who resided in London at the time of collection in 2012.

Measures of hormone receptor activity

A protocol for the estrogen bioassay is described elsewhere[36,37]. Briefly, the stably transfected T47D-kbluc breast cancer cell line, which naturally expresses both ERα and ERβ, was utilized to measure estrogenic activity in plasma samples. All sources of estrogen mimics were

removed from the media one week prior to plasma addition by placing the cells in modified Dulbecco's Modified Eagle Medium and 10% charcoal dextran serum. After one week, cells were seeded at 25,000 cells per well in 96-well plates and incubated for 24 hours. A small volume of plasma (8uL) for both men and women was diluted and then added in quadruplicates directly onto the cells and incubated for another 24-hour period. A luminometer is used to measure luciferase gene expression post incubation and cell lysis. Reporter activity is measured according to the light emitted from each well. Measurements are expressed in relative light units, RLUs, after the quadruplicate measurements are averaged and can be converted to picomolar (pM) equivalents using a 17β-estradiol concentration response standard curve run in duplicate on each plate. This assay has a limit of detection of 1.0pM.

Minor modifications were made to the androgen and glucocorticoid assay protocol described elsewhere[36,38]. Similar to the estrogen bioassay, a stably transfected triple negative breast cancer cell line, MDA-kb2, is used to measure androgenic (A) and glucocorticogenic (G) activity in the plasma samples. Cells were also maintained in Leibovitz's L-15 medium and 10% charcoal dextran serum for one week prior to plasma, then seeded at 25,000 cells per well in 96-well plates. In contract to the estrogen bioassay, the promoter in this cell line, the mouse mammary tumor virus promoter, can simultaneously measure total A and G activities of endogenous and exogenous ligands including testosterone, dihydrotestosterone, corticosterone and aldosterone. To distinguish between the two activities, cells were co-cultured with hydroxyflutamide (OHF), a potent AR antagonist. Unlike the estrogen bioassay, a larger volume of plasma was required to achieve readings above the limit of detection for females (165uL for females and 34uL for males). After plasma is added in quadruplicate to the cells, there is a second 24-hour incubation. Subsequently, cells are lysed and light measurements are obtained with a luminometer. Quadruplicate readings are averaged and reported as RLUs. Additionally, RLUs are converted to picomolar (pM) equivalents using a DHT concentration response standard curve on each plate. The limit of detection for this assay is 100fM.

Persistent Organic Pollutants (POPs) measurements

A subset of the 375 samples (N=198) were previously sent for POPs analysis as described in detail elsewhere[34]. To summarize, sixty six total chemicals, representing 6 chemical classes (15 polyaromatic hydrocarbons, 12 dioxin-like polychlorinated biphenyls, 11 polybrominated diphenylethers, 18 organochlorine pesticides, 5 dioxins and 5 furans), were analyzed using a highly sensitive and specific quadrupole gas chromatography tandem mass spectrometry (GC-MS/MS) method developed at Agilent Technologies. A small volume (<200uL) of plasma was extracted using chemical denaturation, liquid-liquid extraction, solid-phase cleanup and reconstituted with isooctane containing $^{13}C_{12}$-DDT, an internal standard used to account for signal attenuation. Two extraction replicates were included in this process. Additionally, to account for technical variability, a pooled reference samples was injected at several time points and the subject samples were injected in duplicate batches. The limits of detection are as follows: 0.005–0.02 ng/mL for Polychlorinated biphenyls (PCB) and 0.05–0.15 ng/mL for OC pesticides; Linearity Ranges: 0.01–3 ng/mL for PCB and 0.05–80 ng/mL for OC pesticides.

Statistical analysis

In our previous study, the peak signal for each POP was first converted to lipid-adjusted values using standard curves and clinical lipid profiles. POP results were analyzed both individually and as a cumulative sum of the most significant chemicals. Mixed effects regression

models were used to include terms that account for significant sources of technical variability before analyzing variables of interest. Logistic regression analyses were also previously used to assess the relationship between individual POP concentrations and disease status, while controlling for potential confounders including gender, age, smoking status, and ethnicity. Associations between residual endocrine receptor activity (a continuous dependent variable) and POPs (a continuous independent variable) were analyzed using multivariable linear regression in this study. Multivariable models included the following variables: age, being of South Indian Asian descent, smoking, and drinking. Potential confounders that may influence receptor activity were controlled for in the model including age, smoking status, ethnicity, lipid profiles, and fasting glucose concentrations. To increase statistical power, Tamils and Telegus were combined into one group. Results for both receptor activity and POP values were log-transformed to improve normality and homogeneity. Outliers with measures of over two standard deviations above the mean for E activity and non-detectable samples for POPs were removed from the dataset. Samples with missing information for one or more covariates included in the models were also excluded. When possible, analyses were stratified by sex and menopausal status in women. All analyses were performed using R software[39].

Results

Clinical characteristics for the participants in this study are included in Table 1. A total of 375 participants were included in this study. There were 179 European Whites and 196 South Indian Asians with males representing about 52% and 55% of each group, respectively. The average age for the European Whites was 55.6 (standard deviation of 9.6 years), while the South Indian Asians were slightly younger at a mean age of 50.2 (standard deviation of 10.28). There was a statistically significant difference in lifestyle factors between the two groups, specifically for smoking ($p<0.01$)) and drinking ($p<0.001$) prevalence. There was more than a 10% difference European White smokers compared to South Indian Asian smokers (16% vs. 6%), while the difference between the percentage of drinkers exceeded 40% (65% of the European Whites were drinkers vs. 23% of South Indian Asians). With regards to clinical manifestations, the percentage of participants with T2D, prediabetes, and metabolic syndrome was greater in the South Indian Asian group, though the difference was not statistically significant with respect to prediabetes ($p=0.36$). Measurements for body mass index (BMI) and waist circumference were not statistically significant between the two groups; however, waist hip ratio (WHR) was statistically different ($p<0.01$). Measures of cholesterol, triglycerides, high density lipoprotein (HDL) and fasting glucose were statistically different between the groups, while the measurement for low density lipoproteins was not ($p=0.507$).

Estrogenic activity

Table 2 describes the results of E activity, after adjusting for technical variability in our model and stratifying by race. We observed that estrogenic activity is strongly associated with age in both men and women ($p=0.04$ and <0.001, respectively). There were no other statistical findings related to E activity in women. However, raw data (Supplementary Figure 1) clearly depict a difference in women categorized by menopausal status. In men, aside from smoking, all other covariates in the model were statistically significant including BMI (estimate: 0.02, $p=0.04$), being of Indian Asian descent (estimate: 0.26, $p=0.03$), and an inverse relationship with glucose levels (estimate: -0.09, $p<0.001$).

Results assessing the linear association between E activity and POPs in 86 women are presented in Table 3. In the model that was adjusted for technical variability without other covariate data, all chemicals were strongly associated with E activity. However, after adjusting for other covariates that influence POPs and E activity (BMI and lipids), these associations were no longer statistically significant for any of the chemicals.

Data on POPs was only obtained for 86 of the women included in this study. In Table 4, correlations between E activity and POPs in women were examined after stratifying by ethnic group. None of the individual chemicals or the collective sum of all chemicals were statistically significant in either population.

Glucocorticogenic activity

Table 5 provides results for the association between G activity in men and women after adjusting for technical variability. In women, being of Indian descent was inversely associated with G activity ($p<0.001$). In men, an inverse relationship between G activity and being of Indian descent was observed ($p=0.002$). In addition, there was a statistically significant association between G activity and glucose levels (estimate: 0.01, $p=0.05$) and an inverse relationship with BMI (estimate: -0.006, $p=0.01$) in men.

In the 86 women for which POPs information was known, there was a significant relationship between some of the POPs (DDT, DDE and β-HCH) and G activity in the unadjusted model that accounts for technical variability (Table 6). However, in the adjusted model that factors in BMI and lipid levels, only the variable that accounts for the cumulative sum of these chemicals was significant ($p=0.03$).

Androgenic activity

Results for A activity are provided in Table 7. In men, there was a significant association between A activity and age ($p<0.001$), BMI ($p=0.002$), and being of Indian descent ($p=0.072$). In women, A activity was strongly related to age ($p=0.008$), BMI ($p=0.067$), as well as glucose levels ($p=0.045$).

There were no associations between A activity and POPs (data not shown).

Discussion

This study measured hormone receptor activity for the estrogen, androgen, and glucocorticoid receptors in South Indian Asians and European Whites living in West London. Associations between hormone receptor activity and POPs in both populations were also evaluated.

Hormone Receptor Activity

As expected, we found that E activity was positively associated with age in both men and women, despite race/ethnicity. Multiple studies have shown the sex differences in estrogen levels, with decreasing levels observed in women post-menopause[40,41] and an inverse association between estrogen levels and age in men[42–44]. The literature also supports that estrogens levels vary by menopausal status[45,46], which was evident when the women were stratified by menopausal status (Supplementary Figure 1). Our findings in men support an association that is well known (in both men and women) between increasing estrogen levels and increasing BMI[43,47–49], a measure of obesity. Lastly, we observed a negative association between E activity and glucose levels, which

is a controversial finding hypothesized to be related to body measures[50–52] that requires further exploration.

We observed lower levels of G activity in the South Indian Asian group, in both men and women, compared to European Whites. This finding is consistent with another study comparing South Indian Asians with Europeans living in the U.K.[53]. However, there is more compelling evidence in favor of higher cortisol levels in South Indian Asian men and women due to their metabolic phenotype[54]. Therefore, this finding is inconclusive and warrants further investigation.

Similar to previous research findings, we also observed a decrease in A activity with BMI[55], age[56,57], and Indian Asian descent[58,59], in men. This trend is expected as there is more estrogen than androgen production as men age. However, and more importantly, low levels of androgens are also reported to be associated with obesity[55] and diabetes in other male populations[60]. Though not significant, A activity increased with BMI in women. Several studies hypothesize this is due to body composition, which affects circulating androgen levels, and disease risk[61]. The association between A activity and age in women, though not significant in our study, has also been validated elsewhere.[57] Interestingly, our study reveals that A activity increases with glucose levels in women and decreases with glucose levels (not significant) in males. Despite the differences in environment, our results correlate with findings in the Metabolic Syndrome and Atherosclerosis in South-Asian Living in America (MASALA) Study, a cohort study that follows Indian Asian immigrants living in the San Francisco Bay Area[52]. These parallels suggest that genetics or similar lifestyle factors may influence susceptibility in this ethnic population.

Associations between hormone receptor activity and POPs

This is the first study to examine the relationship between environmental pollutants and hormone receptor activity in the LOLIPOP cohort. Prior to adjusting our models, there is an association between some of the POPs and estrogen and glucocorticoid receptor activity. However, as BMI and lipid levels are introduced into the model, these findings are no longer significant. This could be the result of these chemicals accumulating predominantly in adipose tissues and the validity of such adjustments is questionable as a result. Furthermore, of the 66 POPs that were analyzed, only a small group were detectable or significantly associated with T2D in Indian Asians, which reduced the number of POPs that were used in our analysis (only 4 POPs and their sum were included). Perhaps this explains why we did not observe significant associations between the most significant POPs and the receptors. A link between POPs and endocrine hormone levels has been indicated[19,41,42], but our null findings do not fully support this claim. This inconsistency may be due to the difference in chemical classes, the methods used to detect hormone levels, or the small sample size in our study.

Strengths and limitations

To date, few studies have offered a mechanistic connection between POPs, endocrine hormones and T2D, specifically in Indian Asian populations. Although these findings do not support our hypothesis, we offer evidence that persistent pollutants do not influence T2D in Indian Asians via endocrine hormones in the LOLIPOP cohort. Chronic diseases like cardiovascular disease and diabetes have been linked to an imbalance in the gut microbiome[16,62,63], which offers an alternative mechanism linking POPs to T2D that has not been investigated in this population. A recent area of interest, the interaction between gut microbiota and POPs may be a key mechanism that remains to be explored.

One of the strengths of this study is the unique use of multiple -omic technologies that provide both biological and chemical information of the blood exposome. Moreover, the volumes of sample used in these methods were minimal (between 8uL to 200uL) and required less invasive procedures that can enhance subject participation. Associations between hormone receptor activity and POPs were also evaluated and although not significant, these findings rule out one possible mechanism linking POPs, T2D and endocrine hormones.

One of the limitations of this study is that the POPs measurements may not be fully detectable as these chemicals are estimated to be 100 to 1000 times less potent than endogenous hormones in the blood[64]. However, several POPs and other environmental chemicals have been successfully measured in the blood and correlated with T2D in human studies[11,12,28]. Moreover, the concentrations of the 66 POPs analyzed in our study were obtained through a highly sensitive technique, strengthening the number of chemicals and levels detected in our samples. Since this was a cross-sectional study, our conclusions are also limited to a single time point, which may not give an accurate assessment of environmental exposures throughout a person's lifetime. Nonetheless, these findings are informative with regards to multiple hormone receptor activities and the levels of POPs present in the LOLIPOP cohort. Lastly, the sample sizes when stratified by sex and gender were generally small. Despite this, our study provides preliminary findings of possible environmental exposures for both men and women of Indian Asian descent residing in London, which to date, are limited or nonexistent.

Conclusion

We conclude that the group of POPs (DDT, DDE, β-HCH, PCB-118 and their sum) are not acting mainly on the endocrine system via hormone receptor signaling. Future studies should explore alternative mechanisms, such as the gut microbiota and epigenetics to explain the differences in T2D in Indian Asians and European Whites. Greater efforts to determine the untraditional underlying factors leading to T2D are needed, particularly in Indian Asian populations.

Glossary

A	androgenic
AR	androgen receptor
BMI	body mass index
DDT	dichlorodiphenyltrichloroethane
E	estrogenic
EDCs	endocrine disrupting chemicals
ER	estrogen receptor
G	glucocorticogenic
GR	glucocorticoid receptor
HCB	hexachlorocyclohexanes
LOLIPOP	London Life Sciences Population Study
OC	organochlorine
OHF	hydroxyflutamide
pM	picomolar
POPs	persistent organic pollutants
RLU	relative light unit
T2D	type 2 diabetes

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Table 1. Clinical characteristics of all participants (n=375).

	European Whites Mean (SD)	**Indian Asians** Mean (SD)	**p-value**
Sample size	179	196	
Age (years)	55.6 (9.60)	50.2 (10.28)	<0.001
Men (%)	52.51	54.59	0.760
Smoker (%)	16.20	6.12	0.010
Drinker (%)	64.80	22.96	<0.001
Type 2 diabetes (%)	3.35	12.76	0.010
Pre-diabetes (%)	10.61	14.29	0.360
Metabolic Syndrome (%)	17.88	30.61	0.010
BMI (kg/m^2)	27.12 (4.72)	26.86 (3.47)	0.540
Waist (cm)	94.12 (14.70)	94.18 (9.00)	0.954
WHR	0.91 (0.09)	0.93 (0.07)	0.010
Cholesterol (mg/dL)	203.62 (40.52)	192.84 (40.52)	0.010
Triglycerides (mg/dL)	109.13 (63.82)	129.68 (77.26)	0.010
HDL (mg/dL)	63.18 (18.19)	51.01 (13.29)	<0.001
LDL (mg/dL)	119.25 (35.80)	116.72 (37.22)	0.507
Fasting glucose (mg/dL)	93.65 (11.93)	100.93 (31.90)	0.010

Table 2. Estrogenic activity findings in men and women after adjusting for plate effects.

	Men (N=194)		Women (N=172)	
Covariate	**Estimate**	**p-value**	**Estimate**	**p-value**
Age	0.01	0.040	-0.09	<0.001
BMI	0.02	0.040	-0.01	0.620
Indian	0.26	0.030	-0.03	0.880
Glucose	-0.09	<0.001	-0.09	0.950
Smoker	-0.01	0.870	-0.02	0.930

Table 3. Linear association between estrogenic activity and persistent organic pollutants (POPs) in women (N=86).

Chemical	Unadjusted*	p-value	Adjusted**	p-value
DDT	-0.29	0.005	-0.07	0.420
DDE	-0.35	<0.001	-0.09	0.320
Beta-HCH	-0.23	0.030	-0.01	0.860
PCB118	-0.47	0.020	0.09	0.610
Sum of POPs#	-0.35	<0.001	-0.09	0.330

* Adjusted for technical variability only

** Adjusted for technical variability, BMI, and lipids

POPs#: persistent organic pollutants

Table 4. Correlations between estrogenic activity and POPs in women (stratified by ethnic group).

	Indian Asians (N=50)				European Whites (N=36)			
Chemical	**Unadjusted***	**p-value**	**Adjusted****	**p-value**	**Unadjusted***	**p-value**	**Adjusted****	**p-value**
DDT	-0.19	0.400	-0.02	0.870	0.08	0.830	0.32	0.370
DDE	-0.47	0.006	-0.13	0.410	0.08	0.770	0.21	0.450
Beta-HCH	-0.12	0.460	0.01	0.980	0.48	0.200	0.76	0.040
PCB118	-0.57	0.010	0.08	0.740	0.27	0.460	0.46	0.220
Sum of POPs[#]	-0.51	0.010	-0.16	0.390	0.16	0.630	0.34	0.310

* Adjusted for technical variability only

** Adjusted for technical variability, BMI, and lipids

POPs[#]: persistent organic pollutants

Table 5. Glucocorticogenic activity in men and women after adjusting for plate effects.

	Men (N= 194)		Women (N= 165)	
Covariate	**Estimate**	**p-value**	**Estimate**	**p-value**
Age	0.01	0.110	-0.00	0.410
BMI	-0.01	0.010	-0.01	0.100
Indian	-0.07	<0.001	-0.17	<0.001
Glucose	0.01	0.050	0.03	0.140
Smoker	-0.01	0.520	0.01	0.870

Table 6. Linear association between glucocorticogenic activity and POPs in women (N=86).

Chemical	Unadjusted*	p-value	Adjusted**	p-value
DDT	-0.04	<0.001	-0.02	0.400
DDE	-0.03	0.030	0.01	0.700
Beta-HCH	-0.03	0.010	-0.01	0.650
PCB118	0.00	0.860	-0.01	0.820
Sum of POPs[#]	-0.02	0.020	-0.03	0.030

* Adjusted for technical variability only

** Adjusted for technical variability, BMI, and lipids

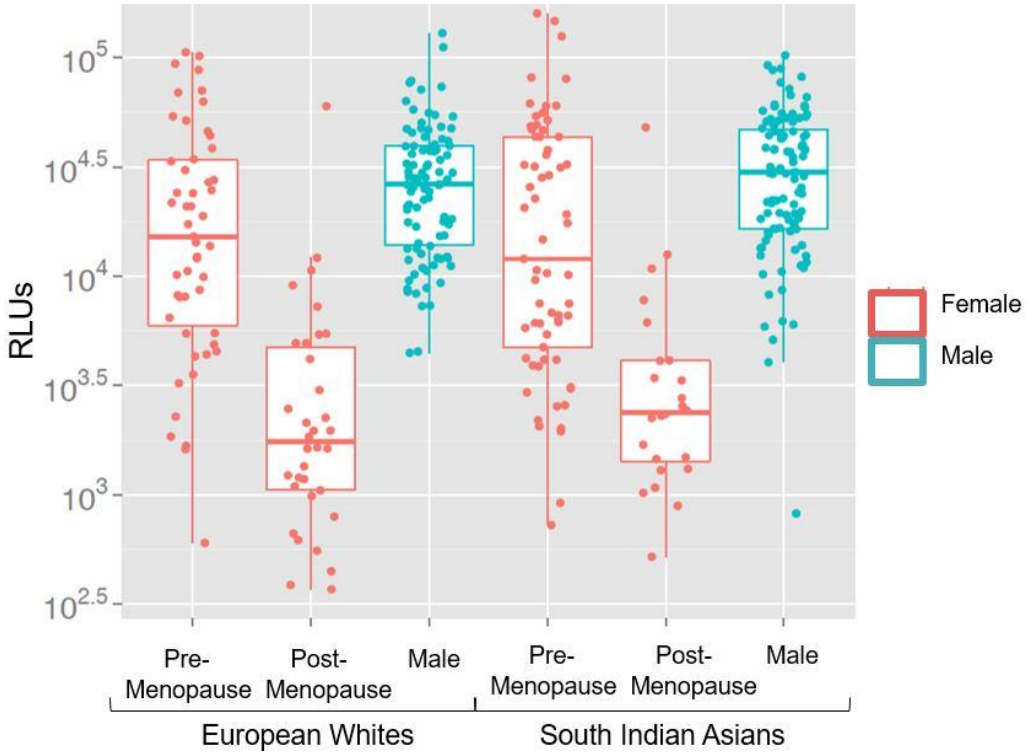
[#]POPs: persistent organic pollutants

Table 7. Androgenic activity in men and women after adjusting for plate effects.

	Men (N= 194)		Women (N= 165)	
Covariate	**Estimate**	**p-value**	**Estimate**	**p-value**
Age	-0.01	<0.001	-0.02	0.008
BMI	-0.02	<0.001	0.03	0.067
Indian	-0.11	0.070	0.04	0.818
Glucose	0.01	0.700	0.18	0.045
Smoker	-0.06	0.270	-0.08	0.680

Supplementary Material

Supplementary Figure 1- Boxplot displaying the greatest variation in estrogenic activity (as reported in relative light units, or RLUs) can be attributed to menopausal status and sex, rather than by ethnic groups.



Chapter 5. Summary and Future Directions

Summary

This dissertation uses an exposomic approach that relies on biological measurements from luciferase gene reporter bioassays to determine the association between hormone receptor activity and chronic disease risk in migrant populations. Overall, this work provides insights on measures of total hormone receptor activity, accounting for both exogenous and endogenous hormone-like compounds that have the potential to modulate chronic disease risk in multiethnic migrant populations living within and outside of the U.S.

In summary, Chapter 1 provides a brief overview and recent statistics of non-communicable diseases (NCDs), particularly breast cancer and type 2 diabetes (T2D), in the U.S. and worldwide. Chapter 1 also highlights the potential role of endocrine disrupting compounds (EDCs) as modifiers of three steroid hormones (estrogens, androgens, and glucocorticoids) and their receptor activities. Disruption to hormone receptor signaling can thereby influence chronic disease risk, especially in migrant populations who encounter a number of environmental exposures, perhaps even different exposures than their country of origin. Lastly, this chapter introduces the exposome approach as a means to investigate the myriad of exposures encountered throughout an individual's lifetime, including environmental relocations as a result of migration.

The pilot study in Chapter 2 investigated the levels of plasma estrogenic (E) and glucocorticogenic (G) activity in Mexican women born both within and outside the U.S., and tested the association with various lifestyle, demographic, and breast cancer risk factors. With minimal amount of sample required, the cell-based bioassay proved to be cost effective and efficient when measuring E and G activity in the plasma samples. In addition to the known associations that were observed between E and G activity with age at blood draw and menopausal status, we also revealed unknown associations with Indigenous American (IA) ancestry, years of U.S. residence, and alcohol intake. These preliminary findings required further replication, which was possible in Chapter 3.

In Chapter 3, a larger sample set of over 500 Hispanic/Latina women was used to validate the results in the previous chapter. The association between E activity and IA ancestry was examined using a luciferase gene reporter bioassay. Although years of U.S. residence was not associated with E activity as in the pilot study, IA ancestry remained significantly and inversely associated with E activity in Hispanic/Latina women. Altogether, these findings provide mechanistic support that both endogenous and exogenous estrogen-like compounds can alter E activity, which may explain the differences in breast cancer incidence rates in different racial/ethnic groups in the U.S.

Chapter 4 features a second migrant population of South Indian Asians living in London who are more susceptible to developing T2D compared to European whites. The association between E, G and androgenic (A) activities and several lifestyle factors and anthropometric measures was determined. Furthermore, a subset of the samples were used to measure the association between the activity of the three aforementioned hormone receptors and measures of persistent organic pollutants (POPs), namely DDT, DDE, and β-HCB, which were previously reported to be a higher burden in the Asian Indians than in the European whites. Although no association beyond the expected difference among women was observed (E activity decreases in

postmenopausal women), it is now evident that the endocrine system defined by our three steroid receptors may not be the causal link between T2D and the POPs burden in our South Indian Asian population.

Overall, the diverse makeup of the study populations throughout these chapters enriches our ability to capture different estrogenic, androgenic, and glucocorticogenic compounds between several different racial/ethnic groups. Additionally, the extensive information on lifestyle factors, ancestry information and other variables strengthen the associations between the variables and hormone receptor activity. Despite these strengths, there are several limitations throughout this dissertation that should be addressed in future studies utilizing the exposome approach. Ideally, researchers should obtain samples from multiple, longitudinal time points throughout the course of a person's life (in utero, early childhood, puberty, pre- and post-migration). This is a large feat and almost impractical in many settings, but it is fundamental to truly evaluate the cumulative effect of environmental exposures and to ascertain the relationship between these exposures and disease risk.

Future Directions

Currently, epidemiologic studies linking breast cancer and T2D to chemical exposures are inconsistent. Future studies could benefit from multiple -omic platforms as a means to address some of the current discrepancies in the field, providing information on both bioactivity as well as chemical features. Metabolomics, or the omics of small molecules, is a valuable tool that can be coupled to the receptorome to identify and quantify small metabolic products of various biological systems including cells, tissues, and human plasma[1]. This holistic, analytical approach gathers data from a specific time point to reveal altered biological and physiological conditions as a response to diet, disease status, genetics, and environmental exposures. Correlations between metabolites and numerous disease outcomes such as pancreatic cancer[2], Parkinson's disease[3], and celiac disease[4], have been established. To illustrate, novel biomarkers related to cardiovascular disease risk were identified using metabolic phenotyping of urine samples of 17 different populations in China, Japan, the United Kingdom and the U.S.[5]. The metabolic profiles displayed specific patterns for East Asian and western populations with contrasting diet and diet-related risk factors as well as different metabolic phenotypes for Chinese and Japanese subgroups whose vegetable/animal protein intake differed. Future studies should utilize this metabolomics platform to generate new hypotheses based on identified features. Moreover, the combination of both methods is most cost effective, less time consuming, and highly sensitive. This technique provides an accurate measurement and identification of potent endogenous and exogenous estrogens in human serum that are capable of activating hormone receptors. This approach is more feasible as compared to performing bioassays and untargeted analyses for the over 80,000 chemicals in commercial use today[6,7]. However, it should be noted that due to the emerging field of metabolomics, there are potential setbacks in the analytical chemistry aspect of the secondary approach. Issues that arise may be due to the inability to identify a matching metabolite feature in a database due to lacking chemical annotations, or features not being commercially available, and/or high limits of detection. In addition, to explore the binding interaction of identified features with key amino acids in the binding pockets of each receptor, future studies should employ molecular docking and molecular dynamic simulation approaches. With the use of molecular docking models, such as Induced fit with Glide[8], a thorough assessment of the binding modes and associated conformational changes between metabolomic features and the various receptor subtypes can be performed.

Conclusions

The concept of the exposome encompasses all changes in an individual's internal and external environments from conception onwards. This approach is essential in epidemiological studies conducting research on migrant population health as it captures a complete environmental exposure assessment. Since chronic diseases like breast cancer and T2D are affected by a combination of both genetic and more importantly, non-genetic risk factors some of which remain uncharacterized, -omic methodologies are crucial in linking environmental risk factors to NCD development. Coupling -omic methodologies that provide information regarding the biological activity and the chemical composition of environmental risk factors through the use of luciferase bioassays and metabolomics, respectively, can provide opportunities to develop novel therapeutic targets and predictive biomarkers related to chronic diseases.

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