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ORIGINAL ARTICLE

Prognostic significance of α- and β2-adrenoceptor gene expression in breast cancer patients

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Aims: Breast cancer is the most frequently diagnosed and leading cause of cancer death among women worldwide. It was classified within molecular *intrinsic* subtypes: luminal A, luminal B, human epidermal growth factor receptor 2-enriched and basal-like. Epinephrine and norepinephrine, released during stress, bind to adrenoceptors. α_2-adrenoceptors are encoded by the *ADRA2A*, *ADRA2B* and *ADRA2C* genes and β_2 by *ADRB2*.

Methods: We compiled several publicly available Affymetrix gene expression datasets, obtaining a large cohort of 1924 patients with distant metastasis-free survival (DMFS) data and evaluated the association between adrenoceptor expression, clinicopathological markers and outcome.

Results: *ADRA2A* high expressing tumours also expressed hormone receptors and presented diminished tumour size, grade and not compromised lymph nodes. *ADRB2* high expression was found in smaller, low grade, oestrogen receptor-positive tumours. Both were significantly associated with the absence of metastasis. High expression of *ADRA2C* was positively associated with increased tumour size and metastatic relapse. We observed a significant increase in DMFS of patients with high *ADRA2A* (hazard ratio 0.54, 95% CI 0.45–0.65, $P < .001$) and *ADRB2* (0.77, 0.64–0.93, $P = .006$) expression and a decrease with *ADRA2C* high expression (1.45, 1.16–1.81, $P = .001$). For patients with luminal tumours, *ADRA2A* was the only factor that retained its significance as an independent predictor of DMFS while *ADRA2C* expression was an independent predictor for worse prognosis in basal-like tumours.

Conclusions: We herein provide new insight for a potential role of *ADRA2A* and *ADRA2C* in breast cancer. In low- and medium-income countries, their incorporation to routine immunohistochemistry analysis of biopsies or tumour samples, could provide additional low-cost prognostic factors.

KEYWORDS

adrenoceptors, breast cancer patients, prognostic factors

The authors confirm that the PI for this paper is Isabel Alicia Lüthy.
All the patients are from microarray gene expression datasets from primary breast cancer samples publicly available at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

1 | INTRODUCTION

Breast cancer is the most frequently diagnosed and leading cause of death by cancer among women worldwide.[1,2] Global incidence is at continuous growth, mainly in less developed countries, due to increasing population number and aging.[1,2] Being a highly heterogeneous disease, breast cancer has been classified by gene expression profiling within molecular *intrinsic* subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, basal-like, and normal breast-like.[3] Additionally, a claudin-low group has been described but, together with the normal breast-like subtype, has found no use in the clinic.[4] The main 4 molecular subtypes, although defined by genomic assay, have a high correlation with the stratification of breast cancer patients that takes place in the clinic according to **oestrogen** (OR) and **progesterone** (PR) receptors and **HER2** expression, very easily assessed by immunohistochemistry (IHC; or *in situ* hybridization in the case of HER2). This classification is relevant, since it determines the treatment selection to be followed and the prognosis of the disease.[5]

Chronic stress has been repeatedly associated with the progression of different cancer types, mainly with the metastatic process.[6] The sympathetic nervous system is a major actor in stress response, releasing catecholamines from nerve fibres and the adrenal medulla.[6] Both epinephrine (also known as adrenaline) and norepinephrine (noradrenaline) bind to adrenoceptors. These receptors belong to the G protein-coupled receptor family, which represent the largest family of cellular receptors and the most common drug targets.[7] Adrenoceptors are divided into 3 groups each of them subdivided in 3 subtypes: α_1 (α_{1A}, α_{1B} and α_{1D}), α_2 (α_{2A} encoded by the *ADRA2A* gene, α_{2B} by the *ADRA2B* gene and α_{2C} by the *ADRA2C* gene) and β (β_1, β_2 encoded by the *ADRB2* gene and β_3), reviewed Lüthy *et al*.[8]

Beta-adrenoceptors were described decades ago in breast human cell lines, normal tissue and tumours.[9,10] The most expressed β-adrenoceptor in breast cancer is the β_2 subtype,[9] whose function in this disease remains controversial. Some reports have associated β_2-adrenoceptor activation with increased tumour growth[10] and metastasis,[6,11] and even with DNA damage.[12] A concomitant stimulatory effect of macrophages has been described in these models.[11,13] Other groups, including our own, found on the contrary, that these receptors are associated with decreased cell proliferation, tumour growth, migration and experimental metastases.[14-18]

The relevance of α_2-adrenoceptors in human breast cancer has been less studied. Our group has described their presence in breast models, in association with increased cell proliferation and tumour growth upon activation with specific agonists.[19-21] However, it has been described that α_2-adrenergic signalling can act through an autoreceptor mechanism inhibiting sympathetic catecholamine release and, thus, indirectly modulating β-adrenergic effects on tumour progression.[22]

Some attempts have been made to link adrenoceptor expression to breast cancer clinical outcome. Characterization of α_{2A}, α_{2C} and β_2-adrenoceptor protein expression on breast cancer tissue samples by IHC was performed by 2 independent groups. A strong staining of α_{2C} was found in high grade, PR negative tumour samples. On the contrary, strong β_2 staining occurred in small-size, low grade luminal tumours.[23] High α_{2A}-adrenoceptor protein expression was inversely associated with HER-2 status and in luminal breast cancer patients, a strong β_2-adrenoceptor expression correlated with increased disease-free survival.[24] These studies suggest, together with experimental evidence, an important role for adrenoceptors in breast cancer clinical outcome. However, due to the limited number of patients usually available for IHC, in most cases no significant associations with disease-free intervals were found, nor stratification in tumour subtypes could be performed.

To assess the prognostic relevance of all 3 subtypes of α_2 (whose individual role in breast cancer is unknown) and β_2-adrenoceptors in the same cohort, and with a sufficient number of patients that assures strong results even upon subtyping, we compiled expression data from a large number of breast cancer samples. Associations between adrenoceptor gene expression, clinical outcome and clinicopathological markers, were statistically evaluated. Our results indicate that adrenoceptors could become new prognostic markers that allow easy and low-cost IHC determination of importance in medium–low income countries in which high-cost methods are not available.

What is already known about this subject

- Beta-adrenoceptors could be important predictors of the clinical outcome of breast cancer patients. Very little is known about α_2-adrenoceptors in breast cancer, apart from experimental work and some immunohistochemical reports. No study has been performed in large databases allowing subtype discrimination.

What this study adds

- The present work provides insight into the potential specific role of α_{2A} and α_{2C}-adrenoceptors in breast cancer and confirms the relevance of the β_2. *ADRA2A* expression is associated with increased disease-free survival in luminal tumours. *ADRA2C* with worse prognosis in basal-like tumours, both independent prognosis factors in these subtypes.
- Clinically, the expression of these adrenoceptors could prove important, mainly in low and medium-income countries, because they could provide additional low-cost prognostic markers. These receptors could be easily incorporated to routine immunohistochemistry analysis of biopsies or tumour samples.

2 | METHODS

We selected 11 microarray gene expression datasets from primary breast cancer samples publicly available at the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/).

We chose only experiments which included patient's follow-up data and carried out with Affymetrix Human Genome U133A Arrays (HG-U133A) for simpler and more accurate compilation. Details for each individual dataset are available in Table S1. Affymetrix raw CEL files from each experiment were downloaded and processed using the RMA algorithm containing the *Affy* package in R/Bioconductor (R version 3.5.0).[25] The obtained individual datasets were later compiled using the R function *merge* into a large dataset including gene expression and phenotypic data from 2142 breast cancer samples. To correct for potential batch effects, the COMBAT method from the *sva* package was applied.[26] Later, the absence of significant batch effects was confirmed by *sva* package's tools and by unsupervised hierarchical clustering. Breast cancer molecular subtype assignment was performed using the PAM50 algorithm from the *genefu* package.[27]

For the analysis of adrenoceptor gene expression, Affymetrix probesets 209869_at, 208544_at, 206128_at and 206170_at were used for *ADRA2A*, *ADRA2B*, *ADRA2C* and *ADRB2*, respectively. Regarding classically used markers for breast cancer patient stratification, from the 9 probesets for the OR gene (ESR1), and the 2 probesets for the HER2 gene (ERBB2), included in HG-U133A arrays, we used only probesets 205225_at (ESR1) and 216836_s_at (ERBB2). It has already been reported that both have the highest correlation with OR and HER2 IHC status (or fluorescence in situ hybridization).[28] Only the probeset 208305_at is available for the PR gene in this array. As for the marker of proliferation KI67 gene, we used the mean value of its 4 different probesets given that they have strong correlation with each other.[28]

Follow-up information was available for 1988 of the 2142 samples. This cohort of 1988 patients was used in subsequent analyses (Table 1). All analyses were performed according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK)[29] and the guidelines for analysis and reporting of microarray data that focus on cancer-related clinical outcomes.[30]

Cut-offs for stratification of patients in high and low expression subsets of each of the stated markers, were calculated using the software Cutoff Finder.[31] For adrenoceptors and KI67 genes, we used the method *survival significance*, which executes multiple survival analyses and defines the optimal cut-off as the point with the lowest log-rank *P*-value. Considering that IHC data were available for OR, PR and HER2, cut-offs for the expression of these markers were derived from the method *outcome significance*. This method correlates the dichotomized gene expression with a binary outcome variable (in this case, IHC positive or negative for the respective biomarker) using logistic regression and selects the optimal cut-off by maximizing the significance assessed by Fisher's exact test. We corroborated that the obtained cut-points have biologically relevant prognostic value in our cohort by performing survival analysis (Figure S1).

The available clinicopathological characteristics of patients from our compiled cohort are shown in Table 1. Association of classical prognostic markers with adrenoceptors expression was analysed using the Fisher's exact test. Survival analysis was performed by the construction of Kaplan–Meier curves and the application of the log-rank (Mantel–Cox) test to determine the univariate significance of each biomarker. For this purpose, being the most prevalent follow-up endpoint in all datasets (1924 of 1988 patients), distant metastasis-free survival (DMFS) was used. Follow-up time was limited to 120 months (10 years). The simultaneous effect of multiple covariates on survival was evaluated using Cox proportional-hazards models (backward elimination: likelihood ratio). Four combinations of covariates were selected that allowed to test a significant number of patients due to their availability in the downloaded data. The individual impact of each variable in survival was evaluated with the Wald test and presented as the hazard ratio (HR) with its 95% confidence interval (95% CI).

Differentially expressed genes between subsets of tumours with high or low expression of each adrenoceptor were identified using the *limma* package in R/Bioconductor.[32] A significance threshold of 5% using the Benjamini and Hochberg method for multiple testing was established. Functional enrichment analysis for the top 250 genes found to be differentially expressed among groups was performed using DAVID v6.7 bioinformatic tool.[33,34] The analysis was limited to gene ontology terms in the *Biological Process (BP)* category and Kyoto Encyclopedia of Genes and Genomes pathways.

Gene expression data from 49 human breast cancer cell lines was obtained from the Cancer Cell Line Encyclopedia (CCLE, available at GEO: accession GSE36133).[35] Affymetrix raw CEL files were downloaded and processed as described above. The following probesets were used for the analysis of adrenoceptors gene expression: 150_at for *ADRA2A*, 151_at for *ADRA2B*, 152_at for *ADRA2C* and 154_at for *ADRB2*.

Statistical analyses were performed with SPSS software version 15.0.1. Reported *P*-values are 2 sided.

2.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[36] and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18.[37,38]

3 | RESULTS

To obtain homogeneous information from a large quantity of breast cancer patients, we compiled several publicly available Affymetrix gene expression datasets (see methods and Table S1). We obtained a cohort of 1988 women with follow-up information whose clinicopathological characteristics are shown in Table 1. We were unable to detect a survival-relevant cut-off for the *ADRA2B* gene. The software used to this end, indicated its expression is not associated to breast cancer outcome. This gene was therefore disregarded in the rest of the work. The only significant split found for this gene, put only 16 samples (0.4%) into the high expression group which did not seem biologically relevant. In fact, during gene enrichment analysis (see ahead), the only available probeset for *ADRA2B* was eliminated upon

TABLE 1 Clinical characteristics of the 1988 samples with follow-up information compiled in this study

Parameter	All		*ADRA2A* high			*ADRA2C* high			*ADRB2* high		
(*n* = 1988)	*n*	%	*n*	%	*P*-value[a]	*n*	%	*P*-value	*n*	%	*P*-value
Age											
≤50 y	523	26.3	262	50.1	n.s.	68	13.0	n.s.	200	38.2	0.005
> 50 y	386	19.4	181	46.9		59	15.3		184	47.7	
Unknown	1079	54.3									
Tumour size											
≤2 cm	326	16.4	199	61.0	<0.001	39	12.0	0.043	139	42.6	0.001
>2 cm	370	18.6	170	45.9		65	17.6		113	30.5	
Unknown	1292	65.0									
Grade											
G1/G2	616	31.0	383	62.2	<0.001	95	15.4	n.s.	277	45.0	0.002
G3	450	22.6	142	31.6		65	14.4		160	35.6	
Unknown	922	46.4									
Lymph node status[b]											
LNN	1231	61.9	651	52.9	0.002	185	15.0	n.s.	511	41.5	n.s.
N+	527	26.5	237	45.0		86	16.3		220	41.7	
Unknown	230	11.6									
OR											
Negative	451	22.7	129	28.6	<0.001	53	11.8	0.003	163	36.1	0.029
Positive	1260	63.4	745	59.1		223	17.7		530	42.1	
Unknown	277	13.9									
PR											
Negative	342	17.2	110	32.2	<0.001	55	16.1	n.s.	120	35.1	n.s.
Positive	331	16.6	204	61.6		63	19.0		128	38.7	
Unknown	1315	66.1									
HER2											
Negative	529	26.6	244	46.1	n.s.	85	16.1	n.s.	218	41.2	n.s.
Positive	50	2.5	19	38.0		9	18.0		22	44.0	
Unknown	1409	70.9									
Metastatic recurrence											
Negative	1416	71.2	778	54.9	<0.001	213	15.0	0.025	592	41.8	0.015
Positive	508	25.6	208	40.9		99	19.5		181	35.6	
Unknown	64	3.2									

[a]n.s. = nonsignificant.

[b]LNN = lymph node negative, N+ = node positive.

HER2 = human epidermal growth factor receptor 2; OR = oestrogen receptor; PR = progesterone receptor

application of a gene filter algorithm to eliminate those regarded as low informative (with no variation among samples). We stratified patients into groups of high and low expression of the other receptors, *ADRA2A*, *ADRA2C* and *ADRB2*.

ADRA2A high expressing tumours are characterized by a bigger fraction of OR (59.1 *vs* 28.6%, $P < .001$) and PR positive cases (61.6 *vs* 32.2%, $P < .001$; Table 1). Accordingly, with these standard good prognosis markers, high expression of this gene negatively correlated with increased tumour size (45.9 *vs* 61.0%, $P < .001$) and grade (31.6 *vs* 62.2%, $P < .001$), and compromised lymph nodes (45.0 *vs* 52.9%, $P = .002$). Similarly, in the whole cohort, *ADRB2* high expression was found in smaller (42.6 *vs* 30.5%, $P = .001$), low grade (45.0 *vs* 35.6%, $P = .002$), OR positive tumours (42.1 *vs* 36.1%, $P = .029$; Table 1). Additionally, high expression of both adrenoceptors is significantly associated with the absence of metastatic recurrence (54.9 *vs* 40.9%, $P < .001$ for *ADRA2A*; 41.8 *vs* 35.6%, $P = .015$ for *ADRB2*). On the contrary, given its positive association with increased tumour size (17.6 *vs* 12.0%, $P = .043$) and the occurrence of metastatic relapse (19.5 *vs*

15.0%, P = .025; Table 1), high expression of *ADRA2C* appears to be found in more aggressive tumours. Not in line with this statement, among high *ADRA2C* tumours there is also a significant increase in the proportion of OR positive cases (17.7 *vs* 11.8%, P = .003).

We next sought to determine whether adrenoceptor gene expression impact on patient survival disregarding differences in treatment, clinical subtype composition or any other clinicopathological parameter. For this, we performed Kaplan–Meier analyses for each of them in the whole cohort of patients (Figure 1). All 3 adrenergic subtype genes showed a significant prognostic value in breast cancer patients. Particularly, we observed a significant increase in DMFS of patients with *ADRA2A* (Univariate HR 0.54, 95% CI 0.45–0.65, P < .001) and *ADRB2* (HR 0.77, 95% CI 0.64–0.93, P = .006) high expression tumours (Figure 1A and C, respectively). On the contrary, and in agreement with the univariate correlations found with worse classical prognostic markers, *ADRA2C* high expression was significantly associated with decreased DMFS (HR 1.45, 95% CI 1.16–1.81, P = .001; Figure 1B).

To investigate the underlying functional biology of these associations, we performed functional enrichment analysis (for gene ontology terms and Kyoto Encyclopedia of Genes and Genomes pathways) of the top 250 differentially up- and downregulated genes in high expression tumours (listed in Table S2). Table 2 shows up to 5 of the most significant terms, for each category, in *ADRA2A* and *ADRB2* high expression samples. No statistically significant enrichment for differentially expressed genes in high *vs* low *ADRA2C* tumours was found. Upregulated genes in *ADRA2A* high expression tumours were involved in cell–cell and focal adhesion, antiangiogenic processes and inhibition of cell proliferation. Congruently, among downregulated genes in these tumours, there is a strong enrichment in processes involved in cell division (DNA replication, G1/S transition, cell cycle, etc.; Table 2). In the case of breast tumours with high levels of *ADRB2*, it is noteworthy that upregulated genes were particularly implied in inflammatory response (T cell activation, leucocyte migration, adaptive immune response, haematopoietic cell lineage), while the downregulated genes were found to be engaged in the catabolic regulation of cell proliferation (Table 2). Overall, the molecular and biological processes enriched in *ADRA2A* and *ADRB2* high expression tumours are consistent with their apparent less aggressive phenotype.

We have reported that activation of α_2-adrenoceptors in several breast cancer cell lines leads to increased proliferation and tumour growth.[15,19-21] Considering these previous results, the strong opposing associations found here between 2 different subtypes of this receptor, were rather unexpected. Since behaviour of human breast cancer experimental models (i.e. cell lines) often diverge from tumours, we hypothesized that the expression ratio of subtypes of the α_2-adrenoceptor family could play an important role in agonist overall response and explain these observations. Therefore, we compared the gene expression levels of the 3 subtypes of α_2 and the β_2-adrenoceptor in tumours from our compiled dataset and in 50 human breast cancer cell lines from the CCLE (Figure 2A). Regarding α_2-adrenoceptors, we observed that among human tumours there is a slight but progressive decrease in expression from the *ADRA2A* gene

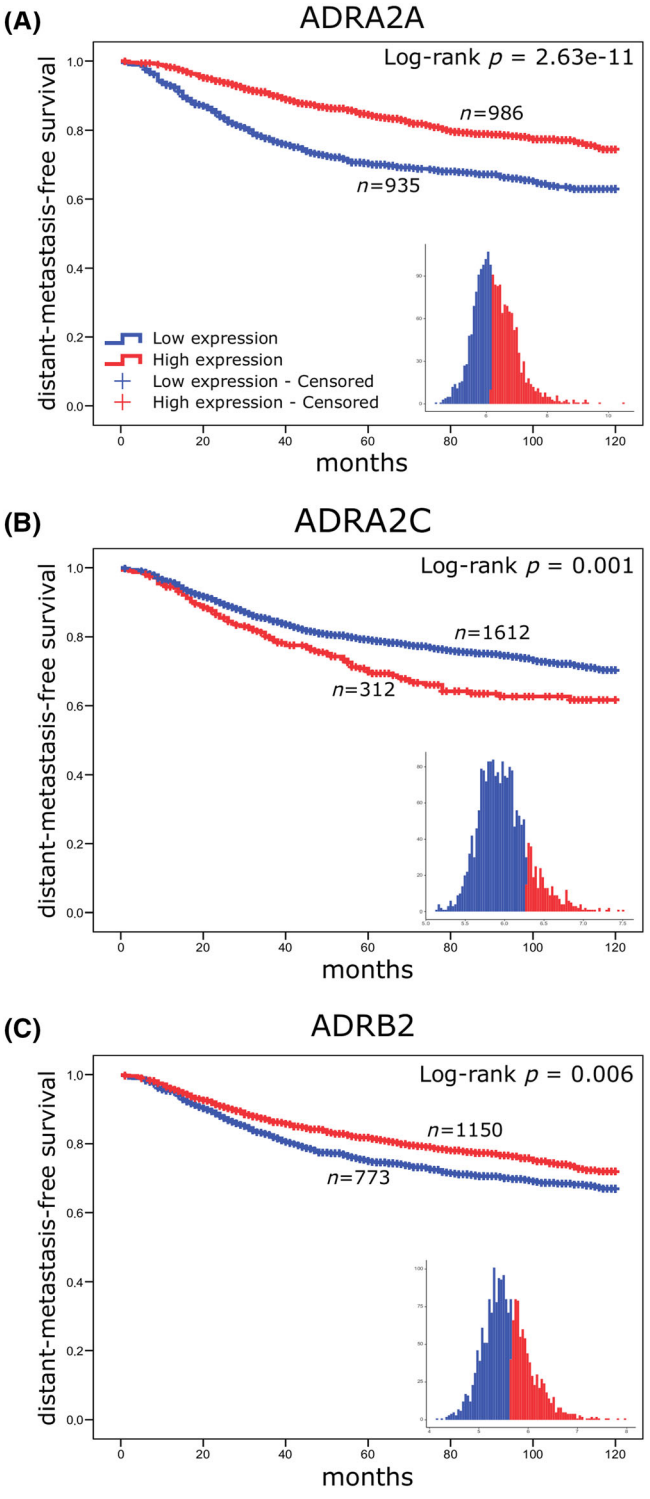

FIGURE 1 Prognostic value of α_{2A}, α_{2C} and β_2-adrenoceptor gene expression in the complete cohort of breast cancer patients compiled in this study. Kaplan–Meier analysis for distant metastasis-free survival was performed for α_{2A} (A), α_{2C} (B), and β_2-adrenoceptors (C). The number of included patients is shown in each curve. Histograms show the distribution of the dichotomized gene expression after applying the cut-offs determined by the software Cutoff Finder. No cut-off was identified for the *ADRA2B* gene

TABLE 2 Gene enrichment analysis of up- and downregulated genes in *ADRA2A* and *ADRB2* high tumours

		Category	Term [a]	Gene count	Fold enrichment	*P*-value[b]	Adjusted *P*-value[c]
***ADRB2*-high tumours**	Upregulated	BP	T cell activation	10	15.81	1.0E-08	1.7E-05
			Leucocyte migration	13	7.92	8.9E-08	1.5E-04
			Inflammatory response	21	4.12	2.0E-07	3.3E-04
			Signal transduction	39	2.50	2.5E-07	4.2E-04
			Transmembrane receptor protein tyrosine kinase signalling pathway	11	8.51	6.3E-07	1.0E-03
		KEGG	Cell adhesion molecules	13	4.94	1.0E-05	1.3E-02
			Hematopoietic cell lineage	10	6.35	2.3E-05	2.9E-02
	Downregulated	BP	Anaphase-promoting complex-dependent catabolic process	15	12.96	7.3E-12	1.2E-08
			Cell division	26	5.07	6.1E-11	1.0E-07
			Negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	13	12.50	4.3E-10	7.0E-07
			mRNA splicing- via spliceosome	20	6.15	6.7E-10	1.1E-06
			Positive regulation of ubiquitin-protein ligase in regulation of mitotic cell cycle transition	13	11.68	9.7E-10	1.6E-06
		KEGG	Proteasome	8	9.38	1.8E-05	2.1E-02
***ADRA2A*-high tumours**	Upregulated	BP	Cell adhesion	32	4.96	3.7E-13	6.1E-10
			Extracellular matrix organization	18	6.53	2.4E-09	4.0E-06
			Negative regulation of cell proliferation	23	4.13	4.2E-08	7.0E-05
			Signal transduction	39	2.39	7.8E-07	1.3E-03
			Negative regulation of angiogenesis	8	9.18	2.5E-05	4.1E-02
		KEGG	Focal adhesion	14	4.52	1.0E-05	1.2E-02
	Downregulated	BP	Cell division	49	9.71	2.5E-33	4.0E-30
			DNA replication	29	12.98	5.1E-23	8.1E-20
			Mitotic nuclear division	34	9.51	1.4E-22	2.2E-19
			G1/S transition of mitotic cell cycle	24	16.33	2.1E-21	3.3E-18
			Anaphase-promoting complex-dependent catabolic process	20	17.57	1.9E-18	3.0E-15
		KEGG	Cell cycle	32	14.04	1.0E-27	1.1E-24
			DNA replication	11	16.63	4.8E-10	5.4E-07
			Oocyte meiosis	12	5.99	4.0E-06	4.5E-03
			Proteasome	8	9.89	1.2E-05	1.4E-02
			p53 signaling pathway	9	7.31	2.6E-05	2.9E-02

[a]Only up to the top 5 terms from each category with a significant adjusted *P*-value are shown.

[b]modified Fisher's exact test.

[c]Benjamini and Hochberg false discovery rate-adjusted *P*-value.

BP, biological processes; KEGG, Kyoto Encyclopedia of Genes and Genomes

to *ADRA2C* (Figure 2A, upper panel), while, in human breast cancer cell lines, the opposite is observed (Figure 2A, inferior panel). It is also remarkable that, in tumours, *ADRB2* expression is lower than that of the 3 α_2-adrenoceptor coding genes, but that this is again reverted in cell lines. This tendency remains even when cell lines are stratified in molecular subtypes (as explained in the legend and shown in Figure S2).

As breast tumour stratification is essential in the clinic to determine patient prognosis and treatment, we next evaluated whether there were differences in adrenoceptor expression, and its relevance in patient survival, among the 4 main intrinsic molecular subtypes (Figure 2B). *ADRA2A* expression levels in luminal A tumours are significantly higher than in all the other subtypes. However, we did not find it among the list of differentially expressed genes in these tumours (data not shown). Moreover, we observed that the positive association between high expression of *ADRA2A* and better prognosis (in terms of DMFS) is maintained in patients with luminal A and B tumours (HR 0.56, 95% CI 0.38–0.82, P = .003 for luminal A; HR 0.71, 95% CI 0.52–0.96, P = .024 for luminal B; Figure 2C). Similarly, the reduction in DMFS found in the whole cohort of patients with high *ADRA2C* tumours was preserved in those from the luminal B (HR 1.56, 95% CI 1.10–2.21, P = .012) and basal-like subgroups (HR 1.72, 95% CI 1.08–2.74, P = .022). High expression of the *ADRB2* gene, which was significantly associated with increased survival in the whole cohort, did not show any significant effect when the tumours were stratified (Figure 2C). Regarding breast cancer treatment, we also found evidence indicating that the expression of adrenoceptors could have implications in response. We observed that high expression of *ADRA2A* in tumours of patients receiving endocrine therapy, was significantly associated with better DMFS (HR 0.38, 95% CI 0.25–0.59, P < .001). Notably, this prognostic value was lost in OR+ untreated patients (Figure S3A). In the case of patients treated with

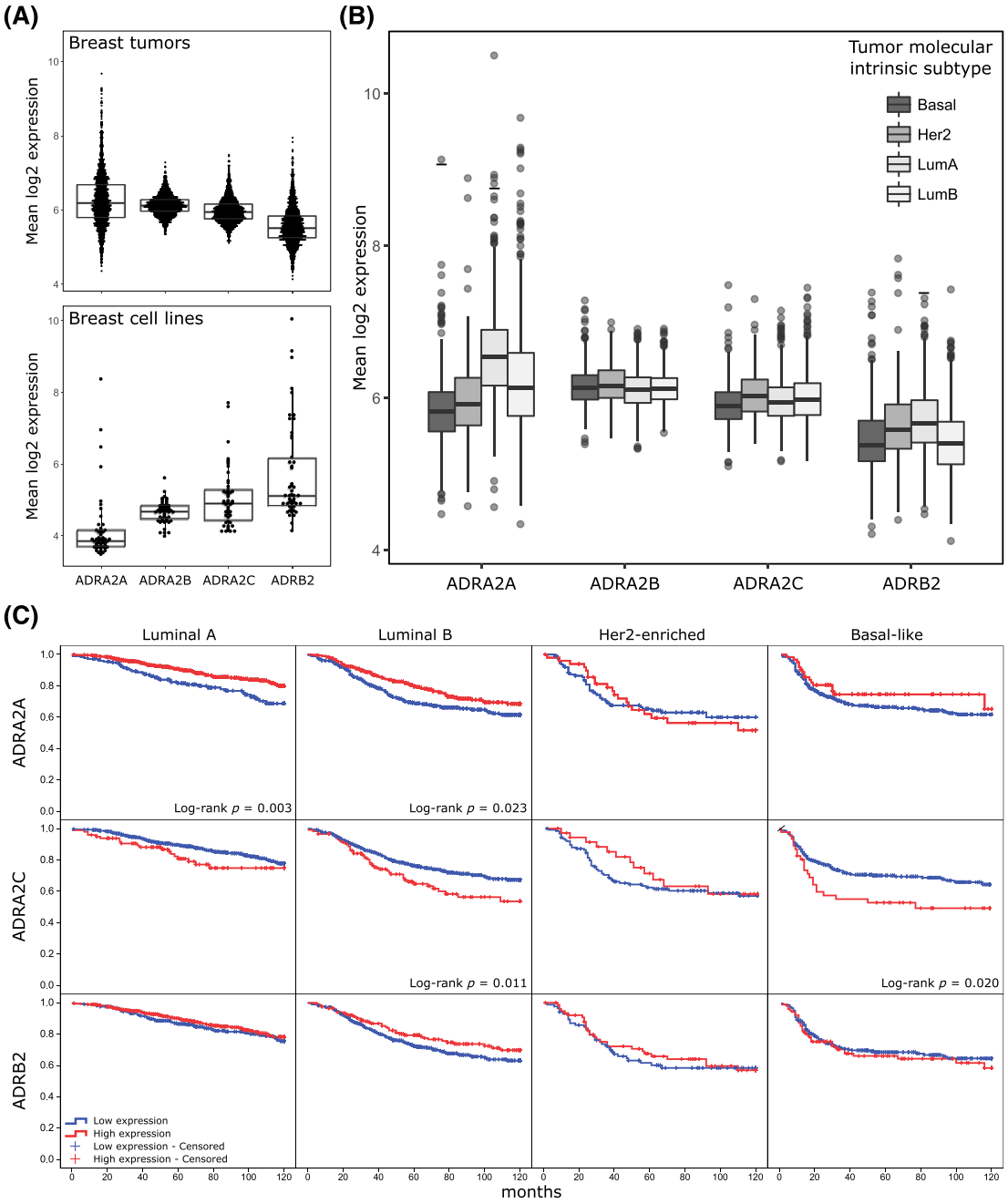

FIGURE 2 Expression levels and significance in patient survival in each of the 4 molecular intrinsic subtypes of α_{2A} and β_2-adrenoceptors in breast cancer samples. (A) Expression levels of each subtype of α_2 and β_2-adrenoceptors in breast tumours (compiled dataset, upper panel) and breast cancer cell lines (CCLE, lower panel). (B) Expression levels of adrenoceptors in tumours of each of the 4 molecular subtypes. (C) Kaplan–Meier analysis for distant metastasis-free survival according to adrenoceptor expression in each of the 4 molecular subtypes. The same cut-off values as for the whole cohort were used. Only significant *P*-values are shown

chemotherapy, also a high expression of *ADRA2A* (HR 0.38, 95% CI 0.21–0.69, *P* = .002), but not of *ADRA2C* or *ADRB2*, was associated with better DMFS (Figure S3B). This suggests that the expression of this particular receptor could have an important influence on treatment response.

To determine if the prognostic value of the 3 adrenoceptors found so far was independent of other clinicopathological factors, we used multivariate Cox regression analysis in the whole cohort and upon stratification in molecular subtypes. For the analysis of the complete cohort, we constructed 4 different models including alternative covariates to maximize the number of analysed patients. Collective information for classical prognostic markers such as tumour size, tumour grade, lymph node status, OR, PR and HER2, was available for only 88 patients. Hence, we divided them into models 1 and 2, together with *ADRA2A*, *ADRA2B* and *ADRB2*, for a total of 592 and 577 samples, respectively (Table 3). Focusing on adrenoceptor genes, in both models, only *ADRA2A* expression remained significant (model 1: HR 0.60, 95% CI 0.42–0.87, *P* = .007; model 2: HR 0.67, 95% CI 0.45–

TABLE 3 Multivariate Cox regression models in the complete cohort

Model 1 (n = 592)[a]		HR	95% CI		P-value[c]
Tumour size	>2 *vs* ≤ 2cm	1.24	0.87	1.76	.233
Grade	G3 *vs* G1/G2	1.51	1.06	2.17	**.023**
Lymph node status	N+ *vs* LNN	2.00	1.13	3.54	**.018**
ADRA2A	High *vs* low	0.60	0.42	0.87	**.007**
ADRA2C	High *vs* low	1.36	0.88	2.11	.172
ADRB2	High *vs* low	0.70	0.48	1.01	.055
Model 2 (n = 577)[a]		**HR**	**95% CI**		**P-value**
OR[b]	Positive *vs* negative	0.56	0.36	0.87	**.010**
PR[b]	Positive *vs* negative	0.57	0.36	0.92	**.022**
HER2[b]	Positive *vs* negative	1.25	0.73	2.13	.412
ADRA2A	High *vs* low	0.67	0.45	0.99	**.043**
ADRA2C	High *vs* low	1.15	0.71	1.87	.566
ADRB2	High *vs* low	1.23	0.86	1.75	.261
Model 3 (n = 592)[a]		**HR**	**95% CI**		**P-value**
Tumour size	>2 *vs* ≤2 cm	1.25	0.88	1.78	.211
Grade	G3 *vs* G1/G2	1.34	0.92	1.95	.124
Lymph node status	N+ *vs* LNN	2.34	1.34	4.12	**.003**
ESR1[b]	High *vs* low	1.02	0.67	1.56	.927
PGR[b]	High *vs* low	0.59	0.40	0.86	**.007**
ERBB2[b]	High *vs* low	0.94	0.57	1.52	.789
ADRA2A	High *vs* low	0.60	0.42	0.86	**.005**
ADRA2C	High *vs* low	1.30	0.84	2.02	.241
ADRB2	High *vs* low	0.69	0.48	0.99	**.046**
Model 4 (n = 1921)[a]		**HR**	**95% CI**		**P-value**
ESR1	High *vs* low	0.85	0.69	1.05	.126
PGR	High *vs* low	0.64	0.51	0.79	**<.001**
ERBB2	High *vs* low	0.91	0.68	1.23	.554
KI67[b]	High *vs* low	1.72	1.38	2.15	<.001
ADRA2A	High *vs* low	0.69	0.57	0.84	**<.001**
ADRA2C	High *vs* low	1.34	1.07	1.67	**.011**
ADRB2	High *vs* low	0.88	0.73	1.07	.203

[a]Number of patients with available information on the parameters included in each Cox regression model.

[b]OR/PR/HER2 = immunohistochemistry data, ESR1/PGR/ERBB2/KI67 = gene expression data.

[c]Significant P-values are shown in bold.

HER2; human epidermal growth factor receptor 2; OR, oestrogen receptor; PR, progesterone receptor

0.99, P = .043), with a trend to significance observed for the *ADRB2* gene (model 1, P = .055). With the objective of increasing the statistical power of this result, in model 3 we included all the previously mentioned classical factors as covariates, obtaining information for 592 of the 1988 samples. We accomplished this by using ESR1, PGR and ERBB2 mRNA expression—dichotomized as described in the methods section—as surrogate for OR, PR and HER2 IHC status. In this case, again *ADRA2A* expression was found to be an independent predictor of DMFS (HR 0.60, 95% CI 0.42–0.86, P = .005), as this was also the case for the *ADRB2* gene (HR 0.69, 95% CI 0.48–0.99, P = .046). Finally, in model 4, we sought to include the majority of the samples from our cohort using only gene expression covariates. For this, we selected the 4 biomarkers used to stratify patients in the clinic (ESR1, PGR, ERBB2 and KI67), obtaining information for 1921 samples. In this model, the 2 genes for α_2-adrenoceptors, *ADRA2A* and *ADRA2C*, retained high significance as independent predictors of better and worse survival, respectively (HR 0.69, 95% CI 0.57–0.84, P < .001 for *ADRA2A*; HR 1.34, 95% CI 1.07–1.67, P = .011; Table 3).

Finally, we performed the same analysis within tumours of each molecular subtype (Table S3). In this case, given that the number of

samples is reduced as a result of stratification, we again used only gene expression covariates, as in model 4 from Table 3, to assure maximum availability. For patients with luminal A tumours, *ADRA2A* was the only factor that retained its significance as an independent predictor of DMFS (HR 0.57, 95% CI 0.39–0.84, P = .004). By contrast, *ADRA2C* expression was an independent predictor for worse prognosis in basal-like tumours (HR 1.79, 95% CI 1.13–2.86, P = .014) and it remained marginally significant in the luminal B subtype (P = .055).

4 | DISCUSSION

Beta-adrenoceptors have been proposed as nonconventional targets for breast cancer metastasis, which is the major cause of deaths by this disease.[39,40] As mentioned, some studies have been published associating adrenoceptor expression with patient's clinicopathological characteristics and outcome. However, due maybe to the limited number of samples analysed, correlations are not always compelling. Moreover, the 3 subtypes of α_2-adrenoceptors, which are generally considered as redundant in cancer, have not been analysed together in the same cohort and an analysis of this nature has not been made in patients stratified in clinically relevant breast tumour subtypes.

The present investigation was undertaken to assess, in a large cohort of almost 2000 breast cancer patients, the prognostic value for gene expression of each of these receptors. Moreover, as some of our experimental results with adrenergic compounds suggest that their effect could be dependent on tumour subtype, we particularly studied the expression of these receptors and its clinical consequences in tumours of the different molecular intrinsic subtypes of breast cancer.

We found strong associations between high expression of *ADRA2A* and biological markers of nonaggressive phenotype, such as hormone receptors and smaller tumour size and grade. Previously, in line with our findings, the expression of this receptor in breast tumours had been only significantly associated with negative HER2 expression, and marginally to OR levels.[24] We also uncovered a highly significant prognostic value for *ADRA2A* expression for all breast cancer patients and among those with tumours of the luminal subtypes. In fact, the expression of this gene remained significant in the 4 tested multivariate Cox regression models in the whole cohort and in the luminal A subtype, highlighting its potential importance as an independent predictor of breast cancer metastasis-free survival. Finally, yet importantly, the expression of *ADRA2A* proved to have prognostic value in endocrine and chemotherapy treated patients, suggesting an interaction between its expression and treatment response, which is currently under investigation in our laboratory.

Similarly, although the results were not as robust as those for *ADRA2A*, *ADRB2* high expression proved to be associated with better prognosis related parameters and increased DMFS in the whole cohort. *ADRB2* expression turned out to be an independent predictor of DMFS in 1 of the performed multivariate analyses. However, this prognostic value was lost upon stratification of patients into the intrinsic molecular subtypes. Multivariate analysis from 1 of the mentioned studies, found that β_2-adrenoceptor expression was an independent predictor of disease-free survival in hormone receptor positive samples.[24] In another study, an improvement in breast cancer-specific survival in β_2 positive, OR positive, **tamoxifen**-treated patients compared to the β_2-negative patients, was also observed, at least during the first 5 years of treatment.[23,40] Likewise, while we did not observe an effect for this gene in molecular subtypes, its expression was significantly associated with augmented survival in both OR+ and ESR1-high samples (data not shown). Associations between OR and the β_2-adrenoceptor are recurrent in the bibliography, suggesting that interactions between their signalling pathways may be essential for the regulation of breast cancer biology.[41] In fact, our group has recently reported that the differentiation of BALB/c mouse mammary glands promoted by the β-agonist isoproterenol, is highly dependent on OR activity.[18]

Although α_2 and β-adrenoceptors has been classically described as antagonistic, in the present investigation their expression was associated with similar outcomes, functional enrichment analysis suggesting distinctive mechanisms. High expression of *ADRA2A* appears to be related to inhibited tumour cell proliferation whereas the *ADRB2* gene seems to be involved in antitumour immunity. It has been already described that β_2-adrenergic activation increases the release of chemotactic factors, such as macrophage colony-stimulating factor and CCL2, from tumour cells, promoting the recruitment of tumour associated macrophages.[11,42] These immune cells may be responsible for the antimetastatic effects observed for some β_2-adrenergic antagonists.[11] Moreover, as the gene expression data used herein were derived from experiments with whole tumour extracts, it is not possible to distinguish between mRNA levels in tumour and microenvironmental cells. Consequently, the effect of adrenoceptors expression in tumour microenvironmental cells cannot be discarded from our results as a relevant modulator of cancer biology and outcome. In this regard, using laser capture microdissection to compare the gene expression profiles of stroma from breast tumours, a stroma-derived prognostic predictor with strong independent associations with clinical outcome was described.[43] Among the 26 genes constituting this predictor, *ADRA2A* was found, suggesting that its expression in stromal cells is fundamental for breast cancer progression.

For the past decade, we have consistently described that α_2-adrenergic activation leads to increased cell proliferation and tumour growth, in several human and murine experimental models of breast cancer.[15,19,20,44] Considering this background, the finding that the expression of a subtype of this receptor is strongly associated with improved outcome in breast cancer patients, was quite unpredictable. However, in line with our experimental results, we found significant associations between *ADRA2C* expression and poor prognosis. Likewise, strong cytoplasmic α_{2C} expression had already been associated with markers of enhanced aggressiveness, such as high grade, HER3/4+ and PR–.[23] Moreover, we observed that the gene expression ratio α_{2A}/α_{2C} is altered in breast cancer cell lines compared to human tumours. When the expression of adrenoceptors was assessed in the whole cohort, all subtypes of α_2 adrenoceptors showed similar expression. On the contrary, in cell lines the expression of *ADRA2A* was extremely low. We have previously described low expression of

α_{2A}-adrenoceptor in several human breast cancer cell lines.[21,44] For example, the human breast cancer cell MCF-7 expressed nearly 200 times more *ADRA2C* than *ADRA2A*.[44] Altogether, these results suggest that the proliferation-enhancing effect we have described for clonidine and dexmedetomidine,[15,19,44] both nonspecific α_2-adrenergic agonists used in clinics in the context of anaesthesia,[45] could be mediated almost exclusively by the α_{2C} subtype. A recent study has also shown that perioperative use of dexmedetomidine increases the metastatic burden of a mammary adenocarcinoma in rats, Lewis lung carcinoma in C57BL/6 mice, and colon adenocarcinoma in BALB/c mice.[46] Rauwolscine, an α_2-adrenergic antagonist that we proved to be a strong inhibitor of breast tumour growth,[15,19,20,44] has a higher affinity for the α_{2C} subtype.[47]

Besides the slight differences in the *in vivo* physiological actions of α_{2A} and α_{2C}-adrenoceptors,[48] until now, no such strong contrast between the 2 had been reported. Unfortunately, as we found no significant enrichment for differentially expressed genes in *ADRA2C* high tumours, we cannot hypothesize about the responsible mechanisms. Whether direct and specific stimulation of α_{2A}-adrenoceptors leads to different biological responses than α_{2C} activation, remains to be proved in breast cancer cell lines. If this were the case, specific agonists/antagonists for each of these receptors (when developed) could find their use in the oncological landscape, as it has been proposed for β-blockers. A recent study shows that perioperative use of clonidine in breast and lung cancer patients, has no incidence in survival.[49] According to our results, α_2-adrenergic agonist response in breast tumours could be dependent on the α_{2A}/α_{2C} ratio, which could be determined by simple IHC.

Finally, we consider it relevant to discuss that gene expression does not necessarily imply receptor activation by circulating catecholamines. For instance, β_2-adrenoceptors are known to have an important basal activity.[50] Moreover, investigating the *in vitro* epinephrine concentration at which α_2 and β-adrenoceptors exerted their action on human breast cancer cells, we found that proliferation is enhanced at around 1 nM concentrations via α_2 receptors, whereas at higher concentrations (0.1 μM) proliferation is inhibited through β-adrenergic activation.[15] Although these results cannot be directly extrapolated to human tumours, it has been described that basal circulating concentrations of epinephrine are in the order of 3–30 nM (and around 300 nM for norepinephrine), increasing to a double value in mild stress.[51,52] We can therefore expect adrenoceptors to be at least partially occupied and activated in control situations, and further occupied in stress conditions.

In conclusion, although our *in silico* results need to be further confirmed experimentally, the present study provides new insight into the potential specific role of α_{2A} and α_{2C}-adrenoceptors in breast cancer and further confirms the relevance of the β_2-adrenoceptor in this disease. As breast cancer is a highly heterogeneous disease, there is continuous need for new prognostic factors that allow for more accurate prediction of patients´ outcome. Considering our results, particularly upon stratification of patients into subtypes, the expression of these 3 adrenoceptors could prove of importance in the clinic. In low and medium-income countries, these receptors could be easily incorporated to routine IHC analysis of biopsies or tumour samples, providing for additional low-cost prognostic factors. From a therapeutic point of view, our results show that high *ADRA2C* expression is found in basal-like tumours of worse prognosis being an independent predictor of DMFS in patients within this subtype. This receptor could become a valuable target in these tumours that lack specific therapy if specific antagonists were to be developed. These results could therefore prove of importance in the prediction of prognosis and treatment for specific subtypes of breast cancer.

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CONTRIBUTORS

E.M.R. performed the study with help from L.M.M. and L.G. in statistics and C.D.B. in the programming. A.B. helped in the supervision of the work. I.A.L. supervised the work and drafted the manuscript, which was corrected and accepted by all authors. The design of the experiments, analysis and interpretation of the data were shared by every author.

COMPETING INTERESTS

There are no competing interests to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in GEO at https://www.ncbi.nlm.nih.gov/geo/, with reference numbers that are listed in Table S1. These data were derived from the resources available in the public domain listed in Table S1.

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SUPPORTING INFORMATION

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