

S-phase fraction, lymph node status and disease staging as the main prognostic factors to differentiate between young and older patients with invasive breast carcinoma

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Abstract. The influence of age on the outcome of patients with invasive breast carcinoma (IBC) has not yet been fully established. The present study investigated two subgroups of patients either side of the age spectrum, and evaluated cytometric, histopathological and molecular characteristics. The series involved 219 patients with IBC that had long-term follow-up, which were divided into two subgroups: Young (≤ 45 years; $n=103$) and old patients (≥ 75 years; $n=116$). Immunohistochemical evaluation of hormonal receptors, Ki67 index and HER2 status (plus HER2 silver *in situ* hybridization in equivocal cases) were used as the basis for surrogate molecular subtyping. Ploidy and S-phase fraction (SPF) were analysed by DNA flow cytometry. Differences between the subgroups' characteristics were assessed by the two proportion Z test. Kaplan-Meier estimation and log-rank test were applied for survival analyses. The median age in the subgroups were 40 years (range, 19-45 years) in the young group and 78 years (range, 75-91 years) in the older subgroup. Young patients exhibited higher lymph node involvement, more advanced disease staging, higher SPF tumour proliferative activity, and a trend of lower incidence of Luminal A and higher incidence of Luminal B tumours. The median SPF value was significantly higher in young patients [7.1% (range, 1.5-23.7%) vs. 4.5% (range, 0.7-26.4%)], whereas the ploidy pattern showed no significant difference. In the whole series, as within IBC of no special type, young patients had a higher rate of recurrence (46.6 vs. 22.4%; $P<0.001$) and deaths from disease (35.9 vs. 20.7%; $P=0.030$), with a statistically significant difference for disease-free survival. In conclusion, the present study

indicated that young patients with IBC exhibited more aggressive disease, with an increased risk of recurrence and shorter disease-free survival. SPF, lymph node status and staging appeared to be the main prognostic factors to differentiate young from older patients with IBC.

Introduction

Invasive breast carcinoma (IBC) is the most common neoplasia that affects women worldwide and is one of the leading causes of cancer-related deaths in this gender (1). In Portugal, about 7,000 new cases of female IBC occur annually, along with 1,800 deaths from the disease (2).

It is widely established that classic features such as TNM status (tumour size, lymph node involvement, distant metastasis) and histological grade have a great influence on the clinical course, prognosis and treatment strategies for IBC. Other risk factors related to disease outcome have been reported, and among them, the patient's age at diagnosis (3-6). Despite being an uncommon disease (1), IBC diagnosed at a young age, when compared to older patients, seems to have a more aggressive biological behaviour. Indeed, younger patients show in general tumours more likely to be of higher grade and detected at a more advanced disease stage, consequently leading to a worse prognosis (7-9). Conversely, most IBCs in the elderly are low-grade and estrogen receptors-positive, consistent with more favourable tumour biology and evolution (10).

In our institution, DNA ploidy and S-phase fraction (SPF), measured by flow cytometry, are usually assessed, whenever possible, in IBC patients to improve the panel of prognostic factors. We have shown that these parameters provide significant prognostic information that is biologically relevant and clinically useful for the management of patients with IBC (11-13). Although potentially related to IBC age-specific differences, the impact of DNA flow cytometry on both extremes of the age spectrum has not yet been fully investigated.

The present study aims to evaluate significant differences in histopathological and molecular characteristics between two subgroups of young (≤ 45 years) vs. older (≥ 75 years) IBC patients, with emphasis on DNA flow cytometry data. We also sought to analyse the influence of age on patients' survival, as

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well as whether the age itself is suitable as a prognostic factor, independently of the common clinicopathological parameters.

Materials and methods

Clinicopathological data. The whole series consisted of 219 patients (≤ 45 years: 103 patients; ≥ 75 years: 116 patients) with primary IBC, diagnosed and treated at the Portuguese Institute of Oncology of Lisbon between January 1992 and December 2017. The present cohort was retrieved from a larger dataset that encompasses DNA flow cytometry information. Beyond the pre-established selective option concerning the age, patients' eligibility criteria included the availability of unfixed fresh/frozen samples for DNA flow cytometry and complete follow-up information. Moreover, patients had no metastatic disease at the time of diagnosis and have not received any type of neoadjuvant treatment. The local institutional ethical committee approved the study. The histological type and pathological staging were evaluated according to WHO classification (14). Tumour differentiation was assessed using the Nottingham grading system (15). Table I shows the different forms of treatment in the two subgroups of IBC patients. A higher proportion of young patients underwent breast-conserving surgery, radiation therapy and chemotherapy, while older patients were submitted mainly to mastectomy and hormonal therapy. Only one patient in each group was treated with trastuzumab.

Immunohistochemistry (IHC) analyses. Estrogen receptors (ER) and progesterone receptors (PR) were re-evaluated in the whole series by IHC analysis on paraffin-embedded material using the peroxidase-indirect-polymer with Ventana ultraview universal DAB detection kit ref 760-500 (Ventana Medical Systems, Inc., Tucson, USA). The primary antibodies used were Ventana anti-estrogen receptor ref 790-4324 (SP1), and Ventana anti-progesterone receptor ref 790-2223 (1E2). The assessment was unavailable in two and nine cases for ER and PR, respectively. The results were recorded as the percentage of positively stained (cut-off $\geq 1\%$) neoplastic cell nuclei (Figs. S1 and S2).

Ki67 index was re-assessed in the whole series on paraffin-embedded material, using Ventana optiview DAB IHC detection kit ref 760-700. The primary antibody used was anti-Ki67 (30-9) ref 790-4286, with antigen retrieval of 40 min CC1 94°C, and 28 min antibody incubation. The assessment was unavailable in 66 cases, due mostly to missing paraffin blocks. A pre-defined 20% cut-off point distinguished low ($< 20\%$) from high ($\geq 20\%$) proliferative tumours (16) (Fig. S3).

Human epidermal growth factor receptor 2 (HER2) expression was first determined by standardised IHC technique (Ventana Pathway anti-HER-2/neu, clone 4B5, ref 790-2991) as a screening test, and by silver in situ hybridization (SISH) (Ventana Silver ISH plus Ventana Red ISH ref 760-512 + ref 760-516, and Ventana Her2 dual ISH dna probe cocktail ref 800-6043) in all IHC equivocal (2+) cases (17,18). HER2 positive status was defined by protein overexpression (3+) (Fig. S4) or gene amplification.

Surrogate IHC molecular classification was based on ER and PR expression, Ki67 proliferation index and HER2 status, including Luminal A, Luminal B, HER2 positive, Triple-negative, and Triple-positive tumours.

DNA flow cytometry. Flow cytometric analysis was performed on fresh/frozen representative tumour samples according to a technique described previously (11,19). Briefly, the tissue samples were mechanically disaggregated using scalpel blades in cold phosphate-buffered saline (PBS). For DNA staining, the nuclei were incubated with propidium iodide (Sigma) 50 $\mu\text{g}/\text{ml}$ in Tris-Mg Cl_2 buffer, for one hour in the dark at 4°C, treated with RNase (Sigma), 1 mg/ml in PBS, and 0.05% Nonidet P40 (Sigma). Usually, a minimum of 20.000 nuclei were acquired and recorded on a single parameter, 256 channel integrated fluorescence histogram. The Multicycle software program (Phoenix Flow Systems, San Diego, CA, USA) was used for cell cycle analysis of DNA histograms. Mixed non-malignant diploid cells from the same tumour sample analysed were used as the internal reference standard. Regarding DNA ploidy pattern, IBCs were classified as diploid vs. aneuploid. Tumours were considered diploid when the DNA index (DI) obtained was 1.0 (range, 0.95-1.05). The aneuploid tumours were further subclassified into several categories based on DI: hypodiploid (DI: < 0.95), hyperdiploid (DI: > 1.05 and < 1.9), tetraploid (DI: 1.9-2.1), hypertetraploid (DI: > 2.1), and multiploid (if more than one aneuploid peak was observed). The SPF was determined from the histogram according to a polynomial model, as the percentage of cells in the S-phase of the cycle (20). Forty-nine (22.4%) of 219 tumours could not be reliably evaluated for SPF, because of the high amount of background debris ($> 20\%$), the overlap of cell populations, or the presence of hypertetraploid or multiploid tumours. The median SPF value (5.65%) in the whole series was used to discriminate between low vs. high SPF proliferative tumours.

Statistical analysis. The statistical differences between histopathological and molecular characteristics within the two subgroups of young (≤ 45 years) and older (≥ 75 years) patients with IBC were evaluated using the two proportion Z test. Chi-squared test with Yates correction and Fisher's exact test were used for assessing differences between treatment modalities and DNA aneuploidy subcategories, respectively. The two-sample Mann-Whitney U test for equality of medians was used for assessing differences between continuous variables (21). Survival analyses were performed using the Kaplan-Meier estimation, and the differences between survival curves were evaluated by the log-rank test. All tests with a P-value of less than 5% were considered statistically significant.

Results

The mean and median values for age in the subgroup of young patients were 39.1 and 40 years (range, 19-45 years) respectively, while the corresponding values in the subgroup of older patients were 78.9 and 78 years (range, 75-91 years).

The median follow-up of the study was 90 months, ranging from two to 252 months. At the end of follow-up time, 74 patients had shown disease recurrences (48 in the younger and 26 in the older subgroup) and 61 patients had died of the disease (37 in the younger and 24 in the older subgroup).

Table II shows the differences in histopathological and molecular characteristics between the young (≤ 45 years) vs. older (≥ 75 years) patients' subgroups. Compared to the

Table I. Treatment modalities among young (≤ 45 years) and older (≥ 75 years) IBC patients in the series.

Variables	Young patients (≤ 45 years), n (%)	Older patients (≥ 75 years), n (%)	P-value ^a
Type of treatment			
Mastectomy	62 (60.2)	87 (75.0)	0.028
Breast-conserving surgery	40 (38.8)	27 (23.3)	0.019
Radiation therapy	65 (63.1)	54 (46.5)	0.020
Chemotherapy	79 (76.7)	16 (13.8)	<0.001
Hormonal therapy	41 (39.8)	69 (59.5)	0.006
Trastuzumab	1 (0.97)	1 (0.86)	NS

IBC, invasive breast carcinoma; NS, not significant. ^aPearson's Chi-squared test for homogeneity (equality of proportions); due to low frequencies, Fisher's exact test was also used to analyze the trastuzumab variable.

subgroup of older patients, young patients showed a higher frequency of IBC of no special type (NST), higher axillary lymph node involvement, more advanced disease staging and higher SPF tumour proliferative activity. The median SPF value (7.1%; range, 1.5-23.7%) in the subgroup of young patients was significantly higher than the corresponding value (4.5%; range, 0.7-26.4%) among older patients ($P=0.017$; Mann-Whitney U test) (Figs. S5 and S6). Concerning DNA ploidy pattern (Table III), no significant difference was observed between the broad dichotomy diploidy vs. aneuploidy in both subgroups, although older patients presented a higher incidence of hypertetraploid tumours ($P=0.016$; Fisher's exact test). Young patients had a higher frequency of hypodiploid and hyperdiploid tumours, but without reaching statistical significance. No statistical differences were found for the histological grade, tumour size, Ki67 index, estrogen receptors, progesterone receptors, and HER2 status. There was a trend ($P=0.058$) toward a lower incidence of Luminal A and higher incidence of Luminal B tumours in the young patients' subgroup.

Concerning the clinical outcome, statistically significant differences were found in the whole series, occurring more recurrences (46.6% vs. 22.4%; $P<0.001$) and deaths from disease (35.9% vs. 20.7%; $P=0.030$) among young patients (Table II). The finding was also observed among patients with IBC of NST ($n=186$), where young patients showed more recurrences (46.8% vs. 22.8%; $P<0.001$) and deaths from disease (35.1% vs. 20.7%; $P=0.028$) than older ones. Table IV illustrates the Kaplan-Meier 5/10-year survival estimates between both patients' subgroups. There is a statistically significant difference in DFS (Fig. 1) between young vs. older IBC patients, with the younger patients showing a less favourable clinical course. For OS, no significant differences were found. Nevertheless, an overlap of OS curves (Fig. 2) up to the first six years since diagnosis was observed, followed by worse long-term clinical evolution in the young patients' subgroup.

Discussion

IBC is a very heterogeneous disease, including distinct molecular subtypes (22,23). However, other patient-related factors, such as age at diagnosis, may affect outcome and influence

prognosis. Our present study sought to determine the impact of age (extremes of age) on patients' survival in two subgroups of young (≤ 45 years) vs. older (≥ 75 years) IBC patients, also assessing significant differences in histopathological and molecular features, with special focus on DNA flow cytometry, that could distinguish both groups of patients.

Our data confirm the view that younger patients have more aggressive disease, with an increased risk of recurrences and shorter disease-free survival (Fig. 1). Furthermore, they appear to show a worse long-term overall survival, mainly after the first six years since diagnosis (Fig. 2). Overall, the findings are clinically relevant because they indicate that age itself may influence prognosis, and thus potentially, the treatment strategies and management of patients with IBC. In this context, Beadle *et al* (4) reported that the risk of recurrence after early-stage IBC decreases with age, and is relatively high in young women, for whom maximizing loco-regional therapy should be a priority. Zavagno *et al* (24), in their study of 1226 IBC patients, analysing the influence of age and menopausal status on pathological features, also showed that the youngest (≤ 40 years) patients had the worst prognostic pattern, which improves as age increases and is the best in patients ≥ 75 years of age. However, in a prospective cohort of 594 women with early IBC, Karihtala *et al* (25) reported comparable survival rates between the age groups of <41 years, 41-69 years, and ≥ 71 years.

In our study, after initially observing the distinct clinical outcome between both subgroups, we sought to investigate further, which were the possible causes. To achieve this task, we hypothesized that tumours arising in young vs. older IBC patients could have differences in histopathological and molecular characteristics, which was confirmed. The main prognostic factors that differentiate young patients from older ones were higher axillary lymph node involvement, more advanced disease stage, and higher SPF proliferative activity. These different key aspects of tumour biology, associated with intrinsic aggressive behaviour (high SPF) and advanced stage, could be considered as constituting a specific phenotype that may explain the distinct prognosis between the two patients' subgroups. On the one hand, it is known that mutations usually accumulate in the various genes that control cell proliferation, accelerating cell division rates or inhibiting normal controls

Table II. Differences in histopathological and molecular characteristics between young (≤ 45 years) vs. older (≥ 75 years) IBC patients.

Characteristics	Young patients (≤ 45 years), n (%)	Older patients (≥ 75 years), n (%)	P-value ^a
Histological type			0.023
Invasive carcinomas of NST	94 (91.3)	92 (79.3)	
Other	9 (8.7)	24 (21.7)	
Grade of differentiation			NS
G1	20 (20.0)	28 (24.8)	
G2	52 (52.0)	59 (52.2)	
G3	28 (28.0)	26 (23.0)	
Tumour size			NS
pT1	35 (35.3)	38 (33.9)	
pT2	57 (57.6)	66 (58.9)	
PT3	7 (7.1)	8 (7.2)	
Lymph node status			<0.001
Negative	44 (44.0)	76 (66.7)	
Positive (≤ 3)	37 (37.0)	34 (29.8)	
Positive (> 3)	19 (19.0)	4 (3.5)	
Disease staging			0.021
Stage I + Stage IIA	54 (55.1)	80 (71.4)	
Stage IIB + Stage III	44 (44.9)	32 (28.6)	
DNA ploidy			NS
Diploid	45 (43.7)	57 (49.1)	
Aneuploid	58 (56.3)	59 (50.9)	
S-Phase fraction			0.021
Low	34 (40.5)	51 (59.3)	
High	50 (59.5)	35 (40.7)	
Ki67 index			NS
Low	39 (60.0)	53 (60.2)	
High	26 (40.0)	35 (39.8)	
Estrogen receptors			NS
Positive	84 (81.6)	86 (75.4)	
Negative	19 (18.4)	28 (24.6)	
Progesterone receptors			NS
Positive	66 (67.3)	67 (61.5)	
Negative	32 (32.7)	42 (38.5)	
HER2 status			NS
Negative	72 (83.7)	89 (84.8)	
Positive	14 (16.3)	16 (15.2)	
Molecular subtyping			0.058
Luminal A	26 (37.1)	43 (47.2)	
Luminal B	24 (34.3)	18 (19.8)	
HER2 positive	3 (4.3)	8 (8.8)	
Triple-negative	10 (14.3)	19 (20.9)	
Triple-positive	7 (10.0)	3 (3.3)	
Death from disease			0.030
No	66 (64.1)	87 (78.4)	
Yes	37 (35.9)	24 (21.6)	
Disease recurrence			<0.001
No	55 (53.4)	85 (76.6)	
Yes	48 (46.6)	26 (23.4)	

IBC, invasive breast carcinoma; NST, no special type; NS, not significant. ^aTwo proportion Z test; due to low frequencies, Fisher's exact test was also used to analyze the molecular subtyping variable.

Table III. DNA ploidy pattern in the subgroups of young (≤ 45 years) vs. older (≥ 75 years) IBC patients.

Variables	Young patients (≤ 45 years), n (%)	Older patients (≥ 75 years), n (%)	P-value ^a
Diploidy	45 (43.7)	57 (49.1)	NS
Aneuploidy	58 (56.3)	59 (50.9)	-
Hypodiploidy	2 (3.4)	0 (0)	NS
Hyperdiploidy	46 (79.3)	39 (66.1)	NS
Tetraploidy	4 (6.9)	6 (10.2)	NS
Hypertetraploidy	2 (3.4)	11 (18.6)	0.016
Multiploidy	4 (6.9)	3 (5.1)	NS

IBC, invasive breast carcinoma; NS, not significant. ^aFisher's exact test.

Table IV. Kaplan-Meier estimates for survival between young (≤ 45 years) vs. older (≥ 75 years) IBC patients.

Variable	5/10-year DFS (%)	P-value ^a	5/10-year OS (%)	P-value ^a
		0.04		NS
Young patients (≤ 45 years)	65.6/56.8		83.2/66.9	
Older patients (≥ 75 years)	78.6/71.7		79.3/74.2	

IBC, invasive breast carcinoma; DFS, disease-free survival; OS, overall survival; NS, not significant. ^aLog-rank test.

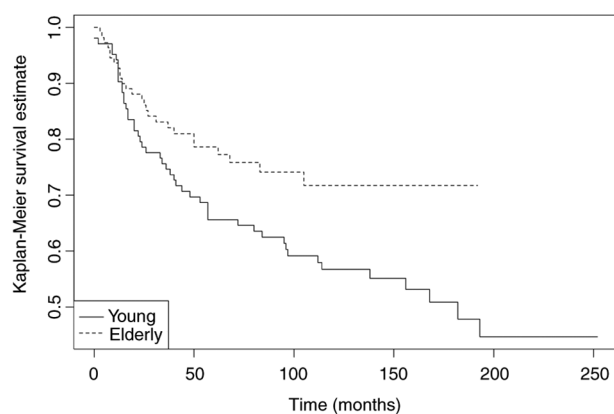


Figure 1. Kaplan-Meier survival curves for DFS between young vs. older IBC patients (P=0.04). DFS, disease-free survival; IBC, invasive breast carcinoma.

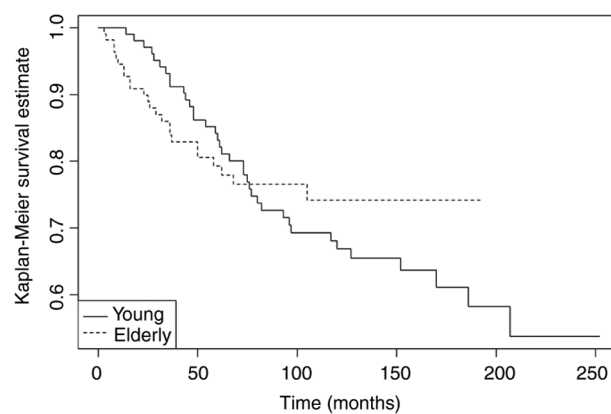


Figure 2. Kaplan-Meier survival curves for OS between young vs. older IBC patients (P=0.70). OS, overall survival; IBC, invasive breast carcinoma.

of the system, such as the cell cycle arrest or apoptosis. The challenge for research would be to identify those mutations that are responsible for the higher proliferative activity, as measured by SPF, in young IBC patients. Indeed, younger age at diagnosis has been associated with higher expression of gene signatures related to proliferation (26), but it requires further elucidation in future studies. Furthermore, Anders *et al* (27), using genomic expression analysis, identified 367 biologically relevant gene sets that could differentiate IBCs of young (≤ 45 years) patients from those of older (≥ 65 years) ones, suggesting that age-specific IBC may be considered a distinct clinical/molecular entity. Zingh *et al* (28) also showed that significantly fewer women with >70 years presented positive

lymph nodes as compared to younger patients. On the other hand, beyond the fact that young women are not routinely included in screening programs (29), a low index of suspicion and a delayed diagnosis could have an impact as compared to older counterparts for a later stage presentation (30).

Regarding surrogate molecular subtyping, we found a trend toward young IBC patients presenting a lower incidence of Luminal A and a higher incidence of Luminal B tumours, which might be related to their clinical aggressiveness. Partridge *et al* (8), studying the effect of age on survival by molecular subtype, concluded that young age seems to be particularly prognostic in patients with Luminal IBCs. In this specific Luminal subtype, Sheridan *et al* (31) showed even that young age is an independent prognostic factor for poor

outcome. In their comprehensive review (32), van Herck *et al* reported that older age is associated, beyond a higher incidence of Luminal tumours, with fewer Triple-negative and HER2-positive subtypes than a younger age. On the contrary, we found no differences in the incidence of Triple-negative and HER2-positive tumours between both groups of patients, which may be explained by missing data and a relatively small sampling size. Nevertheless, it should be noted that other authors (8), like us, have shown that young age is not a predictor of outcome in women with Triple-negative and HER2-positive subtype tumours.

Concerning the histological type, IBCs of NST were more prevalent among the subgroup of younger patients, similarly to data of Azim *et al* and Wang *et al* studies (26,33). Interestingly, within this more common type of IBC, we found that younger patients had also a worse prognosis. The K-M survival estimation was not performed in other histological types (n=33) because it seems superfluous, due to the small number of cases and weak statistical strength. However, contrarily to some studies (7,8,34-36), we could not find significant differences in other classic prognostic features, such as histological grade, tumour size, hormonal receptors, and HER2 status between both patients' subgroups.

Little attention has been paid to the association between the patient's age and tumour cell proliferation in IBC. This was the main reason to perform a thorough analysis of DNA flow cytometry parameters, ploidy and SPF, in our study. Of note, the striking finding is that, as compared to older patients, younger patients have shown tumours with statistically significant higher SPF, since high tumour proliferative rates are usually associated with more aggressive biological behaviour and adverse clinical outcome. However, contrarily to others (37), no differences related to the Ki67 index were observed, which corroborates some data disagreement, previously reported by our group (38), between the two cell proliferation markers. A higher SPF reflects alterations in DNA replication, leading to genomic instability, which has been associated with the development of lymph node metastases and worse disease outcomes (39,40). A significant difference in SPF between young vs. older IBC patients may be a specific indicator of the underlying molecular mechanisms that could differentiate the two age groups. Unfortunately, technical difficulties to assess SPF are well known, being its usefulness limited in clinical practice by a lack of inter-laboratory standardization, which includes the definition of cut-off thresholds that could reliably discriminate low vs. high SPF proliferative tumours.

Regarding the DNA ploidy pattern, no significant differences were found related to the broad dichotomy diploid vs. aneuploid tumours between both subgroups. Nevertheless, further aneuploidy subcategories analysis allowed observing interesting differences. Young patients showed mostly hyperdiploid tumours, an aneuploid category that, following an intermediate stage of tetraploidization (41,42), has been associated with a worse prognosis in IBC. On the other hand, older patients presented a significantly higher proportion of hypertetraploid tumours, a finding that has been considered as a mirror of sequential molecular alterations during life (43), and thus, more prevalent in older patients, although not necessarily related to poor prognosis.

In conclusion, our present data strongly suggest that IBCs in women of different ages could be considered different diseases or clinical entities. To support the view, the finding of distinct clinical evolution and survival, when comparing both subgroups of young (≤ 45 years) vs. older (≥ 75 years) IBC patients. Some histopathological and molecular characteristics, namely SPF, axillary lymph node status, and disease staging, appear as the main factors implicated in the distinction. Therefore, the age of patients, at least at the extremes of the spectrum, seems to be itself a relevant prognostic factor. Because it is an area largely unexplored, further studies are warranted to investigate underlying genomic, transcriptomic, and epigenetic alterations that may differentiate IBCs among patients of different ages.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AEP and SA contributed to the study conception and design, data analysis and interpretation, and wrote the manuscript. JM and TP performed the immunohistochemistry and SISH analyses. GLS performed the statistical analyses. AEP and SA confirm the authenticity of all the raw data. All authors have read, reviewed, discussed and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in this study were in accordance with the ethical standards and approved by the institutional ethical research committee of the Portuguese Institute of Oncology of Lisbon (ref. no. UIC/909). For this type of retrospective study, informed consent is not required.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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