Title: The prognostic role of androgen receptor in patients with early stage breast cancer: A meta-analysis of clinical and gene expression data

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Statement of Translational Relevance

Androgen receptor (AR) recently regain interest as possible therapeutic option in breast cancer (BC) treatment, however little is known about clinical significance of AR in breast carcinogenesis. To our knowledge, we report the largest combined clinical and gene expression meta-analysis, assessing the prognostic significance of AR expression in early stage BC indicating that AR expression at both protein and mRNA level serves as a positive prognosticator for women with early-stage BC. In addition, we explore connection amongst AR and pathological complete response after neo-adjuvant chemotherapy in the gene-expression publicly available data-sets, and also correlation of AR and genes and gene signatures of interest. Additional information about distinct prognostic relevance of AR expression in different BC subtypes was provided, however further studies are warranted to confirm these findings. It is apparent that AR has many effects on the biology of BC and deserves more clinical and translational research attention.
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Abstract

Background: Androgen receptor (AR) expression has been observed in about 70% of breast cancer (BC) patients, but its prognostic role remains uncertain.

Methods: To assess the prognostic role of AR expression in early-stage BC we performed a meta-analysis of studies that evaluated the impact of AR at the protein and gene expression level on disease free survival (DFS) and/or overall survival (OS). Eligible studies were identified by systematic review of electronic databases using the MeSH-terms "breast neoplasm" and "androgen receptor" and were selected after a qualitative assessment based on the REMARK criteria. A pooled gene expression analysis of 35 publicly available microarray data sets was also performed from patients with early-stage BC with available gene expression and clinical outcome data.

Results: 22 out of 33 eligible studies for the clinical meta-analysis, including 10,004 patients, were considered as evaluable for the current study after the qualitative assessment. AR positivity defined by IHC was associated with improved DFS in all BC patients [multivariate (M) analysis, HR 0.46, 95% CI 0.37-0.58, p<0.001] and better OS [M-HR 0.53, 95% CI 0.38-0.73, p<0.001]. 35 datasets including 7,220 patients were eligible for the pooled gene expression analysis. High AR mRNA levels were found to confer positive prognosis overall in terms of DFS (HR 0.82 95%CI 0.72-0.92; p=0.0007) and OS (HR 0.84 95%CI 0.75-0.94; p=0.02) only in univariate analysis.
Conclusion: Our analysis, conducted among more than 17,000 women with early-stage BC included in clinical and gene expression analysis, demonstrates that AR positivity is associated with favourable clinical outcome.
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Running Title: Androgen receptor in early breast cancer: meta-analysis
Introduction

The androgen receptor (AR) is a steroid hormone nuclear receptor, which is frequently expressed in BC. Still, the contribution of AR signalling in BC carcinogenesis and its clinical relevance as potential prognostic factor and therapeutic target remain largely unknown. The AR expression in primary BC was discovered long ago and androgens such as fuoxymesterone showed promising response rates in metastatic BC patients, ranging from 14% to 53% (1). However, due to their adverse effects associated with virilization, lack of solid understanding of their biological mode of action and the development of aromatase inhibitors, androgens were not further pursued as therapeutic modality for BC patients.

*In vitro* evidence indicated that AR has both inhibitory and stimulatory effect on different BC cells lines growth which is considered to be modulated by the presence or absence of oestrogen receptor (ER) expression (2).

Epidemiological studies also provided conflicting results, reporting significant association between androgen serum level and risk for developing BC or no association at all. Those results were dependent on age and more importantly on menopausal status and oestrogen milieu suggesting that androgen could act as anti-oestrogen in premenopausal women, whereas it acts as an oestrogen agonist in postmenopausal women (3). Indeed, some epidemiological studies demonstrate that high circulating androgen level in postmenopausal women was linked with higher risk for developing BC, but it is not known if these effects are mediated through AR signalling (4).
More recent results originated by gene expression profiling studies renewed the interest in AR receptor signalling and its potential clinical relevance for BC. One of these studies identified ER-negative, AR-positive BC as a subtype showing a distinct transcriptome profile, called the molecular apocrine subtype (2, 5). Of note, these studies indicated possible antagonism between AR and ER mediated by competition for the DNA binding sites in the cells and a possible adoption of AR in ER-like oncogenic role in the absence of ER (2, 5).

AR expression by IHC has been observed in up to 90% of primary BC, and in up to 75% of BC metastases, depending on the method, patient’s population and cut-offs used. These studies indicate that the frequency of AR positivity differs between different BC subtypes; the highest positivity observed in ER-positive tumours in up to 80-90% (6-10) and the lowest in triple negative (TN) tumours, in up to 30% (6, 7, 11). Of note, a phase 2 trial assessing enzalutamide in metastatic TNBC, reported AR positivity (defined as ≥1%) in 79% of 404 cases analysed(12). Retrospective clinical studies showed that AR by IHC adds prognostic information beyond the established clinico-pathological parameters in all patient groups and in early-stage ER-positive BC patients (9, 13, 14). Tumours co-expressing AR and ER are smaller, have lower Nottingham grade and low proliferative index (14), lymph-node involvement is less frequent and they are more likely to be found in postmenopausal women (4). However, the clinical significance of AR in ER-negative BC patients is less clear.

To address the prognostic role of AR status in early stage BC we conducted a meta-analysis of published clinical studies to evaluate the impact of AR protein expression defined by immunohistochemistry (IHC) on disease free survival (DFS) and overall survival (OS).
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Furthermore, we conducted a pooled gene expression analysis of publicly available microarray data sets to assess the prognostic significance of mRNA AR status in early stage BC and explore potential associations of AR mRNA expression with the expression of other individual genes and gene signatures (GS) of interest.

Methods

Systematic review of published clinical studies

Meta-analysis of clinical studies that evaluated the impact of AR protein expression on DFS and OS in patients with early-stage BC was performed within all BC subtypes together and, whenever possible, within different BC subtypes, defined as follows: 1) ER-positive (ER+); 2) ER-negative (ER-); 3) HER2+ and 4) TN (ER-HER2-). Patients were considered to have AR-positive tumors according to the cut-off points defined by the authors in each of the eligible studies.

Studies identification and selection

The eligible studies were identified by an electronic search on PubMed/Medline, Cochrane Review and Google Scholar using the MeSH terms "breast neoplasm" and "androgen receptor". The literature search was conducted independently by two investigators (I.B.S. and D.Z.) up to June 2015. Identified studies were eligible for this meta-analysis if they met the following inclusion criteria: studies conducted in patients with early stage BC that assessed AR expression in primary BC, studies that compared clinical outcomes (DFS and/or OS) in association with AR-status, studies published in extenso and reported in the English language.
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Of note, no minimum threshold for either number of patients or duration of follow-up period was mandated. Cross-referencing from relevant studies was performed to confirm retrieval of all potentially eligible studies. To avoid duplication of data, when the same patient population was reported in several publications, only study with the highest number of events was included in the present analysis. In terms of study eligibility, final decisions were taken upon consensus between two investigators (I.B.S. and D.Z.).

Data extraction

Data, such as authors, publication date, number of patients, subgroup analysis, method of AR assessment, AR threshold were extracted by two investigators (I.B.S. and D.Z.) using a predesigned abstraction form. Survival data within all patients and whenever possible within different BC subtypes [including hazard ratio (HR) and p value] were extracted independently by two statisticians (L.A. and M.Pa.).

Quality of the eligible studies

The study methodology of each eligible study was independently scored by three reviewers [two medical doctors (I.B.S. and D.Z.) and one statistician (M.Pa.)], according to REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies)(15). Each of the 20 criteria listed in REMARK was scored for each eligible study, using an ordinal scale with possible values 0, 1 and 2, making a maximal score of 40. The overall score evaluated several scopes of the methodology including the scientific rational and design; the description of the methods used to identify AR expression; data generation, analysis of the study data and discussion of their relevance. The attributed value per item was 2 points if
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clearly defined in the article, 1 point if description incomplete or unclear and 0 point if not defined, inadequate or not applicable. The scores for each individual study were compared and consensus for each item was reached among the three investigators. We did not pre-define a threshold for the REMARK score of each study as inclusion criterion in the meta-analysis. However, scoring was performed as quality assessment of the clinical studies data included in this meta-analysis and used for sensitivity analyses (Table 1 supplementary file).

The studies being eligible for the systematic review are called "eligible" and those providing data for the clinical meta-analysis with at least retrievable HR in one of the defined endpoints are called "evaluable". We excluded studies with insufficient or with unreliable data to estimate clinical outcome. In terms of defining the evaluable studies, final decisions were taken upon consensus between the two physicians (I.B.S. and D.Z.) and the two statisticians (L.A. and M.Pe.).

Pooled gene expression analysis

Thirty-five datasets of gene expression profiling analysis of more than 7,220 primary BC were retrieved from public databases or authors’ websites, 32 previously described in the manuscript of Haibe-Kains et al., with three additional sets: TCGA (TCG Data Portal)(16), PNC (GSE20713) (17) and METABRIC (European Genome-Phenome Archive under accession number EGAS00000000083) (18).

To ensure comparability of expression values across multiple data sets, we performed a 0.95-quantile normalization of all genes and GS of interest.
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AR expression levels were calculated as continuous and categorical variable where classified as low (corresponding to the lower tertile across all available expression values), medium (intermediate tertile) and high (upper tertile).

BC molecular subtypes were defined based on the PAM50 classifier as luminal A, luminal B, HER2-enriched and basal-like. As no clear consensus has ever been established on the existence of this PAM50 group, the normal like subgroup of tumor was not specifically considered in the subgroup analyses. Moreover, as not every study had complete information about ER and HER2 status, ESR1 and ERBB2 status were derived from the bimodal distribution of these two genes expression.

We chose to evaluate potential associations between AR gene expression levels and individual genes and GS that can be classified as follows: oestrogen GS (ESR1), proliferation based prognostic GS (AURKA (19), CIN70 (20), GENE70 (21), GGI (22)), immune-related individual genes and GS (CTLA4 gene, IDO1 gene, IFNG gene, IGKC gene, CD3D gene, CD80 gene, CD8A gene, FOXP3 gene, Immune1 GS (23), Immune2 GS (24), PD1 gene, PDL1 gene), stroma-related genes and GS (CXCL13 gene, CXCL9 gene, RANKL gene, Stroma1 GS (25), Stroma2 GS (24)), as well as other individual genes and GS related to further oncogenic signalling pathways (RAS GS (26), SRC GS (26), MYC GS (26), E2F3 GS (26), BetaCatentin GS (26), BRCA1 and BRCA2 genes, AKT/mTOR GS (27), HER3 gene, IGF1 GS (28), MAPK GS (29), PIK3CA GS (30), PTEN GS (31), VEGFA gene, WNT7B gene).
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Furthermore, we assessed the possible impact of AR expression on pathological complete response (pCR), using neo-adjuvant data sets previously published in Ignatiadis et al (32), with availability of gene expression profiling data, and pCR status.

Statistical analysis

Clinical Meta-analysis

We used as a measure of the prognostic effect of AR protein expression the HR for the comparison of the DFS or OS distributions using AR negative patients as a reference. For each study and each considered subtype (when applicable and possible) we extracted the individual HR estimates and their variances if reported. When only the HR estimates were reported, we calculated their variances using the confidence interval for the HR estimate or the log rank statistic value and the number of events. If none of these methods was applicable, we read the survival curves and calculated back the HRs with their variances. Whenever available, we used both the individual HRs from the univariate and the multivariate analyses in separate analyses. We report combined HRs with 95% confidence intervals (CI) using fixed-effect models or random-effects models depending on the detected heterogeneity between the individual HRs estimates; if heterogeneity was detected random effect was reported. Heterogeneity of HRs between the different studies was assessed using a chi square test for heterogeneity.

Pooled gene-expression mRNA analysis

We calculated potential associations between AR mRNA levels with clinico-pathological variables among all BC patients and within BC subtypes using t-tests. The clinico-pathological
variables that were assessed are the following: age (both as continuous and categorical variable ≤ and > 50 years); tumor size (both as continuous and categorical variable following the UICC-TNM classification); grade (Grade 1, 2 and 3); nodal status (positive versus negative); ER status (positive versus negative); and HER2 status (positive versus negative). Furthermore, we assessed potential associations of AR mRNA expression with the expression levels of the previously mentioned individual genes and GS using a Wilcoxon test.

For the survival analysis, the two endpoints were disease free survival (DFS) and overall survival (OS). Survival plots according to the groups were drawn using the Kaplan–Meier method, and the differences were evaluated with a log-rank test. The median follow-up was calculated with the reversed Kaplan–Meier method and data were censored after 10 years of follow-up.

To compute HR and 95% CI in a univariate analysis we used a Cox linear regression model (adjusting only for the dataset). Multivariate analysis was computed by the linear Cox regression model, adjusted for the ESR1 and ERBB2 gene expression, patient age, tumor grade, tumor size and the lymph node involvement.

In the 8 neo-adjuvant data sets, we looked for the association of AR mRNA expression level among BC subtypes and percentage of pCR by using a logistic regression model (uni- and multi-variate adjusted for dataset, tumor grade and treatment). pCR was defined as loss of the invasive component of the primary tumor in one study (33) and no residual invasive cancer in the breast and axillary lymph nodes in other seven studies (34-38).
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Results

Clinical Meta-analysis

We retrieved a total of 913 references. After applying secondary exclusion criteria (Figure 1A_supp) a total of 33 studies were eligible for the analysis and were scored based on REMARK criteria (15). We then excluded studies where none of the HR of interest was available or retrievable with its variance or where the number of observed events was < 15. (Figure 1A_supp and Table 1_supp).

Out of the 33 eligible studies, 22 studies were found to be evaluable, including 10.004 patients, with 5.860 cases of AR positivity (Table 1). Twenty studies (6-9, 11, 39-52) were evaluable for the DFS and sixteen (7-9, 11, 39-42, 44-49, 53, 54) for the OS (Tables 2a_supp and 2b_supp); among studies only two (44, 53) were prospective, the rest was retrospective.

Of note, differences were noted among the studies concerning the methods of AR status assessment, antibodies used, cut-off values implemented and thresholds for AR positivity selection. There was heterogeneity between evaluable studies in regards to the study population with variation of BC subtypes definition, as well with treatment given and follow-up time.

Prognostic significance of AR expression

For the overall population, univariate analysis showed longer DFS [HR = 0.61; 95% CI 0.52-0.72; p<0.001] and OS [HR 0.62; 95% CI 0.51-0.75; p<0.001] among AR positive patients (Table 2a, Figure 1A). Multivariate analysis was performed based on available information,
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confirming good prognostication conferred by AR positivity in regard to DFS (HR 0.46; 95% CI 0.37-0.58; p<0.001) and OS (HR 0.53; 95% CI 0.38-0.73; p<0.001) (Table 2a, Figure 1B).

Subgroup analysis was performed based on available data, whenever feasible for the following BC subtypes: ER+; ER-; HER2+ER- (representative of the HER2-enriched) and TN (representative the basal-like subtype) (Table 2b). This analysis revealed that AR positivity resulted in significantly improved DFS and OS in ER+ patients, both in univariate (HR 0.53; 95%CI 0.44-0.63, p<0.001 and HR = 0.59; 95%CI 0.49-0.72, p<0.001, respectively) and multivariate analysis (HR 0.40; 95%CI 0.31-0.52, p<0.001 and HR = 0.37; 95%CI 0.16-0.85, p=0.02, respectively). Similar positive association was seen in TN BC patients (HR 0.64 95%CI 0.51-0.81, p<0.001 and HR 0.64 95%CI 0.49-0.88, p<0.001, respectively), in univariate analysis. In HER2+ER- BC AR positivity was not significantly associated with DFS (HR 1.20 95%CI 0.86-1.69, p=0.28) while overall survival was worse (HR 1.50 95%CI 1.01-2.22 p=0.04). No significant association was found either for DFS and OS in ER- BC subgroup (HR 0.33 95%CI 0.04-2.44, p=0.28 and HR 1.32 95%CI 0.98-1.80, p=0.08 respectively).

Pooled gene-expression mRNA analysis

Data sets

A total of 35 data sets with data from 7737 patients and median follow-up time of 10.17 years were available (Figure 1B_supp and Table 3_supp.), including 8 studies with 1.005 patients in the neoadjuvant setting (Table 4_supp.) (32). Clinicopathological characteristics of these patients according to the data sets can be found in Table 3_supp.

Associations of AR mRNA levels with clinic-pathological variables among all BC patients
Associations between AR mRNA expression and clinic-pathological variables were evaluated among all patients: a significant inverse correlation was observed between AR mRNA expression and tumor size ($p=0.03$). Women older than 50 years had higher AR levels ($p<0.001$). Additionally, AR expression was positively correlated with ERBB2 expression ($p<0.001$) (Table 3; Figure 2A_supp). We also evaluated AR expression among different BC subtypes defined by PAM50 classifier (Table 3, Figure 2B_supp.). High AR expression was seen in luminal A tumors with tumors less than 2 cm. High AR expression was found in Luminal B tumors in women older than 50 years and with lymph node negative disease ($p = 0.05$); AR mRNA levels were positively correlated with ESR1 ($p<0.001$).

**Survival analysis according to AR mRNA expression levels**

To address the impact of AR mRNA expression levels on prognosis, we compared the risk of relapse among three subgroups of patients corresponding to low, intermediate and high AR mRNA levels (level 1, 2 and 3 respectively) among all patients, and within different BC subtypes (see methods). Overall, high AR mRNA levels were found to confer better prognosis in terms of DFS (HR 0.82 95%CI 0.72-0.92; $p=0.0007$) and OS (HR 0.84 95%CI 0.75-0.94) $p=0.02$) in univariate analysis, but did not remain significant in the multivariate analysis (DFS: HR 0.97; 95%CI 0.84-1.13 $p=0.72$ and OS HR 0.98 95%CI 0.85-1.12, $p=0.72$) (Table 4).

In contrast, higher AR mRNA expression levels were found to be associated with improved OS in both, uni and multivariate analyses for all women with HER2-enriched BC (HR 0.75, 95%CI 0.56-1.00, $p=0.05$ and HR 0.72; 95% CI 0.53-0.97, $p=0.03$ respectively). Similar results were found in HER2-enriched subgroup treated with hormonotherapy only.
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(multivariate HR 0.50 95%CI 0.26-0.97, p=0.04). We did not find any other significant association with clinical outcome in the luminal A, luminal B and basal-like BC subtypes (Table 5_supplement).

Associations of AR mRNA expression levels with individual genes and gene signatures

We next looked for associations between AR mRNA expression and expression of individual genes and GS of potential clinical relevance. Among all BC subtypes, the following significant associations were found: negative correlation with proliferation-based GS such AURKA GS (19), CIN70 (20), GGI (22) and GENE70 (21) (all p<0.001); negative correlation with immune related genes and GS such as Immune 1 (23) and Immune 2 GS (24) GS (p=0.004 and p<0.001 respectively), as well as CTLA4, IDO1, IFNG, IGKC, PD1, CXCL9, CXCL13 and PDL1 genes (all, p<0.001); positive correlation with stroma1 GS (25) (p<0.001) but not with stroma2 GS (24) (p=0.13); and finally, positive correlations with several oncogenic pathways including WNT7B, ESR1, HER3, PIK3CA, BRCA1 genes (all p<0.001), and negative correlations with IGF1 (28), BetaCatenin, PTEN, MAPK, MYC, RAS, SRC GS (26) and BRCA2, VEGFA, CD80, CD8A, CD3D and SQLE genes (all, p<0.001) (Table 6A_supp). Associations between AR mRNA and expression levels of individual genes and GS among different BC subtypes are presented in Table 6B in supplementary file.

Response to neoadjuvant CT according to AR mRNA levels

We further evaluated whether AR mRNA expression were associated with response to neoadjuvant CT (anthracyclines +/- taxanes). Out of 1005 patients from 8 datasets (33-38), 235 (23%) patients achieved pCR, 765 (76%) did not, while 5 samples were inadequate for
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the analysis. High AR expression levels showed significant association with lower pCR rate (OR 0.57, 95%CI 0.44-0.74, p<0.001) in the univariate analysis while not significant in a multivariate model (OR 0.74, 95%CI 0.54-1.01, p=0.063) (Figure 2).

Discussion

To our knowledge, we report the largest combined clinical and gene expression meta-analysis, assessing the prognostic significance of AR expression in early stage BC indicating that AR expression at both protein and mRNA level serves as a positive prognosticator for women with early-stage BC.

Our results show that AR protein expression confers a DFS and OS advantage among all BC patients. A possible explanation for this is the documented association of AR expression with favourable prognostic factors such as small tumor size (39), low grade (8, 39, 44, 52), negative lymph node status, ER and/or PR positivity (44) and older age (8). These associations were confirmed by our AR mRNA pooled analysis, which showed that higher AR mRNA expression levels serve as positive prognosticator among all women with early stage BC.

In addition, we assessed the prognostic relevance of AR positivity among different BC subtypes, defined differently in the clinical and transcriptomic meta-analysis (IHC and PAM50 based respectively). In the former, a strong better prognostication was conferred by AR positivity among ER+ BC patients both in univariate and multivariate analysis, which is in line with previously published studies (8, 9, 11, 41, 42, 53). Preclinical evidence suggesting an AR-ER cross-talk also support these findings, indicating that AR can antagonize ER-signalling...
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depending on the relative levels of these two steroid receptors (2). Interesting results have been reported by Cochrane et al, assessing the potential prognostic/predictive relevance of AR:ER ration at the protein level assessed through IHC in the setting of ER-positive BC (55). The study was fuelled by previous observations that in ER-positive BC responding to endocrine treatment, AR downregulation at the protein and mRNA level is observed, whereas no such effect is seen in non-responsive tumors (56, 57). In a cohort of 192 patients with early-stage ER-positive BC receiving adjuvant tamoxifen treatment and another one of a randomized phase 2 trial assessing exemestane with or without tamoxifen, high AR:ER ratio was found to predict resistance to endocrine therapy in a statistically significant manner. These findings need further confirmatory studies; however, the data available in our study do not allow further evaluation.

According to the recently proposed “androgen excess” theory, increased androgen activity is one of the most important hormonal abnormalities seen in the patients with both ER+ and ER- BC (58). This theory suggests that androgens could stimulate ER+ tumors by increased conversion into estrogens within the tumor tissue; concentration of estradiol within tumor tissues about 20 times greater than in the circulation has been reported, an observation that could be explained by the local hormone production since androgen precursors are highly present within the tumor. According to this theory, ER- tumors could also be stimulated via androgen activity, and depending on testosterone serum levels and AR positivity, either through EGFR or direct stimulation via androgen pathway (58).

Another notable finding of our clinical meta-analysis is the worse prognostication conferred by AR positivity for women with HER2+ER- (Her2-enriched) disease, albeit the
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Analysis included three studies with 358 patients (7, 11, 44). Similar findings have been reported, with AR positivity associated with worse clinical outcome among HER2+(7) as well as HER2+ER-(44) BC patients. The biologic basis for the interaction between AR and HER2 in BC cell lines was recently studied through an analysis of the androgen regulated gene expression in HER2+ER- BC, indicating that AR signalling results in ligand dependent Wnt and HER2 signalling pathways activation through direct transcriptional induction of WNT7B and HER3, the latter being a dimerization partner to HER2. These findings suggest that anti-androgens merit clinical evaluation as anticancer agents in the setting of HER2+ER- BC (59). There is an ongoing phase 2, single-arm clinical trial assessing the combination of trastuzumab plus enzalutamide in the setting of HER2/AR-positive metastatic pre-treated BC (NCT02091960, https://clinicaltrials.gov/ct2/show/NCT02091960, accessed on July 15, 2016.). However, contrasting to our clinical meta-analysis our gene expression analysis find significantly better OS for AR mRNA expression within all HER2-enriched BCs and within HER2-enriched subgroup treated only with hormonotherapy. Caution is warranted when we compare data from IHC subtyping with data from gene-expression profiling since these two approaches may not produce identical calls for the same tumors and published data suggested modest overlap between AR IHC and transcriptional profiles (2). It must be noted, however, that there is no subset of patients included in the present analysis, for whom information concerning the AR status at both the mRNA and protein level, as assessed through IHC, is available.

We also investigated the prognostic value of AR in the more aggressive TN phenotype, with the clinical meta-analysis indicating that it serves as positive prognosticator
in the univariate analysis, confirming previously published results (6, 8, 39, 41, 44). This might be partly explained by the negative correlation of Ki67 as marker of decreased tumor cell proliferation and AR expression, which was seen in TNBC (60). However, other studies did not show any effect of AR on the TNBC outcome (11, 40, 53). In the gene-expression profiling analysis AR mRNA levels were not prognostic in basal-like BC. Recent findings identified a luminal androgen subtype within basal-like BC confirming the extensive heterogeneity of basal-like BCs (60) and the role of AR within this BC subtype requires further assessment.

The different prognostic relevance of AR positivity found among the different BC subtypes indicates that the broader molecular profile constituting the tumour influences the functional output of AR signalling and its relevance for the clinical outcome of patients. Thus, studies conducted among homogeneous BC populations in terms of subtypes are needed to better delineate the prognostic relevance of AR positivity in early-stage BC. Ideally, analyses should be performed within the context of prospective randomized clinical trials, where the above mentioned conditions are fulfilled, along with homogeneous treatment approaches, along with high-level clinical annotation in terms of clinical outcomes. It is important to have a clear definition of what is considered as AR positivity in such studies, since as mentioned before, different cut-offs were used in the studies we included in this meta-analysis.

We also investigated the potential impact of AR expression in pCR rates among all and different BC subtypes. Only one study, using prospectively collected samples from the GeparTrio trial, in which AR expression was evaluated by IHC on a TMA of 673 core biopsies, evaluated the predictive value of AR for pCR after neoadjuvant chemotherapy in patients.
with primary BC (44). This study found that AR+ tumors had lower rates of pCR in comparison with AR- tumors, 12.8% vs 25.4% respectively p<0.0001, but AR+ tumors had significantly better DFS and OS including those not reaching pCR (44). Moreover, AR, ER and HER2 were independent predictors for pCR in a multivariate model (44). When pCR was analysed across different IHC-assessed BC subtypes, it was found for all subgroups that AR+ tumors had the lower rate of pCR, although the differences were not significant. Hormone receptor positivity is known to be one of the strongest negative predictive biomarkers for pCR after neoadjuvant chemotherapy (61). Indeed, we did confirm that AR positivity was associated with lower pCR rate, but only in univariate analysis.

Finally, we looked for correlations among AR mRNA and other genes and GS of interest; it must be noted that none of these genes or GS is validated and they are therefore not routinely assessed in clinical practice. As expected and in accordance with clinical data AR expression was highly correlated with expression of ESR1. Positive correlations were also found with expression of PIK3CA, recently published as possible target in combination with antiandrogens in AR+ TN BCs (62). Of note, there is an ongoing phase 1/2 trial assessing the combination of taselisib, an alpha-selective PI3K inhibitor, with enzalutamide, in the setting of pre-treated metastatic AR-positive TNBC, with AR positivity defined as AR≥10% (NCT02457910 https://clinicaltrials.gov/ct2/show/NCT02457910, accessed on July 15, 2016.). Other positive correlations of interest were with HER3 and WNT7B, for whom molecular basis for correlation was published by Li at al. (59) and with BRCA1 which requires further investigation.
Recently, a systematic review and meta-analysis of AR prognostic role in early BC has been published (63). Vera-Badillo and co-authors have analysed 19 studies with 7,693 early BC patients, providing data for the pooled analysis evaluating two end points, DFS and OS at 3 and 5 years and AR expression defined by IHC. Of note, out of the 19 and 22 studies included in Vera-Badillo et al and our meta-analysis, 13 studies show overlap between both studies, encompassing 6,015 patients; this corresponds approximately to 60% of the total number of patients included in our meta-analysis. Their results are in line with ours, as they also showed that AR positivity confers better DFS and OS in all early BC patients. AR was a good prognostic factor within ER- patients, contrasting our results, in which ER- patients tended to have worse OS, without reaching statistical significance. Differently, for the ER-subgroup analysis they include six studies (8, 39, 40, 44, 53) in comparison with four (40, 48, 53) in our analysis. One of the explanations could be more restricted criteria for inclusion in our analysis as we excluded from the analysis studies with significant heterogeneity. Of note, currently there is an ongoing program under the umbrella of an international consortium focusing on male breast cancer, the International Male Breast Cancer Program. Both, data and biosamples from 1473 patients have been collected, with the intention being to delineate the clinical relevance of several biomarkers including AR expression, assessed through IHC (64).

Our analysis has some limitations. The studies included in the clinical meta-analysis suffer from methodological issues, such as different populations of patients, different methods and cut-offs used for determine AR positivity, small numbers of patients, retrospective nature of the studies and intergroup heterogeneity. The identification of
Title: The prognostic role of androgen receptor in patients with early stage breast cancer: A meta-analysis of clinical and gene expression data

sources of heterogeneity among different subgroups is not possible, due to lack of published data characterizing them. Additionally, this is a literature-based meta-analytical study without individual patient data, thus limiting our ability to address certain issues, such as interaction of AR expression status with other prognostic factors. Regarding gene expression analysis, we used data from publicly available data sets, which are not without limitations either. Such databases could be biased towards highly-expressed genes.

In conclusion, our study shows that AR expression at both protein and mRNA levels serves as positive prognosticator among more than 17,000 women with early stage BC. Additional information about distinct prognostic relevance of AR expression in different BC subtypes was provided, however further studies are warranted to confirm these findings. It is apparent that androgens and AR have many effects on the biology of BC and deserve more clinical and translational research attention. Currently, AR is being actively investigated as therapeutic target in BC.

REFERENCES
Title: The prognostic role of androgen receptor in patients with early stage breast cancer: A meta-analysis of clinical and gene expression data

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FIGURES LEGEND

I Clinical Meta-analysis

Figure 1A: Forest plots for Disease free survival (DFS) by AR protein expression

Figure 1B: Forest plots for Overall survival (OS) by AR protein expression

II Gene expression analysis

Figure 2: Forest plots of Univariate and Multivariate analysis of AR mRNA expression and pCR in all and in BC subtypes

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Table 2a: Univariate and multivariate analysis of AR prognostic role in all breast cancer (BC) patients

Table 2b: Univariate analysis of AR prognostic role in breast cancer (BC) subtypes and multivariate analysis in ER+ subtype

II Gene expression analysis

Table 3: Correlation of AR level with tumors’ and patients’ characteristics in all and in BC subtypes

Table 4: Disease free survival (DFS) analysis and Overall survival analysis according to AR mRNA expression levels in all BC patients
Title: The prognostic role of androgen receptor in patients with early stage breast cancer: A meta-analysis of clinical and gene expression data

Table 1: Eligible clinical studies for clinical meta-analysis and their characteristics

<table>
<thead>
<tr>
<th>First author</th>
<th>Type of study</th>
<th>N AR+ (total)</th>
<th>Method of AR assessment</th>
<th>Antibody used</th>
<th>AR threshold for positivity (chosen by)</th>
<th>RS</th>
<th>ER threshold for positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collett K. (46) 1996</td>
<td>retrospective analysis</td>
<td>137/269</td>
<td>Dextran-coated charcoal technique</td>
<td>10nM3 H-labelled methyltrienolone R-1881</td>
<td>43 fmol/mg Median value</td>
<td>12</td>
<td>&gt;15 fmol/mg</td>
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<td>Agoff A. (6), 2003</td>
<td>retrospective analysis of random cases</td>
<td>51/88</td>
<td>IHC</td>
<td>F39.4.1</td>
<td>&gt;5% arbitrary</td>
<td>13</td>
<td>&gt;5% of cells</td>
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<td>Rahka EA. (41), 2006</td>
<td>retrospective analysis of consecutive cases</td>
<td>36/282</td>
<td>IHC on TMA</td>
<td>F39.4.1</td>
<td>≥1% arbitrary/ literature based</td>
<td>16</td>
<td>≥1% of cells</td>
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<tr>
<td>Soiland H. (42), 2008</td>
<td>retrospective analysis</td>
<td>174/347</td>
<td>RPPL Microarray</td>
<td>Primary AR antibody - Epitomics</td>
<td>&lt; -0.0852 dichotomized by mean</td>
<td>15</td>
<td>not reported</td>
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<td>Gonzalez-Angulo AM. (8) 2009</td>
<td>retrospective analysis of selected cases/ frozen</td>
<td>116/215</td>
<td>IHC on TMA</td>
<td>AR – U407 antiserum</td>
<td>median % of positive cells 75% (range 0 – 96%)</td>
<td>6</td>
<td>≥10% of cells</td>
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<td>Loibl S., 2010</td>
<td>retrospective analysis of consecutive cases</td>
<td>609/859</td>
<td>IHC on TMA</td>
<td>AR441</td>
<td>≥ 1% literature based</td>
<td>26</td>
<td>≥1% of cells</td>
</tr>
<tr>
<td>Luo X. (39) 2010</td>
<td>retrospective analysis</td>
<td>148/269</td>
<td>IHC</td>
<td>ZYMED PV-6000-G Kit AR antibody not specified</td>
<td>≥ 2 (intensity %) arbitrary</td>
<td>13</td>
<td>not reported</td>
</tr>
<tr>
<td>Micello D. (7) 2010</td>
<td>retrospective analysis of consecutive cases</td>
<td>128/226</td>
<td>IHC</td>
<td>AR27</td>
<td>&gt;10% literature based</td>
<td>16</td>
<td>≥10% of cells</td>
</tr>
<tr>
<td>Peters A. (40) 2009</td>
<td>retrospective analysis of consecutive cases</td>
<td>358/673</td>
<td>IHC on TMA</td>
<td>F39.4.1</td>
<td>&gt;3 (% x intensity)</td>
<td>25</td>
<td>≥10% of cells</td>
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**Title:** The prognostic role of androgen receptor in patients with early stage breast cancer: A meta-analysis of clinical and gene expression data

<table>
<thead>
<tr>
<th>Ref</th>
<th>Year</th>
<th>Study Type</th>
<th>Tissue Collection Method</th>
<th>Method</th>
<th>AR Score</th>
<th>Remmle Score</th>
<th>Data Source</th>
</tr>
</thead>
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<td>(44)</td>
<td>2010</td>
<td>Prospective</td>
<td>541/391</td>
<td>IHC on TMA</td>
<td>AR 441</td>
<td>≥ 10% arbitrary</td>
<td>≥10% of cells</td>
</tr>
<tr>
<td>Park S. (11)</td>
<td>2011</td>
<td>Retrospective</td>
<td>237/327</td>
<td>IHC</td>
<td>AR441</td>
<td>&gt;1%</td>
<td>&gt;1% of cells</td>
</tr>
<tr>
<td>Yu Q. (43)</td>
<td>2011</td>
<td>Retrospective</td>
<td>41/73</td>
<td>IHC on TMA</td>
<td>antiAR (Biocare Medical)</td>
<td>≥1% not reported</td>
<td>not reported</td>
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<tr>
<td>Peters KM. (54)</td>
<td>2012</td>
<td>Retrospective</td>
<td>1154/467</td>
<td>IHC on TMA</td>
<td>AR441</td>
<td>&gt; 1% (1-10%+&gt;10%) arbitrary</td>
<td>not reported</td>
</tr>
<tr>
<td>He J. (47)</td>
<td>2012</td>
<td>Retrospective</td>
<td>73/287</td>
<td>IHC on TMA</td>
<td>AR 441 Dako</td>
<td>≥5% nuclear staining arbitrarily</td>
<td>not reported</td>
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<tr>
<td>Honma N. (48)</td>
<td>2013</td>
<td>Retrospective</td>
<td>212/403</td>
<td>IHC on FFPE</td>
<td>AntiAR Ab clone AR27; Novocastra Laboratories</td>
<td>≥10% nuclear staining literature based</td>
<td>≥10% of cells</td>
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<tr>
<td>Witzel I. (52)</td>
<td>2013</td>
<td>Retrospective</td>
<td>Cohort 1 126/165</td>
<td>AR mRNA</td>
<td>Affymetrix HG-U 133A GeneChip System</td>
<td>Expression value of AR mRNA higher than 75%</td>
<td>not reported</td>
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<tr>
<td>Tokunaga E. (50)</td>
<td>2013</td>
<td>Retrospective</td>
<td>155/250</td>
<td>IHC</td>
<td>AR441; Dako</td>
<td>75% nuclear staining literature based</td>
<td>≥1% of cells</td>
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<tr>
<td>Takeshita T. (65)</td>
<td>2013</td>
<td>Retrospective</td>
<td>295/379</td>
<td>IHC</td>
<td>AR318 Leica Biosystems Newcastle UK</td>
<td>Histoscore&gt;10 literature based</td>
<td>≥1% of cells</td>
</tr>
<tr>
<td>Thike AA. (49)</td>
<td>2013</td>
<td>Retrospective</td>
<td>267/699</td>
<td>IHC on TMA</td>
<td>AR 27 NCL-AR-318</td>
<td>≥1% nuclear staining literature based</td>
<td>≥1% of cells</td>
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</table>
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<table>
<thead>
<tr>
<th>Year</th>
<th>Study Description</th>
<th>Cases</th>
<th>IHC Method</th>
<th>AR Detection</th>
<th>Staining Method</th>
<th>Score</th>
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<td>2013</td>
<td>Tsang J.(51)</td>
<td>549/1144</td>
<td>IHC on TMA</td>
<td>AR 441 Dako</td>
<td>≥1% nuclear staining literature based</td>
<td>12</td>
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<tr>
<td></td>
<td>retrospective analysis of consecutive histologic files of 3 Institutions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>Pistelli M.(45)</td>
<td>15/81</td>
<td>IHC</td>
<td>F39.4.1 Biogenex San Ramon, CA, USA</td>
<td>≥10% nuclear staining arbitrarily</td>
<td>18</td>
</tr>
</tbody>
</table>

AR-androgen receptor; IHC-immunohistochemistry; ARc — cytoplasmic androgen receptor; ARn — nuclear androgen receptor; charc-AR — charcoal androgen receptor; WS — Whole Section; TMA — Tissue Microarray; ROC curve — Receiver Operating Characteristics curve; RPPL — Reverse Phase ProteinLysatemicroarray. RS — REMARK score(15) - reporting recommendations for tumor marker prognostic studies, please refer to Table 1_supplementa
Title: The prognostic role of androgen receptor in patients with early stage breast cancer:
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Table 2a: Univariate and multivariate analyses of AR prognostic role in all breast cancer (BC) patients

<table>
<thead>
<tr>
<th>Analysis</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UNIVARIATE Analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFS (21 studies, N=8036)</td>
<td>0.61</td>
<td>0.52-0.72</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity p=0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS (18 studies, N=8301)</td>
<td>0.62</td>
<td>0.51-0.75</td>
<td></td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity p=0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MULTIVARIATE Analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFS (13 studies, N=5648)</td>
<td>0.46</td>
<td>0.37-0.58</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity p=0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS (8 studies, N=5773)</td>
<td>0.53</td>
<td>0.38-0.73</td>
<td></td>
</tr>
<tr>
<td>Random effect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity p&lt;0.001</td>
<td></td>
<td></td>
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</table>
### Table 2b: Univariate analysis of AR prognostic role in breast cancer (BC) subtypes and multivariate analysis in ER+ subtype

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<tr>
<td><strong>UNIVARIATE analysis</strong></td>
<td><strong>HR</strong></td>
<td><strong>95% CI</strong></td>
<td><strong>p value</strong></td>
<td></td>
<td><strong>MULTIVARIATE analysis</strong></td>
<td><strong>HR</strong></td>
<td><strong>95% CI</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ER+</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFS (9 studies, N=2843) fixed effects, Heterogeneity p=0.53</td>
<td>0.53</td>
<td>0.44-0.63</td>
<td><strong>p&lt;0.001</strong></td>
<td>DFS (5 studies, N=1571) fixed effects, Heterogeneity p=0.65</td>
<td>0.40</td>
<td>0.31-0.52</td>
<td><strong>p&lt;0.001</strong></td>
</tr>
<tr>
<td>OS (6 studies, N=3383) fixed effects, Heterogeneity p=0.26</td>
<td>0.59</td>
<td>0.49-0.72</td>
<td><strong>p&lt;0.001</strong></td>
<td>OS (3 studies, N=2180) random effects, Heterogeneity p=0.003</td>
<td>0.37</td>
<td>0.16-0.85</td>
<td><strong>p=0.02</strong></td>
</tr>
<tr>
<td><strong>ER-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFS (3 studies, N=383) random effects, Heterogeneity p=0.05</td>
<td>0.33</td>
<td>0.04-2.44</td>
<td><strong>p=0.28</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>OS (4 studies, N=687) fixed effects, Heterogeneity p=0.11</td>
<td>1.32</td>
<td>0.98-1.80</td>
<td><strong>p=0.08</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>HER2+/ER-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFS (4 studies, N=410) fixed effects, Heterogeneity p=0.99</td>
<td>1.20</td>
<td>0.86-1.69</td>
<td><strong>p=0.28</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS (3 studies, N=358) fixed effects,</td>
<td>1.50</td>
<td>1.01-2.22</td>
<td><strong>p=0.04</strong></td>
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</tr>
</tbody>
</table>
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|                  |                          |            |            |                |            |            |            |
|------------------|--------------------------|------------|------------|----------------|------------|------------|
|                  |                          | TN         | DFS (9 studies, N=1373) | fixed effects, | Heterogeneity p=0.16 | 0.64 | 0.51 - 0.81 | p<0.001 |
|                  |                          |            | OS (5 studies, N=1285)  | fixed effects, | Heterogeneity p=0.07 | 0.64 | 0.49 - 0.88 | p<0.001 |

Heterogeneity p=0.50

Heterogeneity p=0.16

Heterogeneity p=0.07
Table 3: Correlation of AR mRNA with tumors’ and patients’ characteristics in all and in BC subtypes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ALL</th>
<th>LUMINAL A</th>
<th>LUMINAL B</th>
<th>HER2 enriched</th>
<th>BASAL like</th>
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<tbody>
<tr>
<td></td>
<td>p value</td>
<td>cor</td>
<td>Wilcox p value</td>
<td>Kw p value</td>
<td>p value</td>
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<td>AGE</td>
<td>0</td>
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<td>NA</td>
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<td>AGE CAT</td>
<td>NA</td>
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<td>&lt;0.001</td>
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<td>TU SIZE CAT</td>
<td>0.03</td>
<td>-0.03</td>
<td>NA</td>
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<td>&lt;0.001</td>
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<td>NA</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ERBB2 bimod</td>
<td>NA</td>
<td>NA</td>
<td>&lt;0.001</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Wilcoxon – Wilcoxon test; KW – Kruskal Wallis test; cor – correlation; age cat – age category: <50 vs ≥50 years, TU size cat – tumor size category: <2 vs 2-5cm vs >5cm; ESR1 – estrogen gene level, ERBB2 – erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
Table 4: Disease free survival (DFS) and overall survival (OS) analysis according to AR mRNA expression levels in all BC patients

<table>
<thead>
<tr>
<th>ALL patients N° of patients</th>
<th>Treatment</th>
<th>DFS HR Uni</th>
<th>95%CI</th>
<th>p value</th>
<th>DFS HR Multi</th>
<th>95%CI</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>3190 treated_and_not_treated</td>
<td>0.82</td>
<td>0.72-0.92</td>
<td>0.0007</td>
<td></td>
<td>0.97</td>
<td>0.84-1.13</td>
<td>0.72</td>
</tr>
<tr>
<td>1219 untreated</td>
<td>0.89</td>
<td>0.72-1.09</td>
<td>0.26</td>
<td></td>
<td>0.90</td>
<td>0.69-1.19</td>
<td>0.49</td>
</tr>
<tr>
<td>1268 HT_only</td>
<td>0.91</td>
<td>0.71-1.18</td>
<td>0.12</td>
<td></td>
<td>0.99</td>
<td>0.76-1.32</td>
<td>0.99</td>
</tr>
<tr>
<td>463 CT_only</td>
<td>0.98</td>
<td>0.76-1.28</td>
<td>0.91</td>
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<td>0.87</td>
<td>0.61-1.23</td>
<td>0.43</td>
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<td>240 HT_and_CT</td>
<td>0.82</td>
<td>0.52-1.29</td>
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<td>1.05</td>
<td>0.65-1.71</td>
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Figure 1: Forest plots for A) Disease free survival (DFS) and B) Overall survival (OS) by AR protein expression
Figure 2: Forest plots for Univariate and Multivariate analysis of AR mRNA expression and pCR in all and in BC subtypes

A) Univariate analysis

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B) Multivariate analysis

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Clinical Cancer Research

The prognostic role of androgen receptor in patients with early stage breast cancer: A meta-analysis of clinical and gene expression data

Ivana Bozovic-Spasojevic, Dimitrios Zardavas, Sylvain Brohée, et al.

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