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**Adrenal-permissive *HSD3BI* genetic inheritance and risk of estrogen-driven
postmenopausal breast cancer**

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Background Genetics of estrogen synthesis and breast cancer risk has been elusive. The 1245A→C missense-encoding polymorphism in *HSD3B1*, which is common in White populations, is functionally adrenal permissive and increases synthesis of the aromatase substrate, androstenedione. We hypothesized that homozygous inheritance of the adrenal-permissive *HSD3B1*(1245C) is associated with postmenopausal estrogen receptor (ER)-positive breast cancer.

Methods A prospective study of postmenopausal ER-driven breast cancer was done for determination of *HSD3B1* and circulating steroids. Validation was performed in 2 other cohorts. Adrenal-permissive genotype frequency was compared between postmenopausal ER-positive breast cancer, the general population, and postmenopausal ER-negative breast cancer.

Results Prospective and validation studies had 157 and 538 subjects, respectively, for the primary analysis of genotype frequency by estrogen receptor status in White female breast cancer patients postmenopausal at diagnosis. The adrenal-permissive genotype frequency in postmenopausal White women with estrogen-driven breast cancer in the prospective cohort was 17.5% (21/120) compared with 5.4% (2/37) for ER-negative breast cancer ($p = 0.108$) and 9.6% (429/4451) in the general population ($p = 0.0077$). Adrenal-permissive genotype frequency for estrogen-driven postmenopausal breast cancer was validated using Cambridge and TCGA datasets: 14.4% (56/389) compared with 6.0% (9/149) for ER-negative breast cancer ($p = 0.007$) and the general population ($p = 0.005$). Circulating androstenedione concentration was higher with the adrenal-permissive genotype ($p = 0.03$).

Conclusion Adrenal-permissive genotype is associated with estrogen-driven postmenopausal breast cancer. These findings link genetic inheritance of endogenous estrogen exposure to estrogen-driven breast cancer.

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Introduction

Estrogen exposure increases the risk of estrogen receptor (ER)-driven breast cancer. Data on the effects of exogenous estrogen exposure in postmenopausal women is highly dependent on the type of steroid hormone exposure, for example with hormone therapy in menopause (1, 2). Increased endogenous estrogen exposure may similarly stimulate the development of ER-driven breast cancer (3). However, unlike germline mutations in DNA repair pathway components and certain tumor suppressor genes, inherited genetics of estrogen synthesis has not been causally and reproducibly linked to endogenous estrogen exposure that in turn drives increased risk of breast cancer (4). This may be due to inadequate power for such studies that interrogate multiple loci, the need to account for genetic variability in geography and race, other variations in hormonal physiology that might dilute such an effect, a combination of these factors, or the absence of such a link.

In menopause, adrenal precursor steroids serve as the major source for peripheral metabolism and the generation of endogenous estrogens. The metabolic pathway components culminate with aromatase, which converts androstenedione to estrone and testosterone to estradiol (**Figure 1**) (5). The enzyme that immediately precedes aromatase is 3 β -hydroxysteroid dehydrogenase-1 (3 β HSD1), which is encoded by the *HSD3B1* gene (6). *HSD3B1* has a common functional germline-encoding missense variant (rs1047303) (7). The adrenal-restrictive *HSD3B1*(1245A) allele encodes for an enzyme that is more rapidly degraded, whereas the adrenal-permissive *HSD3B1*(1245C) allele generates an enzyme that is more stable and enables more rapid conversion from adrenal dehydroepiandrosterone (DHEA) to androstenedione and androstenediol to testosterone (8, 9). Previously, we originally identified the mechanistic basis for the two phenotypes in vitro in prostate cancer cells (7). Subsequently, at least 8 clinical studies have shown

that inheritance of the adrenal-permissive *HSD3B1* allele confers worse clinical outcomes in men with advanced prostate cancer treated with medical castration because it enables conversion from extragonadal (i.e., adrenal) precursor steroids to potent androgens, i.e., testosterone and/or dihydrotestosterone (10). Consistently, inheritance of 2 copies of the adrenal permissive allele is associated with the worst outcomes (11-14).

Given that 3β HSD1 is required for synthesis of androstenedione, the immediate precursor to estrogens and potent androgens, we wondered whether the adrenal-permissive *HSD3B1* allele was associated with endocrine-driven cancers in the female sex. Here, we tested the hypothesis that the more rapid metabolism associated with the adrenal-permissive *HSD3B1*(1245C) allele is associated with estrogen-driven breast cancer in postmenopausal women. Our hypothesis was specific to postmenopausal women; in premenopausal women, the relative contribution of estrogens produced peripherally from adrenal precursors and therefore regulated by *HSD3B1* is much smaller due to the large pool of gonadally produced estrogens prior to menopause. Given the worse outcomes in men with prostate cancer who inherit 2 copies of the adrenal-permissive allele and the accompanying cellular metabolic phenotype, our primary hypothesis is that 2 copies of the adrenal-permissive allele [hereafter, the adrenal-permissive genotype] and any associated increase in estrogen synthesis is associated with estrogen-driven postmenopausal breast cancer. Given that the frequency of the adrenal-permissive genotype varies widely by race (C allele frequency ~0.32 in Europeans and ~0.1 or less in Africans and East Asians (15), implying homozygous adrenal-permissive genotype frequencies of ~10%, ~1%, and ~1%, respectively), we tested this hypothesis in White populations because we had sufficient statistical power.

Results

One hundred and seventy-five women who were postmenopausal at diagnosis for stage I-III, HER2-negative, invasive breast cancer with *HSD3B1* genotype were ultimately included in analyses, including 129 with ER-positive and 46 with ER-negative tumors; a flow diagram is shown in **Figure 2**. A cohort of 4451 subjects local to the same institution was used as a general population control for the White population, of which 429 (9.6%) had the adrenal-permissive genotype. This was comparable to other studies in people of European descent and the 1000 Genomes Project (8, 10). Twenty-one of 120 (17.5%) White women with ER-positive breast cancer had the adrenal-permissive genotype, which was significantly higher than the general population ($p=0.0077$; **Table 1**). The adrenal-permissive genotype was found in 2 of 37 (5.4%) women with ER-negative breast cancer. None of the 9 ER-positive or 9 ER-negative Black women had the adrenal-permissive genotype (**Table S2**).

Validation was sought in 2 breast cancer genomic studies. The Cambridge study characterized the genomes of 560 breast cancers with menopausal status and ER status for each tumor recorded (16). Principal component analysis (PCA) was used to infer race. TCGA genetically profiled tumors from 1098 patients with breast cancer (17). The majority of tumors were from an era prior to ER testing standardization (18). Therefore, to exclude the more equivocal cases, only those cases with documentation of at least 50% ER staining were included. The breakdown of patients included and excluded for all cohorts is in **Table S1**. Altogether, the adrenal-permissive genotype was present in 28/199 (14.1%) and 28/190 (14.7%) of White postmenopausal women with ER-positive tumors in Cambridge and TCGA cohorts, respectively. In contrast, the adrenal-permissive genotype was present in 4/60 (6.7%) and 5/89 (5.6%) in ER-negative tumors in Cambridge and TCGA. In the combined validation groups, the adrenal-permissive genotype in ER-positive tumors was significantly more frequent compared with both

the general population ($p=0.0046$) and ER-negative tumors ($p=0.0074$) (**Table 1**). None of 37 Black patients with ER-positive breast cancer and 43 patients with ER-negative breast cancer had the adrenal-permissive genotype. The complete breakdown of genotypes by race, ER and progesterone receptor (PR) status is in **Table S2**.

In the prospective single institution study and the TCGA validation cohort, ancestry was self-described, whereas in the Cambridge validation cohort, ancestry was determined by genetic principal component analysis. To test whether using self-described ancestry might cause unreliable results, we reanalyzed the TCGA data using results from a study that determined genetic ancestry of all TCGA subjects (19). This only marginally changed the results (**Table S3**). Using genetically determined ancestry of European rather than self-described race White, postmenopausal ER-positive breast cancer patients had $26/185 = 14.1\%$ adrenal-permissive genotype and ER-negative had $5/94 = 5.3\%$ adrenal-permissive genotype ($p = 0.0574$ for ER-positive vs. control cohort and $p = 0.0278$ for ER-positive vs. ER-negative).

Because the hypothesis being tested was specific to postmenopausal breast cancer, enrollment in our prospective single institution study was limited to patients who were postmenopausal at diagnosis, but we examined the adrenal-permissive genotype frequencies of premenopausal breast cancer patients in the validation cohorts. In the TCGA cohort, White premenopausal women diagnosed with ER-positive breast cancers had a similar trend of elevated adrenal-permissive genotype frequency ($9/53 = 17.0\%$, $p = 0.0961$ vs. control White cohort), but this was not the case in the Cambridge cohort ($3/66 = 4.5\%$, $p = 0.2060$ vs. control White cohort). In the two validation cohorts together, the adrenal-permissive genotype frequencies in premenopausal White women with ER-positive ($12/119 = 10.1\%$) and ER-negative ($5/52 = 9.6\%$)

tumors were similar to each other and to the control White cohort, but with much smaller sample sizes than for postmenopausal breast cancer (**Table S4**).

The adrenal-permissive genotype increases the conversion from DHEA by 3 β HSD1 to androstenedione, with subsequent conversion to estrogens by aromatase (**Figure 1**). Circulating concentrations of DHEA, androstenedione, estradiol and estrone were determined by mass spectrometry in 13 women who did and 84 women who did not harbor the adrenal-permissive genotype and were not treated with hormonal therapies (**Figure 3**). Androstenedione was significantly higher in the adrenal-permissive group compared with women who were not in the adrenal-permissive group ($p = 0.03$). DHEA, estrone and estradiol did not differ significantly between the two groups.

We previously demonstrated in vitro that prostate cancer cells with the 367T form of 3 β HSD1 encoded by the adrenal-permissive 1245C allele more rapidly convert DHEA to androstenedione than cells with the 367N form encoded by the adrenal-restrictive 1245A allele (7). To confirm that the same would occur in breast cancer cells, we expressed constructs encoding for 3 β HSD1(367N) and 3 β HSD1(367T) in SKBR3 breast cancer cells, which we found were homozygous 1245A in our genotyping assay, and the cells were treated with DHEA. Cells expressing the adrenal-permissive 3 β HSD1(367T) more rapidly converted DHEA to androstenedione than cells expressing the adrenal-restrictive 3 β HSD1(367N) (**Figure 4**).

Discussion

These results demonstrate that the adrenal-permissive genotype is more frequently found in women with postmenopausal estrogen-driven breast cancer compared with the general

population and compared with women who have postmenopausal ER-negative breast cancer. Furthermore, the adrenal-permissive genotype is associated with elevated circulating androstenedione levels. Together, these data suggest that the adrenal-permissive genotype increases risk of estrogen-driven postmenopausal breast cancer.

Our hypothesis was specific to postmenopausal breast cancer, because in postmenopausal women, the adrenals are the sole source of sex steroids, whereas in premenopausal women, estrogen synthesis from adrenally derived precursors would merely supplement the larger estrogen pool produced in the ovaries. For this reason, our prospective study did not include premenopausal women; nonetheless, we analyzed data for premenopausal breast cancer in the validation cohorts and did not find evidence for a similar effect of adrenal-permissive genotype. However, our sample sizes for premenopausal breast cancer were small so these results should be considered preliminary.

Although our hypothesis pertained specifically to ER-positive postmenopausal breast cancer, we also included ER-negative tumors in the prospective study, because if similar trends were found in both ER-positive and ER-negative breast cancer it would contradict our hypothesis that adrenal-permissive genotype specifically increases the risk of ER-positive breast cancer. ER-negative breast cancer is considerably less common than ER-positive, so it was more difficult to obtain sufficient statistical power to draw meaningful conclusions about ER-negative breast cancer, but the observed trend was consistent with adrenal-permissive genotype being enriched in ER-positive but not ER-negative breast cancer. Intriguingly, in all three cohorts (prospective trial and two validation cohorts), there were similar trends of adrenal-permissive genotype frequencies in postmenopausal ER-negative breast cancer being lower than in the general population, the opposite result to postmenopausal ER-positive breast cancer. This suggests the possibility that

there is an interaction between the adrenal-permissive genotype ER-negative breast cancer development. Increased estrogen production in cells expressing *HSD3BI*(1245C) might drive selection for ER positivity, whereas the corresponding lack of estrogen production in cells expressing *HSD3BI*(1245A) might drive selection against ER positivity. Additional cohorts, including larger numbers of ER-negative tumors, should be sought out to validate this observed trend.

Although the adrenal-permissive genotype was associated with higher androstenedione in circulation, the effect size was not large, and circulating estrone and estradiol concentrations did not differ significantly by genotype. However, it is important to note that tissue steroids often differ profoundly compared with levels in circulation. A systematic review of studies on sex steroid hormone levels in circulation and in breast tissue concluded that estrogen levels are consistently found to be higher in tissue than in blood, and additionally that estradiol levels are further elevated in cancerous compared to benign breast tissue in postmenopausal women (20). Furthermore, aromatase expression is known to be specifically up-regulated in the breast in postmenopausal women, which may lead to changes in local estrogen levels without affecting circulating concentrations (21). Taken together, these findings suggest that enhanced androstenedione production with the adrenal-permissive genotype, in circulation and/or at the cellular level in cells expressing *HSD3BI*(1245C), could lead to local increases in breast tissue estrogen levels that may not be reflected in measurements of circulating estrogen concentrations. The effect of adrenal-permissive genotype on tissue steroid concentrations is a topic for further study.

We additionally demonstrated, *in vitro*, that breast cancer cells expressing the adrenal-permissive form of the enzyme more rapidly convert DHEA to androstenedione than cells expressing the adrenal-restrictive form, adding to our previous finding of the same result in

prostate cancer cells (7). The difference we observed in conversion rate between the two forms of the enzyme was fairly modest, which is likely due to overexpression narrowing the difference. In our previous experiments in prostate cancer cells, the difference in conversion rate between cells overexpressing adrenal-permissive and adrenal-restrictive forms was much smaller than the difference between two cell lines that endogenously expressed (at the mRNA level) similar levels of adrenal-permissive or adrenal-restrictive *HSD3B1* (7). However, the current lack of data showing increased DHEA metabolism in breast cancer cells naturally expressing the adrenal-permissive enzyme is a limitation of our study.

A strength of our study is that we tested a single hypothesis (adrenal-permissive genotype would be associated with higher rates of postmenopausal, ER-positive breast cancer) based on an already established mechanism (adrenal-permissive genotype leads to increased synthesis of the estrogen precursor androstenedione). Therefore, the study does not have the issues of multiple hypothesis testing that can make it difficult to separate real results from those occurring by chance in genome-wide association studies (GWAS). In all three cohorts we examined, results consistent with our hypothesis were obtained. A potential weakness is that not all Caucasian populations have identical *HSD3B1*(1245C) allele frequencies, although all fall in a similar range based on comparing different European populations in dbSNP (15) and the gnomAD database (22). Therefore, the control cohort may not be a perfect control for all of the study cohorts. On the other hand, in all three cohorts we observed consistent trends of both elevated adrenal-permissive genotype frequencies in patients with ER-positive tumors and lowered adrenal-permissive genotype frequencies in patients with ER-negative tumors. Any bias in the control population's genotype frequencies would not affect the ER-positive vs. ER-negative comparison, and

furthermore a bias that somewhat weakened the trend for one ER status vs. control would simultaneously somewhat strengthen the trend for the other ER status vs. control.

The *HSD3B1*(1245A/C) SNP (rs1047303) has not previously been identified in GWAS for breast cancer (23). To further explore this, we interrogated publicly available breast cancer GWAS results and found that in the Cancer Genetic Markers of Susceptibility (CGEMS) Breast Cancer GWAS of postmenopausal women of European ancestry (24), rs1047303 was not found in the results, but results for two proxy SNPs (rs6686779 and rs3765945) each correlated with rs1047303 with $R^2 > 0.85$ according to the National Cancer Institute's LDproxy tool (25) were found. Neither proxy SNP had an association with breast cancer risk in this GWAS, but because the GWAS did not break down tumors by ER status, this is unsurprising given our results. The lack of identification of rs1047303 in breast cancer GWAS despite our finding of an association in three independent cohorts may point to advantages of stratifying breast cancer cohorts by all three factors of ER status, menopausal status, and race when testing association with polymorphisms, as well as to advantages of testing a single hypothesis linked to an already identified mechanism. The same SNP had similarly not been identified in GWAS for prostate cancer when we originally identified it (7), but has now been validated in at least 8 different prostate cancer studies (10).

A potential application of these data is incorporation of *HSD3B1* genotype status into breast cancer risk models. This gene is not presently included in any genetic risk scoring systems; however, it may add to risk prediction of estrogen driven breast cancer. Clinical utilization of *HSD3B1* adrenal-permissive variant information could be considered at either a monogenic or polygenic level. At present, polygenic risk scores (PRS) are not yet routinely used in clinical practice for discussion of risk stratification with patients. While polygenic risk is likely a significant contributor to breast cancer risk, estimated to account for 18% of familial risk (26),

clinical application is limited by variability in commercially available panels, lack of consensus about variants of greatest importance, and documented evidence of clinical utility with respect to breast cancer detection/outcomes. Given that the incidence of breast cancer is approximately 5-8% higher in the ER+ breast cancer group compared to the general population in our discovery and validation cohorts, the risk associated with this particular gain of function variant appears higher than that associated with variants considered for inclusion in PRS models but lower than that associated with the moderate- and high-risk genes included in hereditary breast cancer panels. Assessment of this variant independently may be important for clinicians given the hypothesized impact of this variant specifically for postmenopausal breast cancer due to the role of *HSD3B1* in adrenally mediated estrogen biosynthesis. As such, the clinical guidance provided to a premenopausal woman with this variant would likely differ from that given to a postmenopausal woman, particularly if this information is used to inform chemoprevention or screening imaging strategies.

As well established methods of chemoprevention with tamoxifen (27, 28) and aromatase inhibitors (29, 30) are available, identifying a population of patients with disease that is potentially enriched for response to an endocrine-based prevention approach would be clinically valuable. Such information may also be useful for patients previously diagnosed with breast cancer and now considering risks and benefits of adjuvant endocrine therapy. It has been shown that adherence to endocrine therapy is suboptimal with up to 50% of patients stopping prescribed endocrine therapy course prior to planned completion (31). Such patients are frequently worried about the risk of developing a second breast cancer and knowing the *HSD3B1* genotype information may add to an individualized risk/benefit discussion, especially when concerns about endocrine therapy adherence arise.

When considering the factors involved in breast cancer risk, one must keep in mind not only non-modifiable risks such as age and family history but also modifiable factors that may increase exposure to endogenous estrogens such as obesity. Lifestyle modification measures such as weight loss are discussed with all breast cancer survivors and may have an even greater impact in patients with increased sensitivity to endogenous estrogens. Future studies of this genotype are required to determine the relative contributions of *HSD3B1* genotype versus BMI and other modifiable risk factors, particularly when investigating disease related outcomes.

Additional directions include assessing response to various endocrine-based therapies in breast cancer survivors and assessment of disease-related outcomes according to adrenal permissive genotype. Of particular interest would be differential efficacy of tamoxifen or aromatase inhibitors in the adjuvant treatment of breast cancer in women with the adrenal permissive genotype. This may also be relevant with respect to the endocrine therapy partner selected in combination with CDK 4/6 inhibition in the metastatic setting or in selecting which patients may be sensitive to endocrine monotherapy in the metastatic setting as there are currently no biomarkers that predict differential response between endocrine monotherapy and combination endocrine/CDK 4/6 inhibitor therapy (32).

These data also surface a specific mechanism by which race and genetic ancestry may influence the time and type of breast cancer. While the adrenal-permissive genotype is common in White women, it is rare in Black women, who also generally have a lower proportion of estrogen-driven and ER-positive breast cancer (33). On the other hand, the adrenal-permissive genotype is similarly rare in East Asian populations as in Black populations (15), but East Asian women have rates of ER-positive breast cancer more similar to those in White women (34). This is a limitation to the hypothesis that different rates of adrenal-permissive genotype contribute to different rates

of ER-positive vs. negative breast cancer in patients of different ancestries and suggests that other aspects of race and ancestry also influence rates of ER positivity. Furthermore, genetic ancestry can determine the evolutionary trajectory of tumors (35). For example, it is tempting to speculate that the presence or absence of the adrenal-permissive genotype, which regulates production of the stimulus for ER, may determine the likelihood of acquiring subsequent ER-ligand-binding domain mutations as a mechanism of resistance to anti-estrogen therapies (36).

Finally, these data have broad implications for genetic mechanisms that regulate general endocrine physiology. Although our prior work has established that *HSD3B1* genetic inheritance regulates peripheral synthesis of potent androgens and associated prostate cancer clinical outcomes in men absent gonadal sex steroids (8, 10), the current study suggests that *HSD3B1* is also a genetic endocrine regulator in the opposite sex and similarly regulates estrogen-driven clinical outcomes in postmenopausal women. Breast cancer is not the only female hormone-driven cancer (37), and future studies should also interrogate whether there are associations between adrenal-permissive vs. adrenal-restrictive genotype and cancers of the uterus and ovary, which is a limitation of the current study. Moreover, in support of this framework, other clinical outcomes are also associated with *HSD3B1* genetics that speak to a role for *HSD3B1* and sex steroids in inflammatory disease (38).

In conclusion, adrenal-permissive *HSD3B1* genetics is associated with ER-positive breast cancer in postmenopausal women. The results presented here should be validated in additional studies with larger sample sizes, which could also shed light on whether there are associations with more diverse breast cancer subtypes (e.g. luminal A, luminal B, Her2 over-expressing, basal) (39, 40) rather than simply classifying tumors as ER-positive vs. ER-negative. These findings identify

a mechanistic link from germline genetics of sex steroid metabolism to ER-positive breast cancer predisposition and have multiple implications for risk stratification and prevention.

Methods

Prospective Single Institution Study

Eligible patients were women with a history of Stage I-III, HER2-negative invasive breast cancer treated at a tertiary care institution. All patients were postmenopausal at cancer diagnosis and this was confirmed with documentation of last menses ≥ 1 year prior to cancer diagnosis. For ER-positive patients, enrollment was completed prior to start of adjuvant endocrine therapy for purposes of serum steroid hormone analysis. However, the protocol was later amended to allow patients on active adjuvant endocrine therapy (either aromatase inhibitor or tamoxifen). Medical oncology clinics were screened by study coordinators to identify eligible patients, who were then approached for the study by their treating medical oncologist. Consent was obtained per an IRB-approved protocol, and specimens (~15ml blood) were obtained by routine venipuncture for germline DNA extraction and circulating steroid analysis. Patient demographic information, cancer staging information and treatment details including type of endocrine therapy received were recorded and stored in a secure REDCap database. *HSD3B1* genotyping was performed as described previously (11). Once specimen analysis was complete, *HSD3B1*(1245C) genotype information was entered into the REDCap database. A total of 199 subjects were initially entered in the study, 187 of whom were successfully genotyped for *HSD3B1* (**Table S1**). Among postmenopausal subjects, this included 157 White women and 18 Black women; other races were excluded due to small sample sizes.

The population control for the same institution was subjects recruited for elective diagnostic coronary angiography as part of the Cleveland Clinic GeneBank study (41). Peripheral

blood samples were obtained and DNA was genotyped and resulting genotype data underwent quality control and imputation as described (41). Race was self-described and 4451 White subjects were identified and included, with 429 (9.6%) having homozygous adrenal-permissive (CC) genotype, 1993 (44.8%) having heterozygous (AC) genotype, and 2029 (45.6%) having homozygous adrenal-restrictive (AA) genotype. In Hardy-Weinberg equilibrium testing, the control cohort was not found to be out of equilibrium ($p = 0.058$, Chi square test). A comparison of demographic information for White subjects in the breast cancer and control cohorts is shown in **Table S5**. Although the composition of the control cohort differed from the breast cancer cohort by including male subjects as well as marginally in average age, the control cohort was used strictly as a control for adrenal-permissive genotype frequencies in a White population local to the same institution. One would not expect *a priori* that adrenal-permissive genotype frequency would be affected by sex or age, nor did we find evidence for such differences (homozygous adrenal-permissive genotype frequencies in control cohort: male $299/3007 = 9.9\%$, female $130/1444 = 9.0\%$, $p = 0.329$ by Fisher's exact test; average age \pm SD for homozygous adrenal-permissive genotype 64.5 ± 11.5 , for other genotypes 64.2 ± 11.1 , $p = 0.611$ by t-test).

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis of adrenal androgens and estrogens in human serum

Steroid extraction protocol. Serum aliquots stored at -80°C were thawed and briefly inverted multiple times. An equal volume (250 μl) of serum from each aliquot was then transferred to a borosilicate glass tube. Samples were spiked with 10 μl internal standard mix [5ng/ml of $\text{E}_2\text{-}^{13}\text{C}_3$, 25ng/ml, androstene-3, 17-dione-2,3,4- $^{13}\text{C}_3$ and 5α -dihydrotestosterone-d3 (16,17,17-d3)] and briefly vortexed. Methyl-tert-butyl ether, 2 mL (MTBE, Acros) was added to each tube, which was then capped and vortexed for 5 minutes using a multi-tube vortexer (Fisher) and then centrifuged

for 5 minutes at 3000 RPM at 4°C. After centrifugation, the samples were placed on dry ice for 15 minutes, and the extracted steroids in the MTBE fraction were collected in a new glass tube. The liquid-liquid extraction is repeated, and the combined MTBE layers were dried under nitrogen gas and then reconstituted in 150 µl 50% methanol/water (v/v). Reconstituted samples were vortexed 5 minutes on a multi-tube vortexer and centrifuged 1 minute at 3000 RPM at 4°C. Samples were transferred to 1.5ml microcentrifuge tubes and centrifuged at 13,000 RPM for 10 mins at 4°C. Each supernatant was divided in half and collected in separate HPLC vials, one for analysis by mass spectrometry of estrogens and one for analysis of androgens.

Instrumentation and Data Analysis. Extracted steroids are quantified using stable isotope dilution LC/MS/MS. The LC/MS/MS system consists of an ultra-pressure liquid chromatography system (UPLC; Shimadzu Corporation, Japan), composed of two LC-30AD pumps, a DGU-20A5R vacuum degasser, a CTO-30A column oven, SIL-30AC autosampler, and a CBM-20A system controller coupled with a Qtrap 5500 mass spectrometer (AB Sciex, Redwood City, CA).

Estrogen analysis. Briefly, extracted serum samples were injected into a Shimadzu UPLC system and separated through a C18 column (InfinityLab Poroshell 120 EC-C18 column, 4.6 X 75mm, 2.7 µm, Agilent, Santa Clara, CA) using a gradient starting at 20% solvent B (methanol/acetonitrile [90/10, v/v] containing 0.25mM ammonium fluoride) increasing over 3.50 min to 75% solvent B; from 3.51 min to 11 min, solvent B was gradually increased to 97%, and at 11.01 min 100% B was run for 5 min (end time 16 min). The overall total flow rate was 0.3 ml/min except for the period of 7.00 to 12.00 min, when it was adjusted to 0.15 ml/min.

Solvent A was HPLC-grade water containing 0.25 mM ammonium fluoride. The estrogens were detected on a Qtrap 5500 mass spectrometer using electrospray ionization (ESI) in negative ion mode and multiple reaction monitoring (MRM) using characteristic parent → daughter ion

transitions for the specific molecular species monitored. 17β -Estradiol-2, 3, 4- $^{13}\text{C}_3$ (Sigma-Aldrich) was used as an internal standard for calibration of estrogens in human serum.

Androgen analysis. Extracted steroids were injected onto a Shimadzu UPLC system (Shimadzu Corporation, Japan), and the androgens were separated on a C18 column (Zorbax Eclipse Plus C₁₈ column, 150 mm x 2.1 mm, 3.5 μm , Agilent, Santa Clara, CA) using a gradient starting from 20% solvent B [acetonitrile/methanol (90/10, v/v) containing 0.2% formic acid] that was increased over 4 min to 75% solvent B run for 10 min, followed by 95% solvent B for 3 min. Solvent A was HPLC-grade water with 0.2% formic acid. The androgens are quantified on a Qtrap 5500 mass Spectrometer (AB Sciex, Redwood City, CA) using ESI in positive ion mode and MRM. Androstene-3,17-dione-2,3,4- $^{13}\text{C}_3$ and 5α -dihydrotestosterone-d₃ (16,17,17-d₃) (Cerilliant, Round Rock, Texas) are used as internal standards for calibration of androgens in human serum.

Data acquisition and processing for estrogens and androgens were performed using MultiQuant (version 3.0.1) from AB Sciex. Peak area ratio of the analyte over the internal standard was used for quantification. Each sample run included calibration curves with standards for data quantification using the analyte/internal standard peak area ratio.

Cambridge and TCGA Validation

Cambridge Validation Five-hundred-and-sixty patients were recruited from Australia, Belgium, France, Iceland, Italy, the Netherlands, Norway, the UK, the USA, Singapore, South Korea, and Sweden, with breast cancers of differing molecular subtype and stage (16). DNA was extracted from normal somatic tissue (peripheral blood lymphocytes, adjacent normal breast, or skin) for

whole-genome sequencing (WGS) of the germline. Clinical data were recorded according to the International Cancer Genome Consortium (ICGC) guidelines.

Germline WGS was run using 108 base/100 base genomic paired-end sequencing on Illumina GAIIx, HiSeq 2000, or HiSeq 2500 genome analyzers, in accordance with the Illumina Genome Analyzer operating manual. Short insert paired-end reads were aligned to the reference human genome (GRCH37) using Burrows-Wheeler Aligner, BWA (v0.5.9)(42) Cancer Variants Through Expectation Maximization (CaVEMan) was utilised for germline SNP calling (<http://cancerit.github.io/CaVEMan/>). SNP array hybridization was performed using Affymetrix SNP6.0 and Affymetrix protocols. SNP copy number analysis was conducted with ASCAT (v2.1.1), generating tumor profiles of allele-specific copy numbers (43). ASCAT was also applied to WGS data, producing similar results.

Intercontinental ancestry analysis was conducted with principal components analysis (PCA) using 2318 ancestry-associated SNPs utilized by Amos et al (44). Each patient was then plotted using their score on the first two PCs and the ancestry of the resultant clusters were identified by matching the position of the four ancestries previously estimated by Campbell et al (45). The 446 patients with $PC1 < 0$ and $PC2 < 5$ were identified as possessing European ancestry and the remainder were excluded. 259 of these patients were postmenopausal (using the definition of menopausal status or age > 55 years).

TCGA Validation Germline genotype data for breast cancer patients were available from NCI's The Cancer Genome Atlas (TCGA) (17), specifically project "TCGA-BRCA," which contains a mixture of solid tissue breast cancers for 1098 patients. Data were obtained from the NCI Genomic

Data Commons Legacy Archive²¹. Genotype array data (Affymetrix 6.0) for 757 documented Caucasian patients were downloaded and harmonized with the TOPMed Freeze 5 panel using a modified version of the HRC-1000G-check-bim tool (<https://www.well.ox.ac.uk/~wrayner/tools/#Checking>). Chromosome 1 genotypes were imputed with the University of Michigan Imputation Server (phasing with Eagle version 2.4, imputation with Minimac4 version 1.2.4, using the TOPMed Freeze 5 panel) to generate germline genotype calls for rs1047303. The majority of TCGA tumors (diagnosis made from 1988 to 2011)(17) were from pathologic analysis done prior to the 2010 American Society of Clinical Oncology / College of American Pathologists guideline recommendations for ER staining (18). ER staining now requires reporting percent positivity and this was not reported for many of the TCGA tumors. Therefore, for ER-positive tumors, we only included tumors reported as staining at least 50% ER positive to be sure we captured estrogen-driven tumors from a time preceding current pathologic standards. After excluding patients not meeting these criteria, there were 279 White postmenopausal female subjects and 80 Black postmenopausal female subjects (using the definition of menopausal status or age > 55 years). Subjects of other or unknown races were excluded due to small sample sizes.

Steroid Metabolism

SKBR3 breast cancer cells were provided by Dr. Ruth Keri and maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum. For steroid metabolism experiments, ~100,000 cells/well were seeded in a 12-well plate with biological triplicates. 12 hours later, plasmid DNA (20 ng) encoding 3 β HSD1(367N) and 3 β HSD1(367T), as described previously (7), was transfected into the cells using FuGENE HD Transfection Reagent (Promega, Madison, WI). 48 hours later, [³H]-DHEA (~1x10⁶ cpm) was added, and after incubation at 37° C, media samples

were collected at specified time points. Steroids were extracted using 1:1 ethyl acetate:isooctane and dried under nitrogen gas, then dissolved in 50% methanol and injected on a Waters 1525 HPLC system (Waters Corp., Milford, MA). Steroids were separated on a Luna 150 x 4.6 mm, 3.0 μ m particle size C₁₈ reverse-phase column (Phenomenex, Torrance, CA) with a methanol/water gradient at 50°C. Column effluent was mixed with Liquescent scintillation cocktail and analyzed using a β -RAM model 4 in-line radioactivity detector (LabLogic, Brandon, FL).

Statistical Methods

Adrenal-permissive genotype was summarized by gender, menopausal status, and biomarker status using frequencies and percentages. Steroid measurements were summarized using median and range by patient group. Fisher's exact test was used to compare genotype percentages between patient groups. Wilcoxon rank sum test was used to compare steroids between adrenal-permissive and adrenal-restrictive genotypes. All tests were two-sided and p-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS Studio 3.7 (SAS Institute, Cary, NC) and R version 4.0 (R Foundation, Vienna, Austria).

Study Approval

The prospective study was approved by the Cleveland Clinic institutional review board and written informed consent was obtained from participants.

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Declaration of Interests:

N.S. is a co-inventor on patents or patents filed for 3β -hydroxysteroid dehydrogenase in steroid-dependent disease by Cleveland Clinic and may be eligible to receive royalty payments, and has received consulting honoraria from Pfizer and Celgene. S.L.H. reports being named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland HeartLab, a fully owned subsidiary of Quest Diagnostics, and Procter & Gamble. S.L.H. also reports being a paid consultant for Procter & Gamble, and having received research funds from Procter & Gamble and Roche Diagnostics. SC has received research support from Daiichi Sankyo and [Paige.ai](#), clinical trial support from Novartis, Lilly, and Sanofi, and consulting honoraria from Lilly and Novartis. W.H.W.T. is a consultant for Sequana Medical A.G., Owkin Inc, and Relypsa Inc, and has received honorarium from Springer Nature for authorship/editorship and American Board of Internal Medicine for exam writing committee participation, all unrelated to the contents of this paper. The other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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Figures and Figure Legends

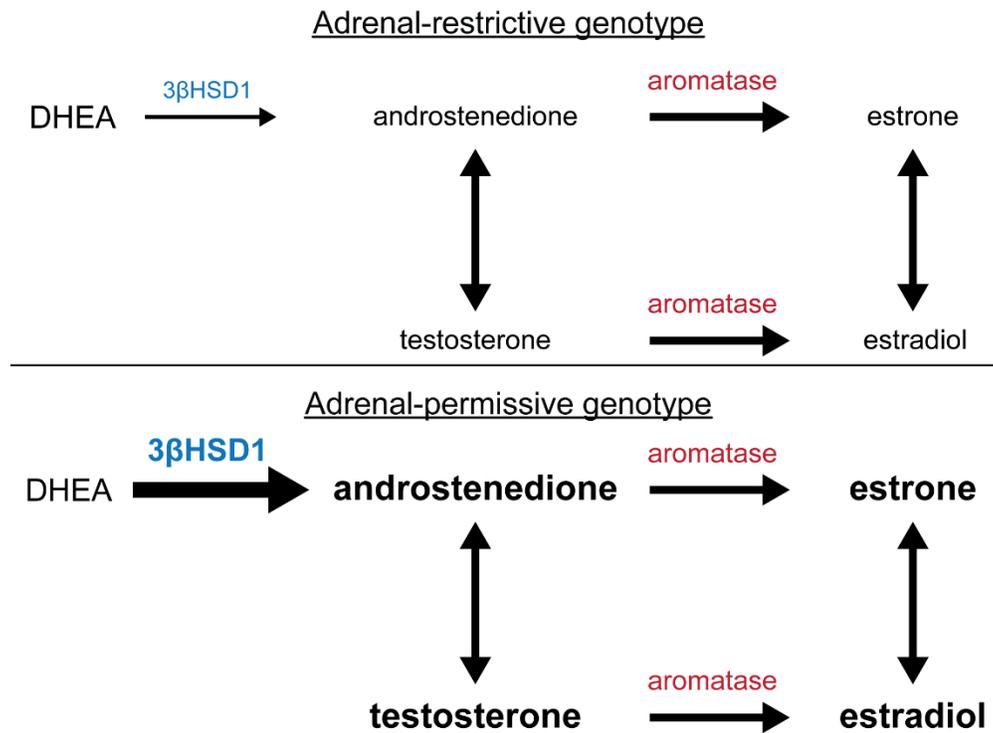


Figure 1. Model and mechanism for the association between adrenal-permissive *HSD3B1* genotype and ER-positive breast cancer. Compared with the adrenal-restrictive genotype (top), with the adrenal-permissive genotype (bottom), increased protein stability of *HSD3B1*-encoded 3βHSD1 in peripheral tissues leads to increased conversion from circulating DHEA to androstenedione, which is subsequently converted by aromatase to estrogens. 3βHSD1 is also necessary for conversion of the adrenal steroid androstenediol (not shown) to testosterone.

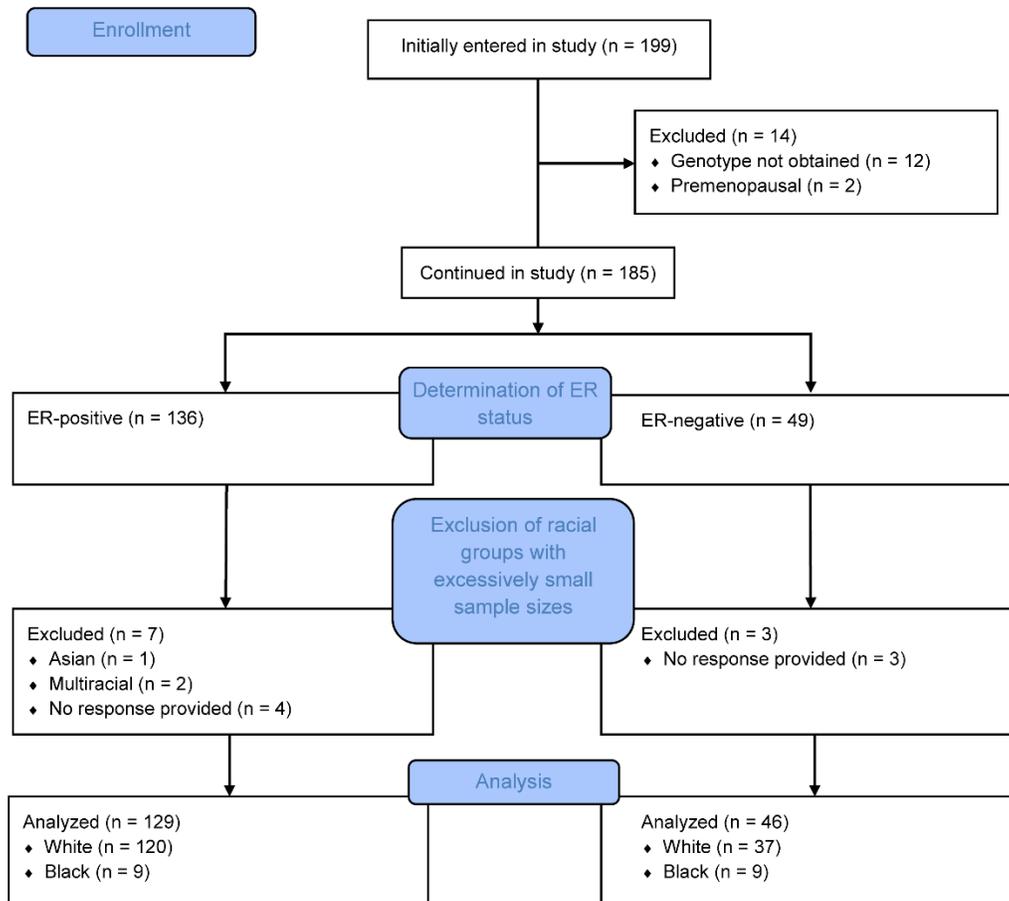


Figure 2. Flow diagram of study participants. From an initial pool of 199 recruited subjects, 175 were ultimately included in analysis, including 157 White women (120 with ER-positive tumors and 37 with ER-negative) and 18 Black women (9 with ER-positive tumors and 9 with ER-negative).

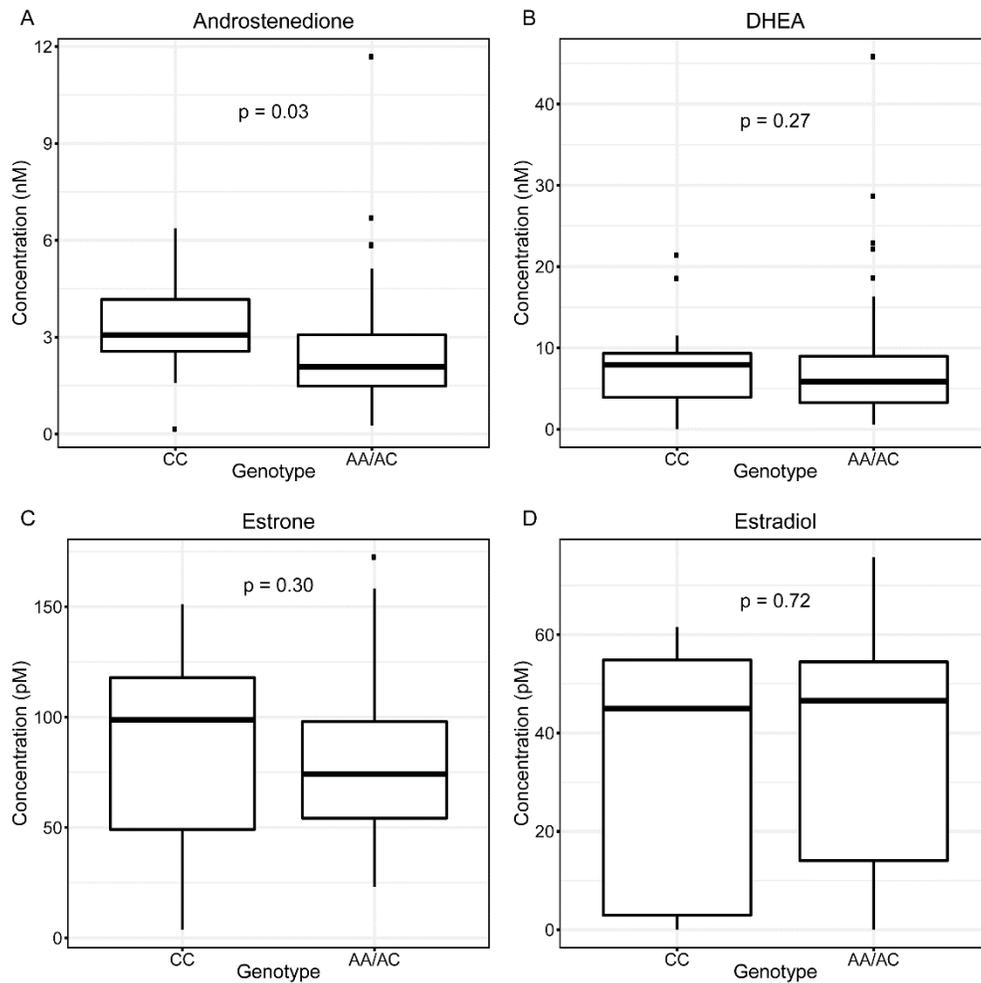


Figure 3. Adrenal-permissive *HSD3B1* genotype is associated with higher circulating levels of androstenedione but not of DHEA, estrone, or estradiol. Box-and-whisker plots showing circulating steroid concentrations assayed by liquid chromatography-tandem mass spectrometry from 13 women of CC (adrenal-permissive) genotype and 84 women not of CC genotype, none of whom was treated with hormonal therapies. Center lines indicate median values, boxes indicate first quartile to third quartile range, whiskers indicate values up to 1.5 times interquartile range, and points indicate outlying values. **(A)** Androstenedione concentrations. **(B)** DHEA concentrations. **(C)** Estrone concentrations. **(D)** Estradiol concentrations. Wilcoxon rank sum test p-values are shown.

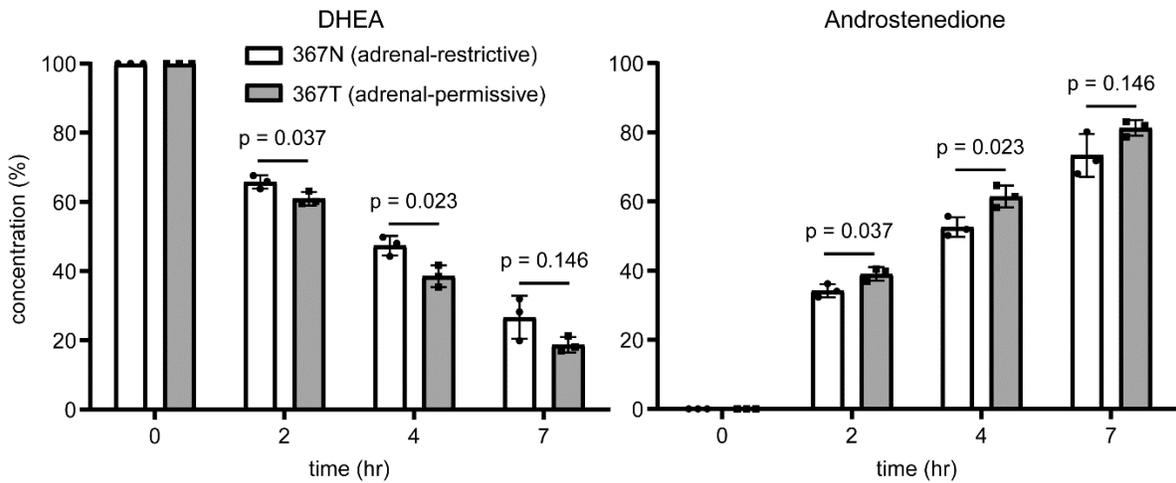


Figure 4. Expression of adrenal-permissive 3 β HSD1(367T) in breast cancer cells results in faster conversion of DHEA to androstenedione than expression of adrenal-restrictive 3 β HSD1(367N). Concentrations (% of total steroid) of DHEA (left) and androstenedione (right) from time zero to 7 hours after SKBR3 breast cancer cells transfected with constructs for 3 β HSD1(367N) or 3 β HSD1(367T) were treated with [3 H]-DHEA. Graphs show individual values and means \pm SD from one experiment with biological triplicates and p-values from t-tests. Similar results were obtained in a second experiment.