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Cytoplasmic Mineralocorticoid Receptor Expression Predicts Dismal Local Relapse-free Survival in Non-triple-negative Breast Cancer

ANNIINA JÄÄSKELÄINEN[1,2], ARJA JUKKOLA[3], KIRSI-MARIA HAAPASAARI[2], PÄIVI AUVINEN[4], YLERMI SOINI[2,5] and PEETER KARIHTALA[1]

[1]*Department of Oncology and Radiotherapy, Medical Research Center Oulu, Oulu University Hospital and University of Oulu, Oulu, Finland;*
[2]*Department of Pathology, Medical Research Center, Oulu University Hospital, Oulu, Finland;*
[3]*Department of Oncology, Tampere University Hospital, Faculty of Medicine and Health Technology, University of Tampere, Tampere, Finland;*
[4]*Department of Oncology and Cancer Center, Kuopio University Hospital, and Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland;*
[5]*Department of Pathology, Kuopio University Hospital, University of Eastern Finland, Kuopio, Finland*

Abstract. *Background/Aim: The aim of the study was to investigate the prognostic role of androgen receptor (AR), mineralocorticoid receptor (MR) and glucocorticoid receptor β (GRβ) expression in HER-2 negative breast cancer patients. Materials and Methods: The study population (n=152) was enriched with triple-negative breast cancers (TNBC) (n=96; 63.2%). The median follow-up time was 100 months. AR, MR and GRβ immunocytochemical staining was compared with that of epithelial-mesenchymal transition (EMT) markers (vimentin, SIP1, ZEB1). Results: High expression of cytoplasmic MR was associated with dismal local relapse-free survival (RR=13.923; 95%CI=1.071-181.045; p=0.044) in tumours with non-TNBC phenotype. AR and GRβ were more frequently expressed in ER+/PR+/HER2- tumours, while cytoplasmic MR was more often expressed in TNBC tumours (for all, p<0.0005). GRβ and AR were associated with decreased vimentin expression (p<0.005), indicating their association with attenuated EMT. Conclusion: Cytoplasmic MR expression is a strong predictor of local recurrence in non-metastatic breast cancer patients with non-TNBC tumour phenotype.*

Correspondence to: Peeter Karihtala, MD, Ph.D., Department of Oncology and Radiotherapy, Medical Research Center, Oulu University Hospital and University of Oulu, P.O. Box 22, 90029 Oulu, Finland. Tel: +35 883152011, e-mail: peeter.karihtala@oulu.fi, ORCID ID 0000-0003-3490-3702

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The expression of oestrogen (ER) and progesterone receptors (PR) and amplification of human epidermal growth factor receptor 2 (HER2) are important prognostic and predictive factors in breast cancer (1). Moreover, androgen receptor (AR), mineralocorticoid receptor (MR) and glucocorticoid receptor β (GR β) belong to the nuclear receptor superfamily of transcription factors (2, 3). AR is expressed in 70-90% of all breast cancers, with a varying expression being reported in TNBC (4-7). The impact of AR in the pathogenesis of TNBC appears to be substantial and AR-positive TNBC forms a unique luminal androgen receptor (LAR) subtype. In relation to breast cancer, less is known about the two other steroid nuclear receptors (SNRs): MR and GRβ.

MR, being a part of the renin-angiotensin-aldosterone-system (RAAS), regulates the physiological sodium and potassium balance and water excretion leading to blood pressure control. It is expressed in both epithelial and non-epithelial tissues, where it binds mainly aldosterone, but also in other mineralocorticoids or glucocorticoids (GC) depending on the expression of 11β-hydroxysteroid dehydrogenase 2 (8, 9). MR is localized in the cytosol in its inactive state, but its expression between the nuclear and the cytoplasmic localization varies, depending on ligands and other factors (10, 11). Considering the newer suggestions for DNA-binding sites, MR and GR both bind glucocorticoid receptor element (GRE) for regulating of gene expression (12, 13). There is also a significant cross-talk between glucocorticoids, mineralocorticoids and PR that has been suspected to be

important in breast pathology (12). MR expression has been reported in certain breast cancer cell lines and some evidence suggests that it may be required for focal adhesion and ductal differentiation of breast cells (12, 14).

The GR has five isoforms, out of which, GRα and GRβ are the most well-known (15, 16). GRβ contains 742 amino acids of which the first 727 amino acids from the N-terminus are identical with GRα (15). The physiologically predominant form, GRα, is mainly responsible for the classical GC-mediated effects, such as the control of stress-related homeostasis, proper organ function, and the immune/inflammatory response (17, 18). The studies about breast cancer and the GR have mainly focused on GRα or they have not specified the GR isoform, yet new evidence on the role of GRβ in breast cancer biology is emerging (19-23). GRβ does not bind GCs and its transcriptional activity is indirect and suppressive compared to GRα (24). It is expressed ubiquitously, both in the cytoplasm and nuclei of human cells (25, 26).

Epithelial-mesenchymal transition (EMT) is physiologically active during wound healing and embryonic development, but it also promotes breast cancer invasion and metastasis (27). EMT can be indirectly assessed by evaluating the expression of proteins such as Zinc finger E-box-binding protein 1 (ZEB1), Smad Interacting Protein 1 (SIP1) and vimentin. ZEB1 and SIP1 are EMT-promoting transcription factors with zinc finger domains in their molecular structure (28-30). Vimentin is a cytoskeleton protein participating in the migration of epithelial cells and is also an indicator of ongoing EMT (31). EMT marker expression also notably differs in TNBC and non-TNBC (32).

Although AR expression is well characterized in both ER-positive and ER-negative breast cancers, the role of MR and GRβ expressions in breast cancer and carcinogenesis are less well-known. In the light of current evidence, the relationship between SNR expression and EMT is significant for breast cancer biology (33). This is the first time that MR and GRβ expression have been studied in breast cancer patients. We also connected in our data AR, MR and GRβ to EMT immunostainings, which were previously assessed. We enriched the cohort with TNBCs to evaluate more specifically the cross-talk between ER/PR and other steroid hormone receptors (33, 34). Based on *in vitro* models, we hypothesized that the biological and prognostic significance of steroid hormone receptors could differ in TNBC compared with non-TNBC.

Materials and Methods

Patients. The study population consisted of 152 breast cancer patients from Oulu and Kuopio University Hospitals (Table I). The patient data was retrospectively collected from the archives of Oulu and Kuopio University Hospitals based on the diagnosis and availability of tissue. Median follow-up period was 100.0 months (mean 95.8 months). Tumours and patients were assessed using histopathological classification and TNM classification according to the WHO (35, 36). Triple-negative breast carcinomas were defined

Table I. *Patient characteristics*.

	N (%)
T class	152 (100%)
T1	58 (38.2%)
T2	84 (55.3%)
T3	7 (4.6%)
T4	3 (2.0%)
N class	152 (100%)
N0	79 (52.0%)
N1	53 (34.9%)
N2	14 (9.2%)
N3	6 (3.9%)
M class	152 (100%)
M0	152 (100%)
M1	0 (0%)
Breast cancer type	152 (100%)
TNBC	96 (63.2%)
Non-TNBC	56 (36.8%)
Histopathology	152 (100%)
Ductal	136 (89.5%)
Lobular	2 (1.3%)
Medullary	9 (5.9%)
Tubular	1 (0.7%)
Other	4 (2.6%)
Histopathological grade	152 (100%)
Grade 1	4 (2.6%)
Grade 2	26 (17.1%)
Grade 3	122 (80.3%)
ER status	152 (100%)
Negative (<9%)	96 (63.2%)
Weak (10-29%)	5 (3.3%)
Moderate (30-59%)	8 (5.3%)
High (>59%)	43 (28.3%)
PR status	152 (100%)
Negative (<9%)	96 (63.2%)
Weak (10-29%)	11 (7.2%)
Moderate (30-59%)	11 (7.2%)
High (>59%)	34 (22.4%)
HER2 status	152 (100%)
HER2-positive (CISH)	0 (0%)
HER2-negative	152 (100%)
Ki67 status	152 (100%)
Negative (<5%)	6 (3.9%)
Weak (5-14%)	22 (14.5%)
Moderate (15-30%)	26 (17.1%)
High (>30%)	47 (30.9%)
Not available	51 (33.6%)
Local relapse	152 (100%)
No local relapse	140 (92.1%)
Local relapse	12 (7.9%)
The site of 1st distant metastasis	152 (100%)
No distant metastases	108 (92.1%)
Bone metastases	9 (5.9%)
Lung metastases	7 (4.6%)
Liver metastases	3 (2.0%)
Multiple metastases	19 (12.5%)
Other distant metastases	6 (4.0%)

as tumours with negative ER, PR and HER2 expression. Ninety-six (63.2%) of the cases were of TNBC phenotype and 56 (36.8%) of $ER^+/PR^+/HER2^-$ (non-TNBC) phenotype.

Immunohistochemistry for ER, PR, Ki-67 and HER2 gene amplification status. TNBC and non-TNBC subtypes were confirmed with immunohistochemistry (IHC) on surgically removed tumours at the Departments of Pathology at Oulu and Kuopio University Hospitals. The formalin-fixed specimens were embedded in paraffin blocks and stored at the Departments of Pathology in Oulu and Kuopio. The expression levels of nuclear ER, PR and Ki-67 were analysed as previously described (37). Tumours expressing nuclear ER or PR in more than 10% of malignant tumour cells were considered as ER/PR receptor-positive. If a specimen was found by IHC to be positive for membranous HER2 (1+ to 3+ on a scale of 0 to 3+), chromogenic *in situ* hybridization was used to determine HER2 gene amplification status. Specimens considered as HER2-positive showed six or more gene copies of HER2 in the cells (38).

Immunohistochemistry and AR, MR and GRβ expression assessment. Expression was recorded only in malignant cells. Cytoplasmic intensity was divided into four categories: 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive). The number of stained tumour cells was reported as percentage (0-100). For a linear comparison of the data, an H score, that is a histological sum score, was calculated for each sample by multiplying the intensity with the percentage (39). All samples received an H score between 0-300.

The staining for AR, MR and GRβ was performed at the Department of Pathology, Oulu University Hospital according to the laboratory's routine protocols. The paraffin-embedded tissue blocks were cut into sections of 3 μm, placed on microscope slides, and de-paraffinized in xylene for three times of three min. Then, sections were rehydrated using graded alcohol solutions and subsequently rinsed in distilled water.

For AR and MR, antigen retrieval was performed using Tris-EDTA buffer (Merck KGaA, Darmstadt, Germany; VWR international, Leuven, Belgium; respectively) (pH 9.0). The sections were heated in a microwave, first for two min (800 W) and then for 15 min (150 W). Then, the AR and MR slides were cooled at room temperature for 20 min and rinsed in distilled water and phosphate-buffered saline with TWEEN (PBS-TWEEN) (Fisher Scientific, Loughborough, UK; Merck KGaA, Darmstadt, Germany; respectively). The endogenous peroxidase was neutralized in all AR, MR and GRβ sections with a blocking solution (Dako S2023, Dako Denmark, Glostrup, Denmark); a five-minute incubation time was applied. For AR and MR, two PBS-TWEEN washes of five min were performed. The AR and MR slides were incubated for 30 min at room temperature with the monoclonal anti-AR (Leica Biosystems Novocastra NCL-AR-318, Newcastle, UK) or anti-MR (Invitrogen Mineralocorticoid Receptor antibody HIOE4C9F, Rockford IL, USA) antibodies (dilutions 1:50 for AR, 1:000 for MR). AR and MR slides were washed with PBS-TWEEN, incubated with the EnVision™ -polymer (DAKO K5007, Dako Denmark) for 30 min and washed again with PSB-TWEEN.

Regarding GRβ, after de-paraffinizing, a second blocking step was performed with bovine serum albumin (BSA; Sigma A-3059, Steinheim, Germany) and phosphate-buffered saline (PBS, Fisher Scientific, Loughborough, UK) solution for 30 min at room temperature. The BSA-PBS solution was trickled from the slides, and the primary antibody (Invitrogen GRβ PA3-514, Rockford IL, USA; dilution 1:1000) was applied. The sections were incubated for two hours at room temperature and left overnight to +4˚C. Then, the GRβ slides were returned to room temperature for two h, washed with PBS solution for ten min and a secondary antibody (Invitrogen biotinylated anti-rabbit IgG BA-1000, Vector Laboratories Inc., Burlingame, CA, USA) was applied. The sections were incubated for 30 min at room temperature. Following a ten-min wash with PBS, the ABC-complex (VECTASTAIN® ABC kit, Elite PK 6100 standard, Vector Laboratories Inc., Burlingame, CA, USA) diluted to 5 ml of 0.5 M NaCl-PBS buffer solution was added. The sections were incubated for 30 min at room temperature and washed with PBS for 10 min.

For AR, MR and GRβ the colour was developed by incubating for 5 min with diaminobenzidine working solution (DAKO K5007 Dako Denmark), after which a rinse with distilled water was performed. The staining was completed by counterstaining the tissue sections with Mayer's haematoxylin (Reagena, Toivala, Finland). Then, the sections were rinsed, dehydrated, cleared and mounted. The methods and results concerning EMT marker immunostaining and assessment in this cohort have been reported earlier (32).

Ethics approval. The local ethics committee of the Hospital District of Northern Ostrobothnia (144/2011, amendment 23.2.2015) and the Finnish National Supervisory Authority for Welfare and Health (1339/05.01.00.06/2009) approved the study design. Following approval from the ethics committee of the Hospital District of Northern Ostrobothnia, written informed consent was not obtained from the patients at the time of sample donation.

Statistical analysis. Statistical analysis was carried out by using IBM® SPSS® Statistics software, v. 25.0.0.0 (IBM Corporation, Armonk, NY, USA). The significance of associations was assessed by using the Mann–Whitney *U*-test. Correlations between AR, MR and GRβ expression were analysed by using the Spearman correlation test. Established prognostic factors were re-formatted as two-class variables for the analyses. T-class was divided into T1 or T2-4 classes, and nodal status to either N0 or N1-3. The histopathological grade was divided into either grade 1-2 or grade 3. Survival was analysed by using Kaplan–Meier curves with the log-rank test. The H-score median of AR, MR and GRβ expression was used as cut-off value in survival. For AR and GRβ expression the median was a division between positive and negative expressions, and for MR an H-score 15 was the median. Overall survival (OS) was calculated from the date of the operation to the time of death from any cause. Relapse-free survival (RFS) was calculated from the date of the operation to the date of the first confirmed local relapse, either ipsilateral or contralateral in axilla, scar or breast. Breast cancer-specific survival (BCSS) was calculated from the date of the operation to the time of death due to breast cancer. Distant disease-free survival (DDFS) was calculated from the date of the operation to the date of the first confirmed distant relapse. For multivariate analyses Cox multivariate regression analysis (co-variates T-class and N-class) was used. Probability of less than 0.05 was considered significant in all analyses.

Results

Expression pattern. Of 152 patients in total, nuclear AR expression was reliably assessed in 139 (91.4%) cases and cytoplasmic AR expression in 135 (88.8%) cases. AR

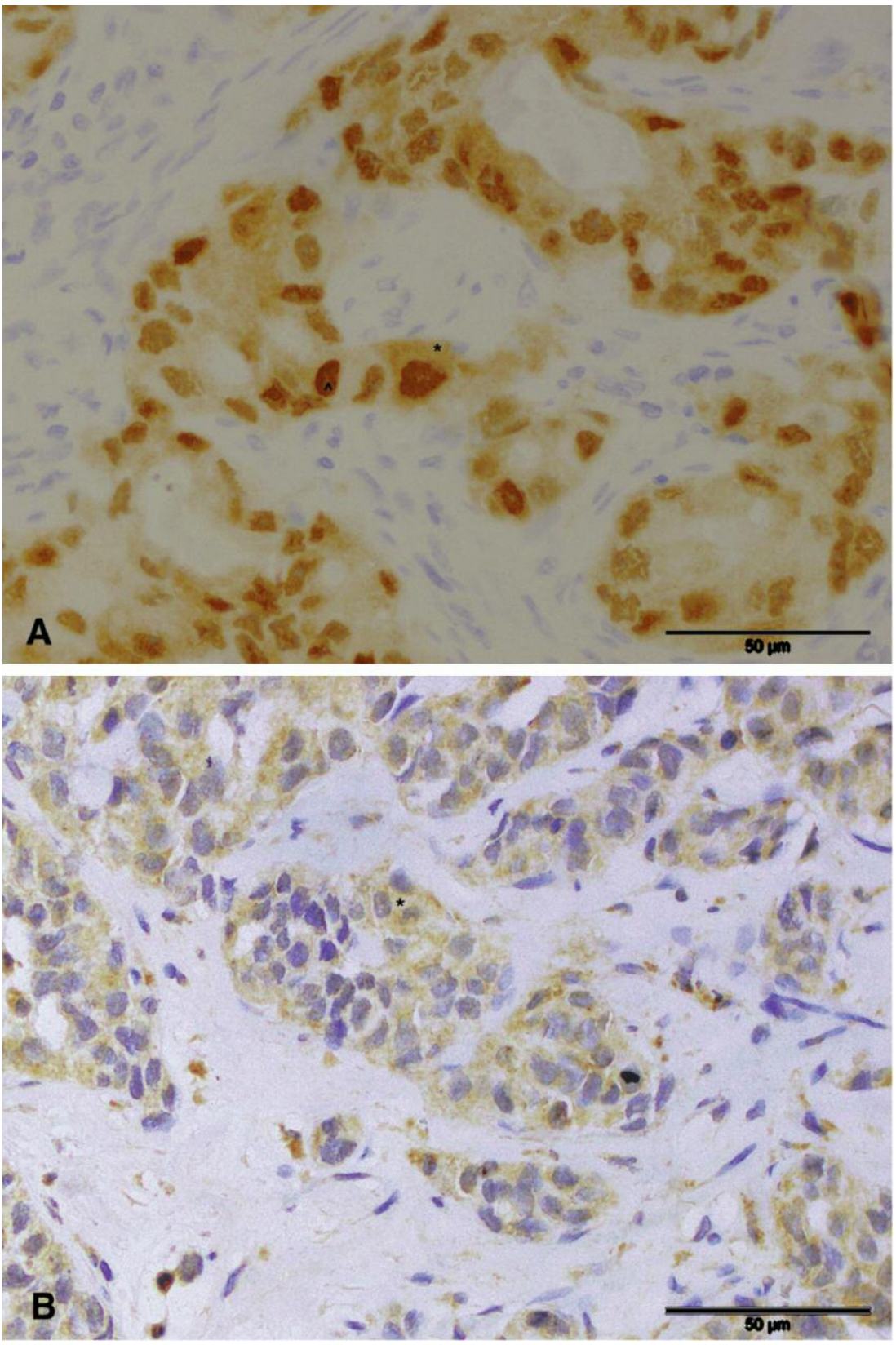


Figure 1. *Continued*

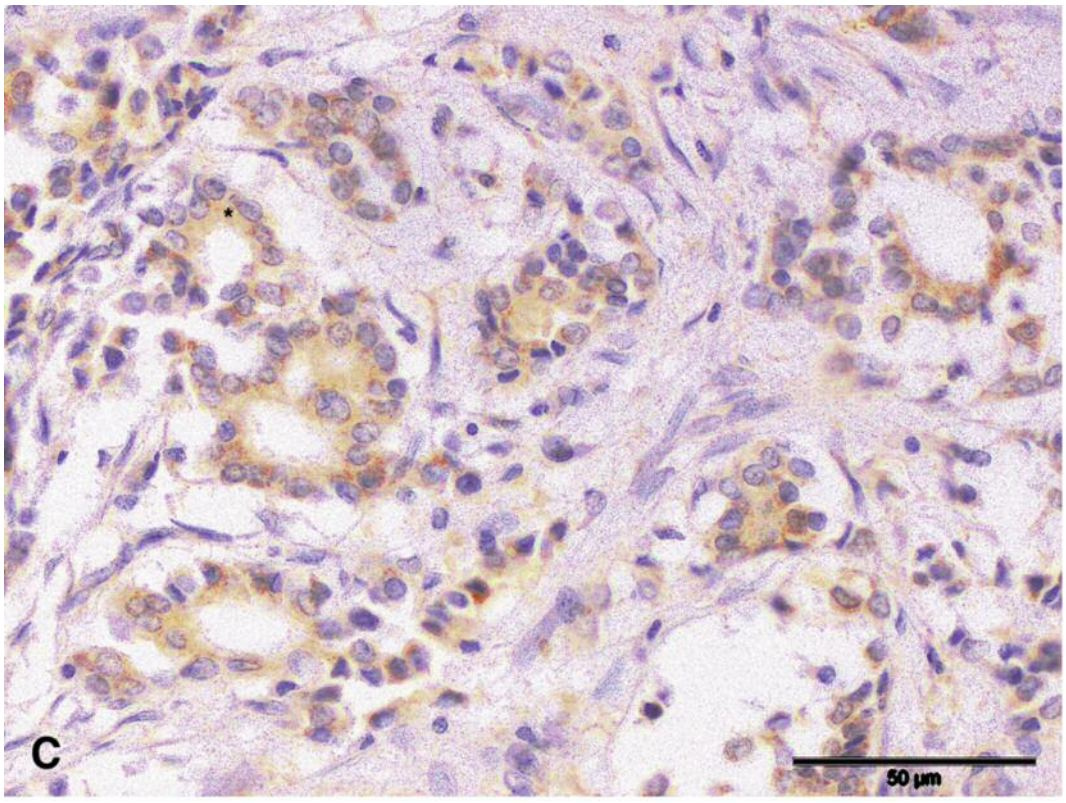


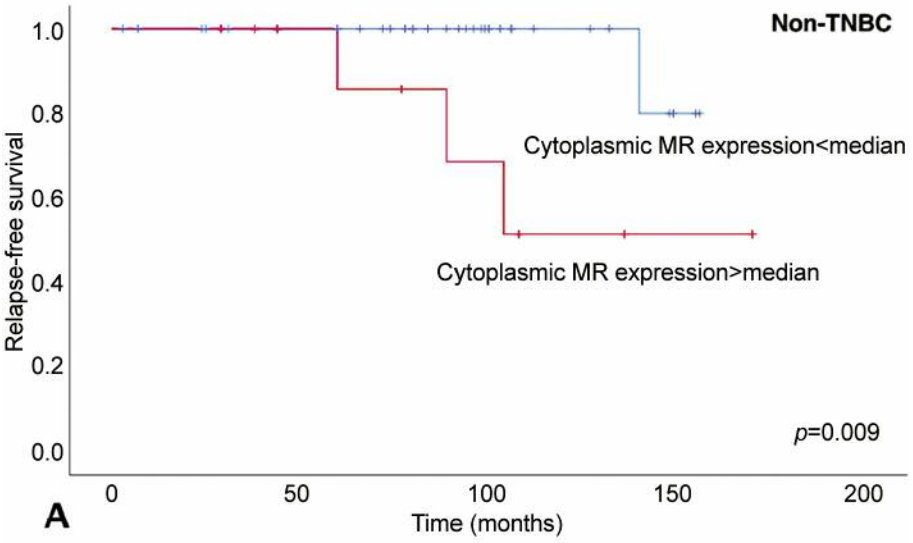
Figure 1. *A-C. Pattern of cytoplasmic and nuclear expression of AR. Positive immunostaining is brown. (A) Strong (3+) nuclear and a weak (1+) to moderate (2+) cytoplasmic expression, with cytoplasmic MR. (B) Moderate expression and cytoplasmic GRβ (C) Moderate expression. Cytoplasmic expression is marked with an asterisk (*) and nuclear expression with an arrow head (^).*

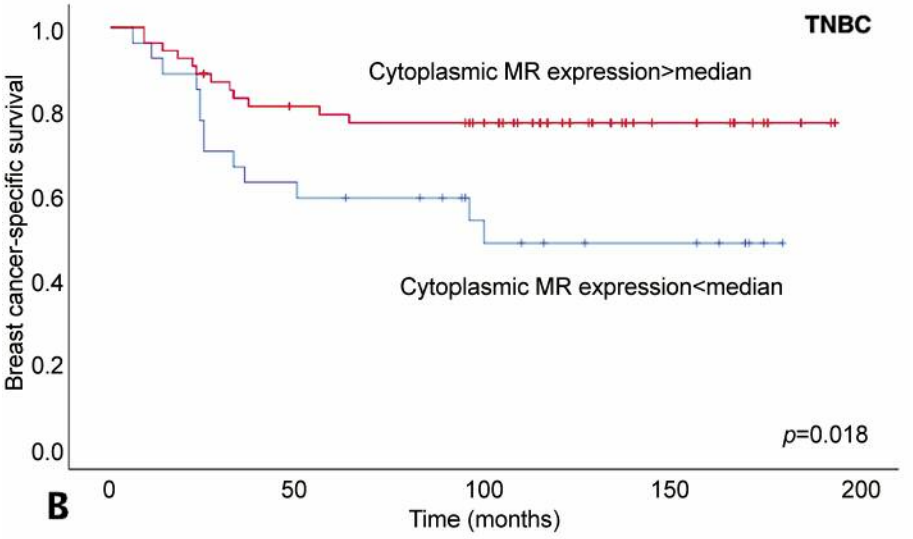
expression was mostly nuclear (97.8%); only 3 samples (2.2%) expressed positive cytoplasmic staining (Figure 1A). Nuclear MR expression was reliably assessed in 123 (80.9%) cases and cytoplasmic MR in 125 (82.2%) cases. Cytoplasmic MR expression was more frequently positive than nuclear expression (Figure 1B). Cytoplasmic GRβ expression was reliably assessed in 140 (92.1%) cases. There was no detectable nuclear GRβ staining in any of the samples (Figure 1C).

Correlations between AR, MR and GRβ expression. Nuclear AR expression was correlated positively with cytoplasmic GRβ expression ($p<0.0050$, correlation coefficient 0.33) when all patients were considered. No other significant correlations were observed. When the correlations between AR, MR and GRβ were also assessed separately in TNBC and non-TNBC subgroups, no significant associations could be detected.

AR, MR and GRβ expression and association with clinicopathological parameters. In the whole cohort, nuclear AR expression was associated with smaller tumour size ($p=0.029$) and better differentiation ($p=0.0010$), whereas cytoplasmic GRβ expression was associated with lower proliferation ($p=0.043$) and cytoplasmic MR expression was associated with node-negativity ($p=0.018$). When the traditional prognostic factors were assessed separately in the subgroups of TNBC and non-TNBC, a similar association was observed with a nuclear AR expression and smaller tumour size in the non-TNBC group ($p=0.024$). In the TNBC group, nuclear AR expression was associated with better differentiation ($p=0.0010$). A cytoplasmic MR expression was associated with node-negativity ($p=0.015$), better differentiation ($p=0.017$), and lesser proliferation ($p=0.033$) in TNBC. When all patients were considered, AR and GRβ were associated with the non-TNBC subgroup ($p<0.0010$ for both) while MR was associated with the TNBC phenotype ($p<0.0010$) (Table II).

AR, MR and GRβ expression and their association with EMT-markers. Nuclear AR and cytoplasmic GRβ expression were associated inversely with vimentin expression ($p<0.0010$) when all patients were considered (Table III). In the TNBC subgroup cytoplasmic MR expression was associated with





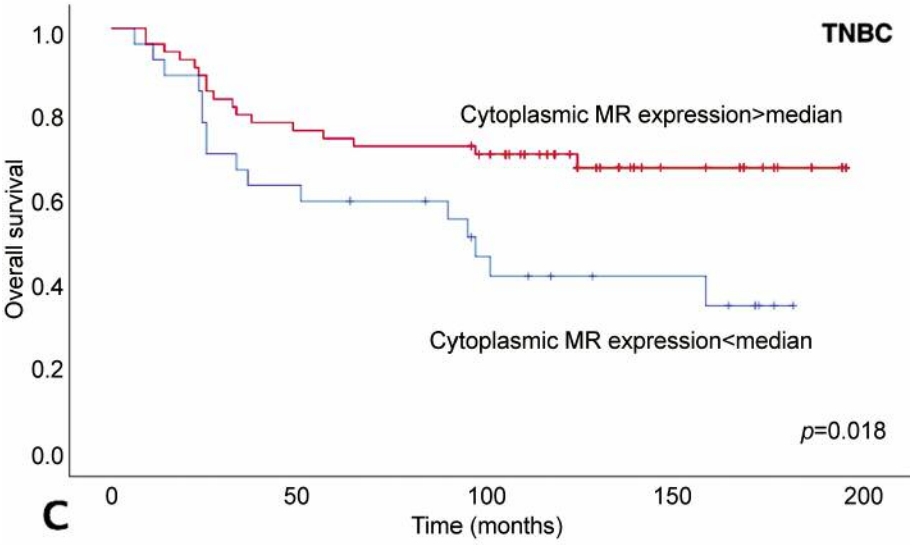


Figure 2. *Continued*

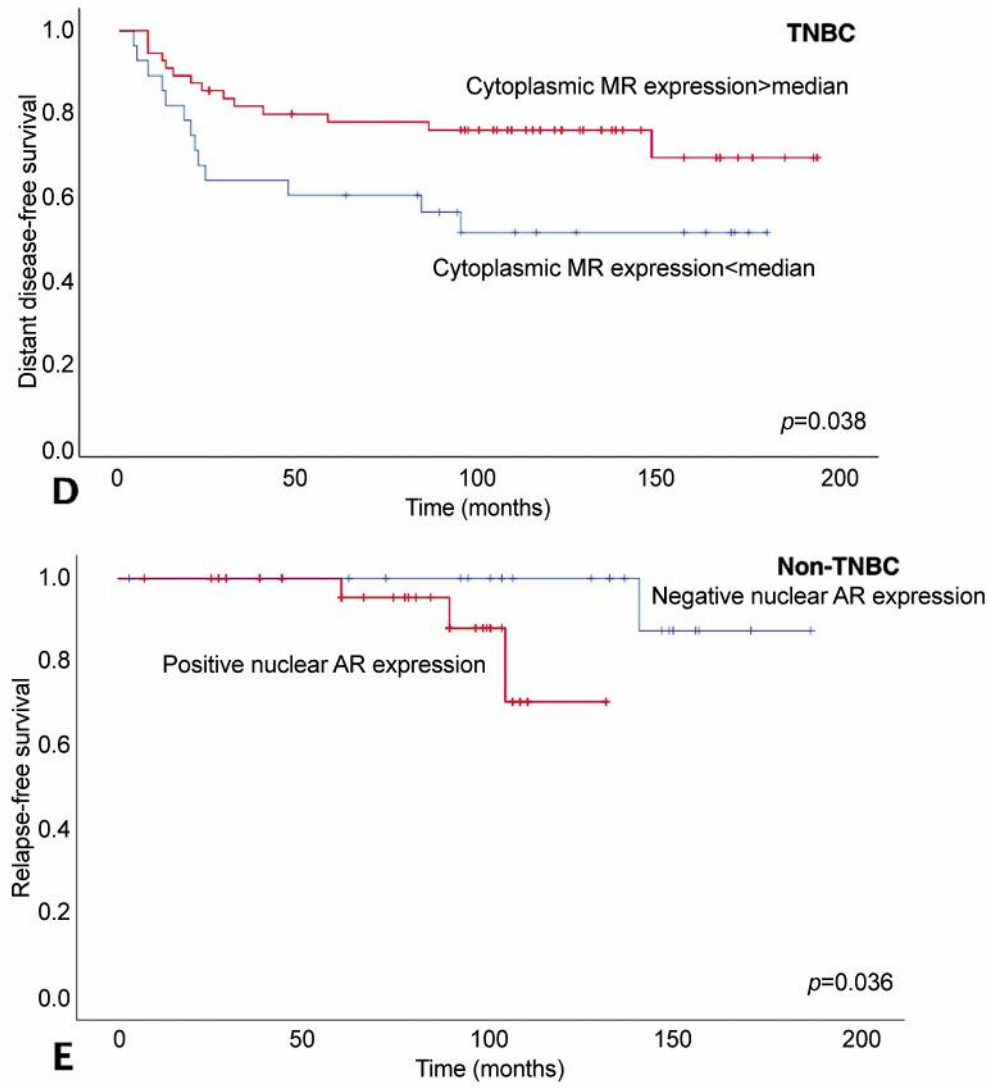


Figure 2. *Cytoplasmic MR expression predicts a dismal relapse-free survival in the non-TNBC subgroup (A). Cytoplasmic MR expression predicts a better breast cancer specific survival (B), overall survival (C) and distant disease-free survival (D) in the patients with TNBC tumour phenotype. Nuclear AR expression was associated with a dismal relapse-free survival in the non-TNBC subgroup (E).*

negative vimentin expression (p=0.038) and stronger SIP1 expression (p=0.036). Nuclear AR expression showed an inverse correlation with vimentin expression (p<0.0010) (Table IV). Cytoplasmic GRβ expression was associated with negative expression of vimentin (p=0.0020) in the non-TNBC subgroup (Table IV).

Survival analysis. An over-median cytoplasmic MR expression was associated with dismal RFS (in univariate analysis, p=0.009) in patients with non-TNBC phenotype (Figure 2A). This was confirmed in Cox regression analysis (RR=13.923; 95%CI=1.071-181.045; p=0.044), when T-class (RR=1.664; 95%CI=0.125-22.213; p=0.700) and nodal status (RR=4.834; 95%CI=0.196-119.113; p=0.335) were included in the analysis. On the other hand, in patients with TNBC phenotype an over-median cytoplasmic MR expression was associated with a better BCSS, DDFS and OS in univariate analysis (p=0.018, p=0.018 and p=0.038, respectively) although this could not be confirmed in multivariate analysis (Figures 2B, C, D). Nuclear AR expression was associated with dismal RFS (in univariate analysis, p=0.036) in non-TNBC phenotype (Figure 2E). This also was not confirmed in Cox regression analysis. GRβ expression did not associate with prognosis.

Table II. *The significant 2-sided p-values of associations between AR, MR and GRβ expression and traditional prognostic factors.*

	Whole cohort	TNBC subgroup	Non-TNBC subgroup
T (T1 *vs.* T2-4)			
AR nuclear	p=0.029 ↓	ns	p=0.024 ↓
MR nuclear	ns	ns	ns
MR cytoplasmic	ns	ns	ns
GRβ cytoplasmic	ns	ns	ns
N (N0 *vs.* N1-3)			
AR nuclear	ns	ns	ns
MR nuclear	p=0.018 ↓	ns	ns
MR cytoplasmic	ns	p=0.015 ↓	ns
GRβ cytoplasmic	ns	ns	ns
Grade (I-II *vs.* III)			
AR nuclear	p=0.001 ↓	p=0.001 ↓	ns
MR nuclear	ns	ns	ns
MR cytoplasmic	ns	p=0.017 ↓	ns
GRβ cytoplasmic	ns	ns	ns
Ki67 (0-14% *vs.* >14%)			
AR nuclear	ns	ns	ns
MR nuclear	ns	ns	ns
MR cytoplasmic	ns	p=0.033 ↓	ns
GRβ cytoplasmic	p=0.043 ↓	ns	ns

↓: Inverse association; ↑: direct association; ns: not statistically significant.

Table III. *The significant 2-sided p-values of associations between AR, MR and GRβ expression and EMT-markers in the whole patient cohort.*

	Vimentin	SIP1
AR nuclear	<0.0010 ↓	ns
MR nuclear	ns	ns
MR cytoplasmic	ns	ns
GRβ cytoplasmic	<0.0010 ↓	ns

↓: Inverse association; ↑: direct association; ns: not statistically significant.

Table IV. *The significant 2-sided p-values of associations between AR, MR and GRβ expression and EMT-markers in the TNBC subgroup.*

	Vimentin	SIP1
AR nuclear	<0.0010 ↓	ns
MR nuclear	ns	ns
MR cytoplasmic	0.038 ↓	0.036 ↑
GRβ cytoplasmic	ns	ns

↓: Inverse association; ↑: direct association; ns: not statistically significant.

Table V. *The significant 2-sided p-values of associations between AR, MR and GRβ expression and EMT-markers in the non-TNBC subgroup.*

	Vimentin	SIP1
AR nuclear	ns	ns
MR nuclear	ns	ns
MR cytoplasmic	ns	ns
GRβ cytoplasmic	0.022 ↓	ns

↓: Inverse association; ↑: direct association; ns: not statistically significant.

Discussion

According to our knowledge, this is the first study to evaluate MR and GRβ expression in breast cancer patients. The main result of the study was that cytoplasmic MR expression predicts worse RFS in patients with non-TNBC ($ER^+/PR^+/HER2^-$) phenotype tumours. Furthermore, our results support previous observations that SNRs interact with EMT regulation.

There is evidence indicating that MR plays a role in cancer progression and prognosis. MR expression appears to be a protective factor in both non-small cell lung cancer and colorectal cancer pathogenesis, and its expression is associated with prolonged OS in both cancers (40). In hepatocellular carcinoma MR expression has been associated with suppression of cell proliferation by metabolic modulation *in vitro* and also with better outcome, although only in univariate analysis (41).

The prognostic value of MR has not been previously assessed in breast cancer. Our results suggest that ER/PR expressions may have an influence on the prognostic role of MR in breast carcinomas. In $ER^+/PR^+/HER2^-$ cancers, high cytoplasmic MR expression predicted dismal outcome in terms of local relapses. Furthermore, multivariate analysis indicated that their prognostic value even exceeded that of tumour size and nodal status which are conventionally considered the most powerful factors. Along with genomic (nuclear) mechanisms, MR plays vital roles in regulating signalling pathways in the cytoplasm (11, 42). The exact mechanism of this rapid secondary messenger signaling or protein kinase activation cascades remains to be elucidated, but they may be related to the current results since nuclear MR has not previously shown to have any prognostic value. Conversely, in TNBCs. MR appeared to predict prolonged OS, BCSS and DDFS, which nevertheless could not be confirmed in multivariate analysis. In line with this, cytoplasmic MR expression was also associated with low

grade, slow proliferation and negative nodal status in the TNBC subgroup. MR expression was significantly more prominent in the patients with TNBC compared with the non-TNBC group.

In the current study, relapses located in ipsilateral or contralateral axilla, scar or breast were recorded as local relapses. MR may promote only local invasion in $ER^+/PR^+/HER2^-$ cancers or may even be linked to radiotherapy resistance. Due to very poor characterization of MR in cancers, and especially in breast cancer, there is no previous data to support interaction between ER and MR in breast carcinogenesis. However, it has been suspected that MR, like the other members of the nuclear superfamily of transcription factors, would have a role in the modulation of EMT, although this has not been thoroughly studied (12). In kidney cells, aldosterone exposure induces EMT *via* MR-mediated mitochondrial-originated, ROS-dependent ERK1/2 activation (33, 43, 44). Aldosterone also mimics PR-like effects in breast cancer *in vitro*, inducing focal adhesion and growth inhibition (12). We did not find any association with MR expression and any of EMT marker expressions in the non-TNBC group. In TNBC patients, higher MR expression was associated with increased vimentin, a cytoskeleton protein, which is considered a marker of EMT. This may partly explain the tendency of poorer survival and distant disease control in TNBC patients. The association of vimentin with ER negative phenotype in breast cancer has been reported decades ago (31). Another possible role for MR in cancer biology could be related to micromilieu and oxidative stress. Activated MR enhances inflammation and leads to reactive oxygen species generation by inducing NADPH oxidase (45). This could result in vascular remodeling *via* an enhanced expression of the multiple genes involved, suggesting that MR activation favors pathogenesis (46).

The role of GCs as well as of GR in cancer biology incorporates a wide range of cellular mechanisms (47). The physiologically dominant isoform, GRα, has an ER-independent prognostic role in breast cancer (23). GRβ has previously attracted only cursory attention in breast cancer literature, but its expression has been reported in ER-positive MCF-7 cells (48). Data from other malignancies suggest that GRβ enhances *in vitro* migration in bladder cancer cell lines, and the proliferation of glioma and prostate cancer cells (49-51). We found an inverse association between GRβ and the EMT-marker vimentin in $ER^+/PR^+/HER2^-$ tumours. In line with this, GRβ was associated with a slightly lower proliferation rate. According to our knowledge, GRβ protein expression has not been previously evaluated in any cancer.

AR is by far the best characterized SNR in breast cancer. In our study, nuclear AR expression was associated with worse RFS in non-TNBC phenotypes, although we could not confirm this association in multivariate analysis. Søiland et al have previously measured AR cytoplasmic expression with the dextran-coated charcoal method and reported that it predicts prolonged RFS in patients with high AR levels (52). Some meta-analyses have demonstrated longer disease-free survival (DFS) (but not overall survival) in AR-positive breast cancers (53, 54). We were unable to replicate this result in the current study, which is not very surprising since the effect of AR on survival in previous studies has been quite small and the size of our sample was quite limited. On the other hand, in our whole cohort, AR was associated with a smaller tumour size and lower tumour grade, confirming recent findings (55, 56). The possible therapeutic role of AR in breast cancer is currently under evaluation. Regarding EMT markers, we observed strong suppression in vimentin in patients with high AR expression and the TNBC phenotype. The role of AR and EMT is not explicitly clear, it is possible that dihydrotestosterone, an AR ligand, reduces E-cadherin, triggers EMT and promotes tumour metastasis (56). High AR expression, detected by immunohistochemistry, may be the result of high AR stability (57, 58). Finally, in line with previous literature, AR was prominently overexpressed in non-TNBC breast cancers (54).

Our study has some weaknesses. The prognostic role of MR expression needs to be viewed in proportion with the relatively small number of cases in the non-TNBC group, although the result was indeed confirmed in multivariate analysis. In the future, we would welcome a study which will combine immunostaining of GRα and GRβ to clarify the role of GRβ expression in breast cancer, since the biochemical pathways of GRα are quite well-known. Cytoplasmic MR expression may be a new prognostic marker for non-metastatic breast cancer, but additional research is needed to establish its role in breast cancer biology. In future studies, receptors, ligands and ligand-receptor interactions should be evaluated.

We conclude that cytoplasmic MR protein expression is tightly associated with worse RFS, independent of tumour size and nodal status, if the primary tumour has $ER^+/PR^+/HER2^-$ phenotype. This is likely to be mediated by a mechanism other than EMT. Whether poor local relapse control is related to the effect of aldosterone or another ligand of MR or radioresistance, remains to be evaluated. The current results encourage clarification of the possible prognostic value of MR expression in a prospective study.

Conflicts of Interest

The Authors report no conflicts of interest regarding this study.

Authors' Contributions

All Authors contributed to the study design and conception. AJä, KMH and AJu collected the data on Oulu University Hospital patients. YS and PA collected the data on Kuopio University Hospital patients. Immunohistochemical stainings were coordinated and examined by KMH and YS. PK and AJä were responsible for

assessing statistical analyses. AJä and PK were major contributors in writing the manuscript. All Authors provided comments on drafts of the manuscript. All Authors read and approved the final manuscript.

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