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Androgen Receptor htSNPs in Relation to Androgen Levels and OC Use in Young Women from High-Risk Breast Cancer Families

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Abstract

High testosterone levels have been associated with breast cancer. BRCA1 may function as an androgen receptor (AR) co-regulator. We aimed to examine AR haplotype-tagging single-nucleotide polymorphisms (AR htSNPs) and diplotypes in relation to in vivo androgen levels, combined OC use, CAG and GGC genotypes, and BRCA1/2/X family status in 269 young healthy women from breast cancer high-risk families and 56 additional BRCA1/2 mutation carriers. Testosterone, androstenedione, dehydroepiandrosterone sulfate and body constitution were measured on cycle days 18-23. Six AR htSNPs, and CAG and GGC repeat lengths were genotyped. Most OC users had lower androgen levels than non-users (all Ps<0.0001). Rare variant diplotypes were associated with higher testosterone levels in OC users than in non-users (Pinteraction=0.011). The interaction remained after adjustment for family clustering. Neither individual AR htSNPs nor other diplotypes were significantly associated with androgen levels and did not tag for CAG or GGC genotypes. In the first included woman from each family, the odds of having the most common diplotype was lower in BRCA1 families compared to other families OR 0.41 (95%CI 0.22-0.78). In conclusion we found few associations between AR htSNPs or diplotypes and androgen levels in women. Diplotypes cannot replace genotyping of microsatellites CAG or GGC. Since testosterone levels are not affected the same way by combined OC use among all women, young women who have higher testosterone levels during combined OC use may belong to the subgroup of women who will not be helped by combined OCs for treatment of androgen dependent conditions and may be at higher risk for early-onset breast cancer. Whether these women can be identified with AR genotyping needs to be confirmed in an independent cohort.

Key words: androgen receptor polymorphisms / androgens / oral contraceptives / BRCA / premenopausal women
Introduction
Androgens play an important role in several conditions affecting women, such as acne, hirsutism, alopecia, oligo-amenorrhea [1, 2] and breast cancer [3]. Approximately 25% of testosterone in women is produced in the ovaries, 25% in the adrenal glands and 50% via conversion of the proandrogens dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and androstenedione in the peripheral tissues [4, 5]. Circulating testosterone can be converted to dihydrotestosterone (DHT) and estradiol. High testosterone, androstenedione and DHEAS levels have been linked to higher breast cancer risk in premenopausal [3, 6, 7] as well as postmenopausal women [3, 8-10]. However, others have not found an increased breast cancer risk with high androgen levels [11]. Both growth inhibitory and stimulatory effects of androgens have been described in human breast cancer cell lines [4, 12]. Testosterone and DHT exert their effect in the mammary tissue via the androgen receptor (AR) and the effects depend on the type and dose of the androgen hormone, the structure of the AR, and the expression of co-regulators [4], as well as possible interactions between testosterone and estrogen, progesterone and insulin-like growth factor (IGF-I) [12, 13].

Breast cancer is diagnosed in approximately 7000 women in Sweden every year [14]. Hereditary factors are believed to play a role in 5-10% of breast cancers [15-17]. Mutations in the dominant, high-penetrance genes \textit{BRCA1} and \textit{BRCA2} confer a substantially increased risk of developing breast and ovarian cancer [15, 16]. In non-\textit{BRCA1/2} families, referred to as \textit{BRCAX} families, the pathogenesis is believed to depend on polymorphisms in several low-penetrance and high-prevalence genes, modified by environmental factors [15, 17, 18]. The \textit{BRCA1} may function as an AR co-regulator and enhance the AR transactivation in breast cancer cells [19].

One risk modifying, low-penetrance, high-prevalence susceptibility gene might be the \textit{AR} [16, 20], located on the X-chromosome. The AR protein is located in steroid hormone-sensitive cells, such as the normal breast epithelium, and in the majority of breast cancer cells [11, 12]. It functions as a transcription regulator [11]. Two highly polymorphic microsatellites located on the N-terminal of the AR, a polyglutamin tract encoded by (CAG) repeats and a polyglycine tract encoded by (GGT)$_3$GGG(GGT)$_2$(GGC)$_n$ repeats have been studied in relation to testosterone levels in women [21-24]. The CAG and GGC repeat lengths have also been studied in relation to breast cancer, both in the general population [20, 25-27] as well as among women from high-risk families [28]. However, the results have been inconsistent. The risk associated with different CAG and GGC repeat lengths might be modified by OC use [20, 24]. OC use lowers testosterone levels in most, but not all women [24, 29]. Moreover, OC use is associated with higher breast cancer risk in the general population [30], as well as in women from high-risk families [31, 32]. It is possible that young women who have higher testosterone levels during OC use belong to the subgroup of women who are at higher risk for early-onset breast cancer if they use OCs. Genetic polymorphisms in the \textit{AR} other than the CAG and GGC microsatellites may also modify androgen levels and breast cancer risk. Cox \textit{et al.} recently studied \textit{AR} haplotype-tagging single-nucleotide polymorphisms (htSNPs), haplotypes and CAG repeat in a large case control study, but none of the \textit{AR} haplotypes, individual htSNPs or the CAG repeat lengths were associated with breast cancer risk [33]. To our knowledge, \textit{AR} htSNP diplotype data have never been examined in relation to \textit{in vivo} androgen levels, combined OC use, CAG and GGC genotype or \textit{BRCA1/2} and \textit{BRCAX} family status among young women with a family history of breast cancer.
We aimed to examine AR htSNP diplotype data in relation to in vivo androgen levels, combined OC use, CAG and GGC genotypes and BRCA1/2 and BRCAX family status. The first aim was to analyze the frequency of the AR htSNPs, which have been identified to capture 95% of the haplotypes found in Swedish men in the Cancer Prostate in Sweden (CAPS) study [34]. The second aim was to determine whether any specific AR htSNP diplotypes were related to testosterone, androstenedione and DHEAS levels among all women as well as stratified according to current use of combined OCs. The third aim was to determine whether AR htSNP diplotypes were associated with AR microsatellite CAG and GGC repeat length genotypes. The fourth aim was to determine whether any specific diplotypes co-segregated within BRCA1/2 and BRCAX families.
Materials and Methods

Study population
Eligible participants had to belong to high-risk breast cancer families and be either known BRCA1 or BRCA2 mutation carriers or first or second-degree relatives of a breast cancer case or a known male or female BRCA1 or BRCA2 mutation carrier. We considered a family to be at high-risk for breast cancer if at least three first-degree relatives were diagnosed with breast cancer and one diagnosed before age 50; two first-degree relatives with breast cancer and one diagnosed before age 40; or one first-degree relative with breast cancer diagnosed before age 30. Participants had to be 40 years of age or younger and be menstruating. Exclusion criteria were a previous prophylactic mastectomy, a bilateral oophorectomy or any type of cancer diagnosis at the time of enrollment between 1996 and 2006. Two hundred and sixty nine young women from 161 breast cancer high-risk families volunteered to participate in this study, and 267 of them consented to genetic testing of single nucleotide polymorphisms (SNPs). Potential participants were identified from charts and pedigrees from the Lund Oncogenetic Clinic. Individuals who themselves had been to the Clinic were contacted, first by a letter providing brief information on the study, then by phone. In cases where the index individuals were not eligible, they were asked whether they would be willing to inform relatives of the study and then inform us as to whether we might contact their relatives directly. The Lund University Ethics Committee approved the study.

A letter including an extensive epidemiologic questionnaire and a written consent form was mailed to women who verbally agreed to participate. The questionnaire included questions on lifestyle and reproductive factors, the use of oral contraceptives and other medications, etc. A trained research nurse collected blood samples between 7:15 am and 12:15 pm and took body measurements twice during each menstrual cycle, days 5-10 and 5-10 days before the predicted onset of the following menstrual period, i.e. cycle days 18-23 in most women. All women were asked to call back with the date of the first day of their next menstrual period. Body measurements included height, weight, waist and hip circumferences, and breast volumes. The plasma and blood cells were separated and frozen at minus 70 degrees Celsius at our laboratory at the Department of Oncology, Lund.

In addition to the 269 healthy women, we also included 40 BRCA1 and 16 BRCA2 carriers born between 1950 and 1988, irrespective of their cancer status, who had consented to genetic testing and undergone BRCA1/2 mutation testing at the laboratory of the Department of Oncology, Lund. Nine of the BRCA1/2 mutation carriers had a prior breast cancer diagnosis at the time of BRCA1/2 mutation testing. The total cohort therefore consisted of 325 women from 194 families and the vast majority was Swedish. Some, but not all of these 56 extra BRCA1/2 carriers had filled-out questionnaires regarding lifestyle factors and prophylactic mastectomies at the Oncogenetic clinic. Our cohort therefore consisted of all BRCA1 and BRCA2 carriers from the South Swedish Health Care Region born between 1950 and 1988 with DNA available for SNP testing. All BRCA1/2 mutation testing in the South Swedish Health Care Region is performed in our laboratory. Mutation testing of the BRCA1 and BRCA2 genes was not performed as part of this study. The data on mutation status was obtained from clinical records from the Oncogenetic Clinic of the Department of Oncology in Lund. BRCA1 and BRCA2 mutation testing is offered at the Oncogenetic Clinic of the Department of Oncology in Lund if an individual is considered to be at high-risk for breast cancer as defined above. Women are usually not offered testing before age 25. BRCA gene mutation carriers included only those with confirmed deleterious alterations, i.e. nonsense or frameshift indel mutations that cause protein
truncation, or known disease-associated missense mutations. Full mutation screening was performed according to denaturing high-performance liquid chromatography (DHPLC), sequencing, and multiplex ligation-dependent probed amplification (MLPA).

Genotyping of AR CAG, GGC and htSNPs
Genomic DNA was extracted from 300 µl of peripheral blood using Wizard, Genomic DNA Purification Kit, (Promega, Madison, WI, USA). The AR polymorphisms CAG and GGC were analyzed as previously described [24]. A CAG allele with <22 repeats was classified as short (S) and with ≥22 repeats as long (L). The GGC repeat <17 repeats was classified as short and ≥17 as long. The selection of AR htSNPs was based on the six reported SNPs from a Swedish male cohort and these SNPs are estimated to cover over 95% of the haplotypic variation among the men with and without prostate cancer in Sweden [34]. The positions of the htSNPs are presented in the paper by Lindström et al. [34], figure 1.

Figure 1. Shows the distribution of the htSNPs in the AR gene.

The genotyping of AR htSNPs was performed at Region Skåne Competence Centre (RSKC Malmö), Malmö University Hospital, Malmö, Sweden. The SNPs were genotyped according to the manufacturers’ protocol with TaqMan® assay AR rs1337080 by allelic discrimination based on real-time PCR on ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The AR SNPs rs17302090, rs6152, rs7061037, rs1337080, rs5031002, and rs5964607 analyses were performed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Sequenom MassARRAY® platform (Sequenom, San Diego, CA, USA), using iPLEX reagents according to the manufacturers’ protocol. The Sequenom MassARRAY® designer software was used for multiplex SNP analysis design. For quality control, over 10% of the samples were run in duplicate. The concordance was 100% for the validated samples. The call rates varied between 96.6% and 100%.

Construction of haplotypes and diplotypes
Since the AR is located on the X-chromosome and women have two, we constructed diplotypes instead of haplotypes. First each SNP was cross-tabulated against the other five SNPs. This procedure showed that certain combinations did not exist or were very rare. Secondly, we constructed the haplotypes and diplotypes based on the most likely combinations. Thirdly, we compared our results to the haplotypes obtained from the Swedish male cohort and our haplotypes were in agreement with their haplotypes. Rs6152 had 10 missing values. However, the kappa value between rs6152 and rs7061037 was 0.967. For the diplotype analyses we therefore completed the missing rs6152 SNPs based on the rs7061037.
Similarly, rs1337980 was missing for 11 women and based on the remaining five SNPs and most likely haplotypes we were able to complete these missing values. We could, however, not complete one missing value for rs17302090 and two missing values for rs5964607. Diplotype variants present in fewer than five women were classified as rare variants and combined into a single category called rare variants. This group included 18 women with ten different diplotype variants.

**Hormone analysis**

Testosterone, androstenedione and DHEAS levels were analyzed for 258 women from EDTA plasma obtained 5-10 days prior to the predicted onset of the next menstrual period, i.e. during menstrual cycle days 18-23 in most women. Testosterone was also analyzed for 257 women from EDTA plasma obtained during menstrual cycle days 5-10.

**Testosterone** in EDTA plasma was measured by electrochemiluminescent immunoassay by Elecsys 1010/2010 Modular analytics E170 analyzer with the Roche Elecsys 1010/2010 (Roche Diagnostics, Mannheim, Germany). This system is based on competitive binding analysis principle using monoclonal antibodies against testosterone. The maximal allowed immunoassay variation was ≤20% for testosterone and according to the manufacturer the detection limits were 0.069-52.00 nmol/L. The limits of detection were defined by the lowest detectable value and the highest value on a master curve,. Values above the curve were reported as >52nmol/L.

**Androstenedione** (4-androsten-3,17-dione) and **Dehydroepiandrosterone sulphate** (DHEAS) in EDTA-plasma was analysed with a Coat-a-Count Direct Androstenedione or DHEA-SO₄ radioimmunoassay in-vitro diagnostic test kit (DPC Skafe, Mölndal, Sweden) which is based on competitive binding of androstenedione and DHEAS, respectively, on polyclonal antibodies. MultiCalc Advanced program, version 2.60 linked to the radio count was used to measure the radioactivity. The maximal allowed variation was 10% for androstenedione and the detection limits 0.2-35 nmol/L. For DHEAS, the maximal allowed variation was 13% and the measure limits were 0.3-27.1 micromol/L.

**Follow-up**

Women were followed until the development of a first breast cancer according to the regional cancer registries, until the date of a self-reported prophylactic mastectomy or until May 31 2009, whichever came first. The report rate of cancer diagnoses to the Swedish cancer registries is close to a 100 percent. The clinical follow-up of high-risk women includes annual mammograms, ultrasounds and MRIs of the breasts in addition to a physical examination and annual follow-ups of the ovaries by ultrasound, CA-125 and a gynecological examination.

**Data analysis**

The statistical software PASW Statistics 17.0 and STATA were used for all statistical analyses. Testosterone, androstenedione and DHEAS levels were not normally distributed, and the values were transformed using the natural logarithm (ln) to obtain a better distribution. Multivariate linear regression models were used to compare circulating ln-transformed androgen levels in relation to combined OC use, AR htSNPs and the diplotype. To investigate whether there were differences according to which type of current OC formula was used, three dummy variables were created for the most common formulas. Another dummy variable was created for progestins with less or no androgenic properties (desogestrel, norgestimate, and drospirenon) compared to those with partially or unclassified androgenic
properties (levonorgestrel, noretisteron, and lynestrenol). The models were standardized for age (29 years), ln-transformed weight (67 kg), waist-to hip ratio (0.77), number of days to next menstrual period (7 days), and for non-current use of combined OCs. For htSNPs, each model also included two dummy variables for heterozygous and homozygous variants of the htSHP. The normal variant was used as reference. For the diplotypes models each model included the diplotype as a dummy variable. The multivariate linear regression models were also adjusted for family clustering by using the cluster option of the regress command in STATA. An interaction term was created between OC use and rare variant diplotypes to test for interactions on androgen levels. The odds of diplotype frequencies were compared between women from families with different mutations, BRCA1, BRCA2, BRCAX or untested family was calculated with Chi-square. Non-standardized geometric means with 95% confidence intervals (CI) for androgen levels for each diplotype were obtained using one-sample t-tests. The diplotype frequencies were compared between women from families with different mutations, BRCA1, BRCA2, BRCAX or untested family. Cox regression models were used to investigate whether the htSNPs or diplotypes were associated with an increased Hazard Ratio of incident breast cancer after 18 years of age. Nominal P-values are presented. All P-values were two-tailed.
Results
DNA for genotyping was available for 323 women and hormone analyses were done for 258 women. When we examined AR htSNPs in relation to androgen levels and OC use, we excluded women who were breast-feeding at the time of the blood draw (n=4), women using hormone contraceptives other than combined OCs, e.g. progesterone-only pills, injectable, intrauterine, and implantable hormone contraceptives (n=19) or both (n=1), one woman with missing information on hormonal contraceptive use, as well as women with missing information on number of days to the next menstrual cycle (n=2). The characteristics for the 325 women with genotyping data and the 231 users of combined OCs and non-users with androgen levels are presented in table 1.

Table 1. Characteristics of the 325 women included in the study and of the 231 non-users and users of combined OCs with androgen levels available.

<table>
<thead>
<tr>
<th></th>
<th>All n=325</th>
<th>Androgen analyses n=231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs Medians IQR n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>30 25-36 325</td>
<td>29 24-35 231</td>
</tr>
<tr>
<td>Weight, kgs</td>
<td>64.4 58.3-74.0 269</td>
<td>63.8 57.8-72.8 231</td>
</tr>
<tr>
<td>Height, cms</td>
<td>168 164-172 269</td>
<td>168 164-172 231</td>
</tr>
<tr>
<td>Body mass index</td>
<td>22.8 20.9-25.6 269</td>
<td>22.7 20.8-25.2 231</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.76 0.73-0.80 269</td>
<td>0.76 0.73-0.80 231</td>
</tr>
<tr>
<td>Age at menarche</td>
<td>13 12-14 312</td>
<td>13 12-14 230</td>
</tr>
<tr>
<td>Age at first full term pregnancy</td>
<td>25 22-28 302</td>
<td>24 22-27 124</td>
</tr>
<tr>
<td>Percent Parous</td>
<td>54.4% 305</td>
<td>46.3% 231</td>
</tr>
<tr>
<td>Ever use of oral contraception</td>
<td>92.2% 269</td>
<td>91.3% 231</td>
</tr>
<tr>
<td>Current use of combined oral contraception</td>
<td>34.2% 269</td>
<td>40.0% 231</td>
</tr>
<tr>
<td>Current use of progestin-only pills</td>
<td>4.5% 269</td>
<td>0 231</td>
</tr>
<tr>
<td>Current use of other hormonal contraception</td>
<td>3.3% 268</td>
<td>0 231</td>
</tr>
<tr>
<td>Current smoker</td>
<td>22.8% 268</td>
<td>23.0% 230</td>
</tr>
<tr>
<td>Number of women BRCA1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation carrier</td>
<td>134 325</td>
<td>76 231</td>
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<td>Negative</td>
<td>70 269</td>
<td>23 23</td>
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<td>Untested</td>
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<tr>
<td>BRCA2 family</td>
<td></td>
<td></td>
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<tr>
<td>Mutation carrier</td>
<td>34 325</td>
<td>13 231</td>
</tr>
<tr>
<td>Negative</td>
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<td>Untested</td>
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<tr>
<td>BRCAX family</td>
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<tr>
<td>Mutation carrier</td>
<td>112 325</td>
<td>101 231</td>
</tr>
<tr>
<td>Untested family</td>
<td>45 325</td>
<td>41 231</td>
</tr>
</tbody>
</table>

IQR interquartile range

Androgen levels in relation to combined OC use, AR htSNPs and AR diplotypes
Standardized androgen levels during cycle days 18-23 were lower among most current OC users compared with non-users. The models were standardized for age (29 years), ln-transformed weight
(67 kg), waist-to hip ratio (0.77), and number of days to next menstrual period (7 days). In current OC users, compared with non-users, standardized geometric mean testosterone levels were 1.01 versus 1.59 nmol/L, androstenedione levels were 5.91 versus 9.08 nmol/L, and DHEAS levels were 3.16 versus 4.51 µmol/L (all $P$s<0.0001). Thirteen different formulas of combined OCs were used. There were no significant differences in testosterone, androstenedione or DHEAS levels according to the androgenic properties of the progestin. The three most common formulas were Desolett® (n=24; monophasic pill containing ethinylestradiol and desogestrel), Follimin® (n=17; monophasic pill containing ethinylestradiol and levonorgestrel), and Trionetta® (n=10; triphasic pill containing ethinylestradiol and levonorgestrel). There were no significant differences in testosterone, androstenedione or DHEAS levels between women using the above most common formulas and other formulas.

The individual htSNPs were not associated with androstenedione or DHEAS levels among all women, current OC users or non-users (data not shown). Only one htSNP, heterozygous rs5031002, was associated with lower standardized testosterone levels among non-users ($P$=0.03), table 2.

We then analyzed androgen levels during cycle days 18-23 in relation to diplotypes in all women, and stratified for current combined OC use, figure 2. Only rare variant diplotypes were associated with testosterone levels, but not with androstenedione or DHEAS levels. The rare variant diplotypes were associated with non-significantly higher testosterone levels among current OC users compared with the other diplotypes (adjusted $P$=0.067) and lower testosterone levels among non-users compared with the other diplotypes (adjusted $P$=0.045). There was a strong interaction between the rare variant diplotypes and combined OC use on testosterone levels during cycle days 18-23, ($\beta$=0.61; $P_{interaction}=0.011$), adjusted for age, ln-transformed weight, waist-to hip ratio, and number of days to next menstrual period. The interaction between the rare diplotype variants and combined OC use on testosterone levels was also present during menstrual cycle days 5-10 ($\beta$=0.44; adjusted $P_{interaction}=0.048$). When we adjusted the models for family clustering, the rare variant diplotypes were still associated with higher testosterone levels during cycle days 18-23 among current OC users compared with the other diplotypes (adjusted $P$<0.001) and lower testosterone levels among non-users compared with the other diplotypes (adjusted $P$=0.012). Similarly, the interaction between the rare variant diplotypes and combined OC use on testosterone levels during cycle days 18-23 and during cycle days 5-10 remained significant after adjustment for family clustering (adjusted $P_{interaction}<0.001$ and adjusted $P_{interaction}=0.003$; respectively).

**AR diplotypes in relation to CAG and GGC**

As previously reported [24], the AR microsatellite CAG and GGC repeat lengths were genotyped for a subset of the cohort (254 and 253 young women, respectively). The frequencies of CAG and GGC genotypes in relation to AR diplotypes are shown in figure 3. The AR CAG (L/L) genotype did not co-segregate with GGAAGC/GAGGGT. The AR CAG (S/S) genotype did not co-segregate with GGAAGC/GGAAAC. AR GGC S/S genotype co-segregated only with GGAAGC/GGAAGC, the rare variant diplotypes, and GGAAGC/AAGAGT diplotypes. Since OC users had lower testosterone levels than non-users with all AR CAG and GGC genotypes, except for the GGC (S/S) genotype as reported in our previous study, we combined the three diplotypes that co-segregated with the GGC (S/S) genotype. However, these three diplotypes were not associated with testosterone levels in current OC users or non-users when compared with the remaining three diplotypes.
**Figure 2**

Geometric means with 95% CI of testosterone in relation to AR diplotypes in A) all women, B) non-users, and C) current OC users during menstrual cycle days 18-23. The number of women with each diplotype is indicated. The squares indicate non-standardized testosterone levels and the round dots indicate testosterone levels standardized for age (29 years), ln-transformed weight (67 kg), waist-to-hip ratio (0.77), number of days to next menstrual period (7 days), and for non-current use of combined OCs. In A) the continuous line represents the geometric mean for all women, and in B) and C) the dotted line represents the geometric mean for all women and the continuous line the geometric mean for non-users and current OC users, respectively. The rare variant diplotypes were associated with non-significantly higher testosterone levels among current OC users compared with the other diplotypes (adjusted $P=0.067$) and lower testosterone levels among non-users compared with the other diplotypes (adjusted $P=0.045$). There was an interaction between the rare variant diplotypes and combined OC use on testosterone levels during cycle days 18-23, ($\beta=0.61$; $P_{\text{interaction}}=0.011$), adjusted for age, ln-transformed weight, waist-to-hip ratio, and number of days to next menstrual period.
Table 2. AR ht SNPs in relation to standardized testosterone levels. The levels were standardized for age (29 years), ln-transformed weight (67 kg), waist-to-hip ratio (0.77), and number of days to next menstrual period (7 days). In the model including all women the levels were standardized to non-current use of combined OCs. Heterozygous and homozygous variants of the htSHPs were entered as dummy variables. The normal variant was used as reference.

<table>
<thead>
<tr>
<th>AR htSNP</th>
<th>Genotype</th>
<th>All women N (n=325)</th>
<th>% (n=231)</th>
<th>All women Testosterone nmol/l P-value</th>
<th>N (n=143)</th>
<th>No OC use Testosterone nmol/l P-value</th>
<th>N (n=88)</th>
<th>Current OC use Testosterone nmol/l P-value</th>
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<td>85.8</td>
<td>197</td>
<td>1.59</td>
<td>ref</td>
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<td>1.60</td>
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<tr>
<td></td>
<td>GA</td>
<td>41</td>
<td>12.6</td>
<td>29</td>
<td>1.68</td>
<td>0.48</td>
<td>25</td>
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<td></td>
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<td>2</td>
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<td></td>
<td></td>
<td>2</td>
<td></td>
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Figure 3
A) CAG repeat length frequencies among women (n=254) for each AR diplotype. The CAG alleles with <22 were classified as short (S) and ≥22 as long (L). The AR CAG (L/L) genotype did not co-segregate with GGAAGC/GAGGGT. The AR CAG (S/S) genotype did not co-segregate with GGAAGC/GGAAGT.

B) GGC repeat length frequencies among women (n=253) for each AR diplotype. The GGC alleles with <17 were classified as short (S) and ≥17 as long (L). AR GGC S/S genotype co-segregated only with GGAAGC/GGAAGC, the rare variant diplotypes, and GGAAGC/AAGAGT diplotypes.
**Figure 4**

A) Frequency distribution of $AR$ diploposes among all women (n=320) from $BRCA1$, $BRCA2$ and $BRCAX$ families. In the $BRCA1$ families, the odds of having a GGAAGC/GGAAGC diplotype was lower than in other families, irrespective of mutation status, OR 0.63 (95%CI 0.40-1.00; $P$=0.051). The GGAAGC/GGAAAC was not present in women belonging to $BRCA2$ families. None of the other diploposes was associated with $BRCA1/2$ or $BRCAX$ family status.

B) Frequency distribution of $AR$ diploposes for the women first enrolled from $BRCA1$, $BRCA2$ and $BRCAX$ families (n=192). The odds of having a GGAAGC/GGAAGC diplotype was lower in women from $BRCA1$ compared to women from the other family types, OR 0.41 (95%CI 0.22-0.78; $P$=0.005), whereas the odds of having a GGAAGC/AAGAGT diplotype was higher in $BRCA1$ families OR 3.28 (95%CI 1.22-8.81; $P$=0.014). The GGAAGC/GGAAAC was not present in women belonging to $BRCA2$ families. None of the other diploposes was associated with $BRCA1/2$ or $BRCAX$ family status. The GGAAGC/GGAAAC was not present in women belonging to $BRCA2$ families.
**AR diplotypes in relation to BRCA-family status**

The frequency distribution of AR diplotypes among all women and women from BRCA1, BRCA2 and BRCAX families, and among the women first enrolled from each family (n=192) are presented in figure 4. In the BRCA1 families, the GGAAGC/GGAAGC diplotype was less common than in other families, irrespective of mutation status, OR 0.63 (95%CI 0.40-1.00; \(P=0.051\)). Since some women belonged to the same families, we restricted the analyses to include only to the first enrolled woman from each family. In this subgroup analysis, the odds of having a GGAAGC/GGAAGC diplotype was lower in women from BRCA1 compared to women from the other family types, OR 0.41 (95%CI 0.22-0.78; \(P=0.005\)), whereas the odds of having a GGAAGC/AAGAGT diplotype was higher in BRCA1 families OR 3.28 (95%CI 1.22-8.81; \(P=0.014\)). None of the other diplotypes was associated with BRCA1/2 or BRCAX family status.

Among the 325 women, 23 have developed breast cancer after age 18 years. Eleven incident cancers were found in the original cohort of 269 women, three incident cancers were diagnosed after mutation testing in the additional 56 BRCA1/2 mutation carriers and nine of the carriers had a breast cancer diagnosis prior to mutation testing. Thirteen women were known BRCA1 mutation carriers, five were known BRCA2 mutation carriers, four belonged to BRCAX families, and one woman belonged to an untested family. The median age at diagnosis was 39 years (range 28 to 50 years). Breast cancer was not significantly associated with any of the hSNPs or the diplotypes. Of the 23 breast cancer cases, 17 women had the most common GGAAGC/GGAAGC diplotype, three women had the GGAAGC/AAGAGT diplotype, two women had the GGAAGC/GAGGGT diplotype, and one woman had a rare variant diplotype. The hazard of an incident breast cancer was non-significantly higher with the three diplotypes that co-segregated with the GGC (S/S) genotype compared with the remaining diplotypes, HR 2.79 (95% CI 0.65-11.99; \(P=0.17\)), adjusted for BRCA1 and BRCA2 mutation status.
**Discussion**

This is the first study to examine AR htSNP diplotype data in relation to in vivo androgen levels, combined OC use, CAG and GGC genotypes, and *BRCA1/2* and *BRCAX* family status in young women from breast cancer high-risk families. Our main findings were that OC status affected testosterone levels differently among women who had a rare variant diplotype compared to women with other diplotypes, and that the odds of having the most common diplotype GGAAGC/GGAAGC was lower in *BRCA1* families compared to other families.

In the current study, there was a strong interaction between the rare variant diplotypes and combined OC use on testosterone levels during both cycle days 18-23 and cycle days 5-10. Women with rare variant diplotypes may not be helped by combined OC treatment of acne or other androgen dependent conditions, but this needs to be confirmed in other studies. Since many of the women were from same families, we expected that some of the diplotype variants would be more common among women from the same families and the testosterone levels would differ between the families. We therefore adjusted the models for family clustering. Surprisingly, the interaction between rare variant diplotypes and combined OC use on testosterone levels became stronger after adjustment for family clustering. However, there were few women with the rare variant diplotypes and this group consisted of ten different diplotype variants. There is therefore a risk of an inflated finding due to small-sample error. An alternative explanation would be that the rare variant diplotypes actually do reflect rare AR genotypes with altered functionality due to other functional polymorphisms and different androgen levels. We found that androgen levels during cycle days 18-23 were lower among most current OC users compared with non-users, which is in line with previous findings [24, 29]. OCs suppress gonadotropins, which leads to lower endogenous androgen and estrogen production in the ovaries [11, 35, 36].

It is easier to perform high throughput genotyping of htSNPs than of microsatellites. Since some polymorphisms in the AR have been linked to testosterone levels in both women and men, as well as breast and prostate cancer [20, 21, 23, 25-28, 37, 38] it is possible that the htSNPs that co-segregate with these polymorphisms may modify androgen levels and breast cancer risk. It is also possible that young women who have higher testosterone levels during OC use belong to the subgroup of women who are at higher risk for early-onset breast cancer if they use OCs. While it has been established that OC use is associated with higher breast cancer risk in the general population [30], as well as in women from high-risk families [31, 32] and that ethinylestradiol is carcinogenic to humans [39], the role of testosterone in breast cancer is less clear [3, 6, 7, 11]. In a previous study, we found a significant interaction between the cumulative number of short GGC alleles and testosterone levels among current OC users during cycle days 5-10 and a near significant interaction during cycle days 18-23 [24]. The testosterone levels were increasing with increasing number of short GGC alleles among current OC users during both cycle phases [24]. In the current study, GGC (S/S) genotype co-segregated only with rare variant diplotypes and two other diplotypes. However, only the rare variant diplotypes were associated with testosterone levels in the current study. We do not know whether this interaction was driven by the rare variant diplotypes or the GGC genotype.

The individual AR htSNPs were not associated with androstenedione or DHEAS levels among current OC users or non-users. Only one AR htSNPs (rs5031002) was associated with lower testosterone levels among non-users. Due to the large number of analyses, this may be due to chance. The selected htSNPs were from a large Swedish male cohort of prostate cancer patients and their controls, and were estimated to cover over 95% of the genetic variation in the AR gene [34]. After construction of the haplotypes and diplotypes in the current study, we had the same haplotypes as Lindström *et al.* [34]. However, the association between haplotypes and long AR CAG allele in the study by Lindström *et al.* [34] was not the same as in our study. In the study by Lindström *et al.* a
long CAG allele co-segregated only with GGAAGC and GGAAGT haplotypes, while the short CAG allele was present among individuals with all haplotypes [34], in contrast to our results. Lindström et al. [34] did not genotype the GGC repeat and our study is the first one to report that the GGC S/S genotype co-segregated with three AR diplotype variants.

We also studied the frequency of the different AR diplotype in relation to BRCA family status and among the first women enrolled from each family (BRCA1, BRCA2 and BRCAX families). The odds of having the most common diplotype in the whole study population (GGAAGC/GGAAGC) was lower in the BRCA1 families, irrespective of mutation status, than in the other families. The odds of having the GGAAGC/AAGAGT was higher in BRCA1 families. This diplotype had the highest testosterone levels, although not statistically significant. Since the GGAAGC/GGAAGC was a less common diplotype among BRCA1 families than among other families, one may hypothesize that this combination confers some survival disadvantage. In the study by Lindström et al. both the most common AR haplotype (GGAAGC) and the AAGAGT haplotypes were associated with increased prostate cancer risk [34]. Another study showed that CAG with ≥ 28 repeats was associated with younger age at breast cancer diagnosis [28]. In our study, the most common AR diplotype GGAAGC/GGAAGC was more commonly represented among women with over 28 CAG repeats (data not shown). No significant association was found between incident breast cancer and the different AR diplotype variants or individual htSNPs. However, the number of cases with incident breast cancer was low (23 of 325 women). Moreover, we only had information on OC use for half of the cases. A recent large study investigated the relationship between AR htSNPs, haplotypes and CAG repeats among 5603 breast cancer cases and 7480 controls and no association was found between any genetic variants and breast cancer [33].

Women are mosaics for two different cell types, cells with an active X-chromosome from the mother and cells with an active X-chromosome from the father. Skewed X-inactivation is arbitrarily defined as deviation from the binomial inactivation, with 80% or more of the cells having preferential inactivation of only one X-chromosome. Although the inactivation is thought to be permanent and passed on to the cell descendants, the pattern changes with age and older women have increased frequency of skewed inactivation in peripheral blood cells [40]. One study reported a higher frequency of skewed X-inactivation in young breast cancer patients with a family history of breast cancer, but no BRCA1/2 mutations, compared to controls. However, skewed X-inactivation frequency did not differ significantly between BRCA1/2 mutation carriers and age-matched healthy controls [41]. We did not perform X-inactivation analysis in our study. All diplotype variants, except for a subgroup of the rare variants, contained at least one copy of the most common haplotype.

There are limitations to our study. There were few incident breast cancer cases during follow-up and a larger cohort with complete information on OC use would have been preferable. However, our cohort consisted of all BRCA1/2 mutation carriers from the South Swedish Health Care Region born between 1950 and 1988 with DNA available for SNP testing. We also lacked information on androgen dependent conditions such as acne, and were therefore unable to assess whether AR diplotype was associated with these conditions. Women have low testosterone levels. Compared to the assays used in the current study, more sensitive experimental methods based on chromatography followed by tandem mass spectrometry have since been developed [3].

In conclusion, this is the first study to examine AR htSNP diplotype data in relation to in vivo androgen hormone levels, combined OC use, CAG and GGC repeat lengths, and BRCA1/2 and BRCAX family status. Neither individual AR htSNPs nor diplotype tagged for CAG or GGC genotypes. Combined OC use affected testosterone levels differently among women who had a rare variant diplotype compared to women with other diplotypes, but this finding needs confirmation in
independent cohorts. Apart from the rare variant diplotypes, there was no significant association between AR diplotypes and androgen or proandrogen levels. The most common diplotype was less common in families with BRCA1 mutations and the clinical significance of this finding needs elucidation. Since testosterone levels are not affected the same way by combined OC use among all women, young women who have higher testosterone levels during combined OC use may belong to the subgroup of women who will not be helped by combined OCs for treatment of androgen dependent conditions and may be at higher risk for early-onset breast cancer. Whether these women can be identified with AR genotyping needs to be confirmed in an independent cohort.
Author contributions
Maria Hietala provided background information, performed the statistical analyses and prepared the manuscript. Maria Henningson abstracted data and clinical information on study participants, performed data entry and provided comments on the manuscript. Therese Töngren performed the CAG and GGC genotype analyses. Håkan Olsson provided background information and comments on the manuscript. Helena Jernström initiated this study, provided background information, and supervised the statistical analyses and the writing of the manuscript. All authors approved the final manuscript.

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