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Comprehensive clinical and molecular analyses of neuroendocrine carcinomas of the breast

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ABSTRACT

Neuroendocrine breast carcinomas represent a rare subtype of breast cancer. Their definition, prevalence and prognosis remain controversial in the literature. The 2012 WHO classification of breast cancer categorizes neuroendocrine carcinomas into three morphologically distinct subtypes: well-differentiated neuroendocrine tumors, poorly-differentiated neuroendocrine carcinomas and invasive breast carcinomas with neuroendocrine differentiation. We aimed to gain insight into the clinical, morphologic, phenotypic and molecular features of 47 neuroendocrine breast carcinomas. Targeted next generation sequencing by an AmpliSeq 22 cancer gene hotspot panel and the Prosigna® assay were performed on 42/47 and 35/47 cases, respectively. Average age at diagnosis was 69 years old. All tumors were estrogen receptor positive and the large majority expressed progesterone receptor (89%), GATA3 (98%), FOXA1 (96%) and CK8/18 (98%). There was an almost equal distribution of luminal A (52%) and B (48%) carcinomas. Almost half of the cohort (49%) displayed a high risk of recurrence with the Prosigna® test. Patients with a neuroendocrine carcinoma had a shorter disease free survival compared to those affected by carcinomas of no special type matched for age, size, grade and estrogen receptor status. No significant differences were observed in terms of overall survival. Stratification of neuroendocrine carcinomas using the 2012 WHO criteria did not reveal statistically significant differences among the distinct categories (well differentiated neuroendocrine tumors, poorly differentiated neuroendocrine carcinomas and invasive breast carcinomas with neuroendocrine differentiation), in terms of either progression free or overall survival. Our targeted sequencing analysis found three cases (7%) harboring a *PIK3CA* mutation and in three other cases (7%) *TP53* mutations were detected. This study showed that neuroendocrine breast carcinoma is a distinct subtype of luminal carcinoma with a low rate of *PIK3CA* mutations and with an aggressive clinical behavior. An

accurate identification of neuroendocrine differentiation may be useful to better tailor patient adjuvant therapy within luminal carcinomas.

Keywords: breast carcinoma, neuroendocrine differentiation, prognosis, DNA sequencing, *PIK3CA* mutations, GATA3, FOXA1.

INTRODUCTION

Neuroendocrine breast carcinoma is a rare special histologic type of breast cancer that has similar morphologic and phenotypic characteristics to digestive and pulmonary neuroendocrine tumors (1) but also shows a certain degree of heterogeneity, including some features often indistinguishable from those of invasive carcinomas of no special type (2). They also exceptionally present with a functional syndrome (3).

In the breast, Feyrter and Hartmann recognized neuroendocrine carcinomas in 1963 (1). Using Grimelius silver staining techniques, they suggested the endocrine nature of some primary breast carcinomas. In 2003, the WHO classification of tumors of the breast and female genital organs defined neuroendocrine carcinomas as mammary carcinomas showing more than 50% of neoplastic cells expressing neuroendocrine markers by immunohistochemistry (synaptophysin, chromogranin A) (4). The revised 2012 WHO classification (5) includes three categories of neuroendocrine carcinomas: i) well differentiated carcinoma (densely cellular, solid nests or trabeculae of cells that vary from spindle to plasmocytoid and large clear cells separated by fine vascular stroma); ii) poorly differentiated/small cell carcinoma (morphologically indistinguishable from its counterpart in the lung); iii) carcinoma of no special type or for instance mucinous hypercellular variant invasive breast carcinoma with neuroendocrine differentiation demonstrated on immunohistochemistry. In this new edition, the definition of neuroendocrine carcinomas is unclear since there is no minimal percentage of tumor cell expressing neuroendocrine markers required for a diagnosis of neuroendocrine breast carcinoma.

Changes in classifications between 2003 and 2012 affect the comparison between different studies in the literature since different terminologies have been used. Some followed their own classification before 2003, or later, finding the WHO classification inadequate (6-8).

The lack of uniformity in the definition and classification of neuroendocrine carcinomas hampers an exact estimate of the prevalence of these tumors, ranging from 0.1% to 15% depending on the series (9-13). This may affect also the controversial data on the prognostic implication of neuroendocrine differentiation in breast cancer. Some have reported a better prognosis in neuroendocrine carcinomas than in patients with invasive carcinomas of no special type (9, 11, 14, 15), whereas more recently a worse prognosis has been observed in larger series of neuroendocrine carcinomas as compared to carcinomas of no special type (9, 11, 14, 15).

Molecular data on neuroendocrine carcinomas are scarce. Weigelt *et al* (16) showed that these are luminal tumors with transcriptomic profiles similar to type B mucinous carcinomas (hypercellular variant). Two studies have investigated the mutational profiling of small cohorts of neuroendocrine carcinomas by using targeted sequencing panels (17, 18) and identified recurrent mutations affecting *PIK3CA* (17, 18), the *FGFR* family (17) and chromatin remodeling genes (18).

The aims of our study were i) to characterize the molecular and phenotypic aspects of neuroendocrine carcinomas of the breast, ii) to assess differences between the three main morphologic subtypes of neuroendocrine carcinomas, and iii) to assess the prognostic impact of the distinct categories of this rare subtype.

MATERIALS AND METHODS

Cases

A series of 47 neuroendocrine carcinomas were retrieved from the files of Institut Curie, France and The University Hospital of Geneve, Switzerland by searching in the pathology files of the authors' institutions using the following terms: "Neuroendocrine; Solid papillary;

Mucinous; Chromogranin; Synaptophysin" by text recognition system within the informatics medical files of the patients.

Patients' identification was anonymized prior to analysis. Representative H&E (hematoxylin and eosin) slides were reviewed by three pathologists (ML, EM, AVS) at a multi-headed microscope to confirm the diagnosis and to categorize the cases into one of the morphologic subtypes: well differentiated, poorly differentiated/small cell carcinoma and invasive breast carcinoma with neuroendocrine differentiation according to the histologic criteria described above. For each tumor, histologic grade was assessed using the Nottingham grading system (19). The presence of associated ductal carcinoma *in situ*, lymphovascular invasion, necrosis and lymph node metastasis, were also recorded. To assess the potential impact of neuroendocrine differentiation on overall survival and disease-free survival, we strictly matched each of our cases with patients affected by invasive carcinomas of no special type, based on age at diagnosis, histologic tumor size, histologic grade and hormone receptor status. A maximum of two controls for each neuroendocrine carcinoma was included.

This series of matched invasive carcinomas of no special type that served as a control group was retrieved from the files of Institut Curie.

Samples and Immunohistochemistry

Following slide review, the most representative paraffin block was selected for immunohistochemical analysis. Details about antibodies, clones, dilutions, antigen retrieval methods used for each single antibody are reported in Supplementary Table 1. For each case, six immunohistochemical staining were performed on full sections to confirm the diagnosis of neuroendocrine carcinoma and evaluate prognostic factors: estrogen receptor, progesterone receptor, HER2, Ki67, chromogranin A and synaptophysin.

Furthermore, tissue microarrays were constructed from paraffin blocks with duplicate tumor cores. Normal peritumoral tissue was included in the tissue microarrays as a control. Immunohistochemistry was performed on three µm thick tissue microarray sections, using a panel of antibodies against androgen receptor, Forkhead Box A1 (FOXA1), GATA binding protein 3 (GATA3), Cytokeratin (CK) 8/18, CK14, Epidermal Growth Factor Receptor (EGFR), Thyroid Transcription factor 1 (TTF1), Caudal type Homeobox 2 (CDX2). Positive and negative controls were included in each experiment.

FOXA1, GATA3, AR and CDX2 expression were also investigated in the control group of invasive carcinomas of no special type matched with neuroendocrine carcinomas based on age at diagnosis, histologic tumor size, histologic grade and hormone receptor status (available samples: n= 30).

The stainings were interpreted semi-quantitatively by a pathologist (ML), who was blinded to genomic data. Inclusion criteria were the expression of at least one neuroendocrine marker (synaptophysin or chromogranin A) in at least 50% of tumor cells. The expression of estrogen and progesterone receptors was interpreted following ASCO recommendations (20). Ki67, chromogranin A, synaptophysin, androgen receptor, CK14, EGFR, CDX2, TTF1 were reported as a percentage of positive tumor cells. For GATA3, FOXA1, androgen receptor and CK8/18, percentage of staining was categorized as ‘‘0’’ if there was no nuclear expression (or cytoplasmic for CK8/18), ‘‘1’’ for up to 10% positive tumor nuclei, ‘‘2’’ for 11% to 20% and so on till a maximum score of ‘‘10.’’ Intensity was scored as ‘‘1+,’’ ‘‘2+,’’ and ‘‘3+’’ for weak, moderate, and strong staining, respectively. Percentage and intensity of nuclear expression were multiplied to generate a score (Score= Percentage x Intensity).

The scoring system updated according to 2013 ASCO guidelines (21) was used for HER2 assessment and fluorescence *in situ* hybridization was performed in case of an equivocal score 2+ result.

Tumors were then classified into molecular subtypes described by Goldhirsh *et al* (22) into luminal A, luminal B, HER2 enriched and basal-like, according to their hormone receptor status, HER2 status and proliferation assessed by the Ki67 index.

Macrodissection and DNA extraction

DNA extraction was possible for 45 cases (for two biopsy cases, the representative tumor material in the paraffin blocks was scarce). Enrichment in tumor cell content was performed with a punch-biopsy in order to ensure a percentage of tumor cells greater than 30%. Five samples showed a tumor cellularity of 30%, whereas all of the others displayed a tumor cell content ranging from 40% to 80%. DNA was then extracted using NucleoSpin® 8 tissue kit (Macherey-Nagel, Germany). Double-strand DNA concentration was measured by fluorimetric method, using Qubit dsDNA HS Assay (Life Technology). DNA quality was assessed with qPCR EGFR using Light Cycler 480 technology.

Out of 45 microdissected carcinomas, 42 yielded DNA of sufficient quantity and quality for sequencing.

Targeted Next Generation Sequencing

Forty-two cases were tested on the Ion PGM (Personal Genome Machine) Sequencer (Life Technologies).

We used an Ion AmpliSeq Cancer Panel that covers mutational hot-spots of 22 genes: *KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, FGFR3, NOTCH1, ERBB4, FGFR1, FGFR2*.

In brief, 10 ng of DNA was amplified by PCR using Ion Ampliseq Library Kit 2.0. The 90 multiplexed amplicons generated were then ligated to adapters from the Ion Xpress Barcode Adapters Kits according to the manufacturer's instructions (Ion Torrent).

Multiplexed barcoded libraries were amplified by emulsion PCR on Ion Sphere particles (ISPs, Ion PGM Template OT2 200 Kit). Sequencing was performed on a PGM sequencer (ION Torrent) using the PGM Sequencing 200 Kit V2 according to the manufacturer's instructions. Torrent Suite software version 3.4.2 was used to parse barcode reads, to align reads to the reference genome, and to generate run metrics.

Comparisons with invasive breast carcinomas from the TCGA and the METABRIC datasets

To compare the mutational frequencies of neuroendocrine carcinomas to those of estrogen receptor positive and PAM50-defined luminal A and B carcinomas we interrogated the TCGA and METABRIC datasets. For the TCGA dataset (23) we retrieved the clinicopathologic data from the UCSC Xena Browser (24), and extracted case lists based on estrogen receptor ("ER_Status_nature2012"), HER2 ("HER2_Final_nature2012"), PAM50 ("PAM50_mRNA_nature2012") and histologic type ("histological_type"). Cases with "Infiltrating Lobular Carcinoma", "Mixed Histology", "Mucinous Carcinoma", "Other" or missing histology were excluded. Somatic mutations of genes found to be mutated in neuroendocrine carcinomas were retrieved for estrogen receptor positive (n=477), luminal A (n=183), luminal B (n=111), and estrogen receptor+/HER2- (n=376) from the cBioPortal website (25).

For the METABRIC dataset we retrieved the estrogen receptor and HER2 status, histology and somatic mutations from the appropriate repository of Pereira *et al* (26, 27). PAM50 status was retrieved from the supplement of Curtis *et al* (28). Cases with "Mixed", "Lobular", "Other", "NA", "Mucinous", "Benign" or missing histology were excluded. Somatic mutations of genes found to be mutated in neuroendocrine carcinomas were extracted for

estrogen receptor positive (n=1282), luminal A (n=504), luminal B (n=378), and estrogen receptor+/HER2- (n=1125).

Since the METABRIC dataset provides information on histologic grade, we also compared the mutational frequencies of neuroendocrine carcinomas to those of estrogen receptor/HER2/grade-matched invasive carcinomas of no special type. METABRIC cases were estrogen receptor, HER2 and grade-matched to neuroendocrine carcinomas at a 1:5 ratio.

For all analyses, only mutations within the regions interrogated by the Ion AmpliSeq 22 cancer gene hotspot panel were considered. Comparisons were performed using Fisher's exact tests. p-values <0.05 were considered statistically significant.

Prosigna® assay

For 35 cases, a four µm thick section was prepared and stained with H&E (hematoxylin and eosin). A breast pathologist (JCT) identified the invasive tumor area and assessed tumor cellularity. Tumor cellularity was comprised between 20% and 80%. According to the manufacturer instruction, depending on the surface area of the tumor a series of 10µm sections were mounted onto Superfrost slides. RNA extraction and nCounter analysis were performed according to the Prosigna® instructions.

Statistical Analysis

The R (version 3.2.1, cran, http://cran.r-project.org/) and R Studio software (version 7.6, http://www.rstudio.com, R Studio, Boston, USA) were used for the statistical analysis of immunohistochemical data. Statistical comparisons were performed using Student test, Pearson correlation and Fisher's exact test. Survival curves are constructed with the Kaplan-Meyer method. Progression free survival was defined as the time between treatment and any

recurrence or evidence of metastasis. Univariate survival analyses were done according to the Cox model with log rank test. Two-sided tests with $p < 0.05$ were considered statistically significant. The Cox proportional hazards regression model was used in the multivariate survival analysis that included lymph node status and therapy.

RESULTS

Clinical analysis

The clinical features of the 47 neuroendocrine carcinomas are described in Table 1. The mean age at diagnosis was 69 years, with a median of 67 years. Forty one patients (87%) were postmenopausal. Seven patients (15%) had a history of ipsilateral or controlateral invasive carcinoma of no special type. Average tumor size was 23 mm (standard deviation: 6-70), with a median of 20 mm. During the initial management, 43 patients (91%) underwent partial or total mastectomy as treatment. Three of the four patients who did not receive a first surgery had chemotherapy first; the other was treated with radiation and hormone therapy. Axillary dissection was performed in 39 patients (83%), there was a sentinel node biopsy for 14 of them, completed in six cases with axillary dissection. Radiotherapy was performed in 36 patients. Thirty-nine patients (83%) received hormonal therapy. Two patients received targeted therapy with Herceptin. One of these two patients showed a primary tumor with *HER2* amplification confirmed by fluorescent *in situ* hybridization, the other showed a metastatic site harboring *HER2* amplification.

Local recurrence was observed in one patient; metastatic disease was present in 12 patients, of whom three at time of initial diagnosis. The most frequent metastatic sites were bone, followed by lung, bone marrow, liver, pleura, skin and two more unusual sites included the adrenal gland and pancreas.

Morphologic features

The 47 neuroendocrine carcinomas were classified as follows: 30 well differentiated carcinomas, seven poorly differentiated/small cell carcinomas and 10 invasive breast carcinomas with neuroendocrine differentiation (two lobular carcinomas, five solid papillary carcinomas, three mucinous carcinomas of type B) (Figure 1). Regarding the morphologic classification of our series: 79% were of no specific histologic type (other than showing neuroendocrine differentiation), 4% were lobular carcinomas, 11% were solid papillary carcinomas and 6% were mucinous type B carcinomas (Figure 2). Twenty nine (62%) were histologic grade 2, fifteen (32%) were grade 3 and three (6%) grade 1. The mean of mitotic count was 13 mitoses for 10 high power fields, with a range of 1-111. Vascular invasion was observed in 25% of cases and three tumors showed perineural invasion. Necrosis was not observed in this series.

Six patients received neoadjuvant chemotherapy and the corresponding tumors were neuroendocrine carcinomas of no special type, where the tumor cellularity was high despite neoadjuvant chemotherapy. Cytotoxic effects were minimal in residual cells.

Immunophenotypic analysis

Details on the immunophenotype of the 47 neuroendocrine carcinomas and comparison between different subtypes are described in detail in Table 2, Figure 2 and Supplementary Table 2.

Thirty-three tumors showed expression of chromogranin A (70%), 45 for synaptophysin (96%) and 31 both (66%) (Figure 3). The mean expression of chromogranin A was 48% of the tumor cells with values ranging from 0% to 100% and a median of 50%. Mean synaptophysin expression was 84% of the tumor cells with values ranging from 1% to 100% and a median of 100%. All cases were estrogen receptor positive (25% -100% positive

tumor cells) and the large majority (N=42, 89%) was also progesterone receptor positive (1% - 100% positive tumor cells). One tumor (2%) was *HER2* amplified and showed expression of both estrogen and progesterone receptors. The mean Ki67 proliferation index was 18% (range: 1% - 90%).

When classified into molecular subtypes by immunohistochemistry, 24 cases (52%) were estrogen receptor+/HER2-/Ki67<14% (luminal A), 22 (48%) were estrogen receptor+/HER2-/Ki67>14% (luminal B-HER2 neg) and 1 case was estrogen receptor+/HER2+/Ki67>14% (luminal B-HER2 pos).

The group of poorly differentiated neuroendocrine carcinomas was significantly more frequently of luminal B subtype (86%) compared to well differentiated carcinomas (42%) (*p=0.034*).

All tumors except one (98%) expressed CK8/18. Similarly, the large majority of tumors expressed GATA3 and FOXA1 (98% and 96% respectively), similarly to the control group of invasive carcinomas of no special type (Figure 3, Supplementary Table 3). None of the cases expressed basal markers (CK14, EGFR) nor TTF1.

All cases but one were CDX2 negative. The tumor that intensely expressed CDX2 was weakly estrogen receptor positive and showed peripheral foci of ductal carcinoma *in situ*.

Androgen receptor was significantly less frequently expressed in neuroendocrine carcinomas compared to the control group of invasive carcinomas of no special type (15% *versus* 100%, *p< 0.001*, Supplementary Table 3) and whenever expressed, the intensity ranged from 1 to 3 and the percentage tumor cells showing an expression never exceeded 50%.

Clinical and immunohistochemical variables such as size, grade, nodal status, Ki67 were not associated with a particular neuroendocrine subgroup or to GATA3, FOXA1 expression.

Survival analysis

Stratification of patients by histologic grade was statistically significant: the subset of grade 2 neuroendocrine carcinomas showed a better progression free survival compared to that of grade 3 cases (HR= 14.83, 95% CI 2.539-86.58, *p= 0.002*; Figure 4A). Overall survival was not significantly different between these two groups. The survival analysis showed that patients with a neuroendocrine carcinoma had a progression free survival significantly shorter than matched control series of patients with invasive carcinomas of no special type (HR= 7.349, 95% CI 2.399-22.51, *p= 0.0005*) (Figure 4B). However there were no differences in terms of overall survival (*p= 0.52*). Similar results for these two comparisons were obtained when these analyses were performed after excluding the patients with history of ipsilateral or contralateral invasive carcinoma of no special type as well as the patients affected by a carcinoma of histologic special type (Figure 5 A, B).

We did not observe any differences in overall survival (*p=0.4*) or progression free survival (*p= 0.42*) between well differentiated, poorly differentiated neuroendocrine carcinomas and invasive carcinomas with neuroendocrine differentiation (Figure 4C). Similar results were obtained when the analysis was run excluding the patients with history of ipsilateral or contralateral invasive carcinoma of no special type (Figure 5C).

The multivariate survival analysis that included as variables lymph node status and therapy showed that neuroendocrine differentiation was an independent predictor of poor prognosis when we assessed progression free survival (Supplementary Table 4). This analysis was performed on the cohort after exclusion of patients with history of ipsilateral or contralateral invasive carcinoma of no special type as well as the patients affected by a carcinoma of histologic special type.

Molecular analysis

Targeted sequencing using the Ion AmpliSeq 22 cancer gene hotspot panel

Targeted sequencing analysis was performed at a mean depth of 2256X (Supplementary Table 5) and 85% of analyzed cases showed a depth above 500X.

PIK3CA mutations were observed in three cases (3/42, 7%) with classical somatic activating hot-spot mutations described in the COSMIC repository, of which two were in exon 20 (kinase domain), namely a H1047R and a H1047L. The third mutation was located in exon 9 in the helical domain of the catalytic subunit p110α, i.e. a E542K. The detected allele frequencies were 10%, 40% and 5%, respectively. The three *PIK3CA* mutations were all found in well differentiated neuroendocrine tumors.

Three other cases (3/42, 7%) harbored a *TP53* mutation (C277Y, Y220C, H193R) with an allele frequency of 5%, 30% and 9%, respectively. These three *TP53* mutations are described in the COSMIC repository. Two out of the three *TP53* mutated cases were poorly differentiated neuroendocrine carcinomas (Figure 2).

All of other tested genes were not mutated in the current cohort, in the limit of the analyzed sequences.

We next compared the *PIK3CA* and *TP53* mutational frequencies found in neuroendocrine carcinomas with those of invasive breast carcinomas from TCGA and METABRIC, considering only the mutations within the regions interrogated by the Ion AmpliSeq 22 cancer gene hotspot panel. *PIK3CA* mutations were significantly less frequent in neuroendocrine carcinomas compared to luminal A, luminal B and estrogen receptor positive carcinomas from both TCGA and METABRIC, as well as from a cohort of invasive carcinomas of no special type matched for estrogen receptor/HER2 status and histologic grade extracted from the METABRIC dataset (all *p values <0.05*, Fisher's exact tests, Table 3).

TP53 mutations were significantly less prevalent in neuroendocrine carcinomas compared to luminal B from both TCGA and METABRIC ($p=0.006$ and 0.001, respectively, Fisher's exact test), as well as from estrogen receptor positive carcinomas from TCGA ($p= 0.01$, Fisher's exact test) and from the cohort of invasive carcinomas of no special type matched for estrogen receptor/HER2 status and histologic grade ($p=0.048$, Fisher's exact test).

Prosigna® analysis and assessment of risk of recurrence

For the 35 neuroendocrine carcinomas tested with Prosigna®, the distribution of molecular classes resulted in 18 luminal A tumors (51%), 16 luminal B tumors (46%), one basal-like tumor (3%). The CDX2 positive carcinoma was classified as a luminal A carcinoma. The concordance rate of molecular and immunohistochemical classification was 86%.

The five cases (14%) differently classified by Prosigna® consisted of two IHC-luminal A carcinomas re-classified as luminal B; three IHC-luminal B re-classified as two luminal A and one basal-like (Figure 2). The latter showed estrogen receptor expression in about 25% of tumor cells and was a poorly differentiated neuroendocrine carcinoma, whereas the others were all well differentiated neuroendocrine carcinomas. One of the two cases re-classified as luminal A by Prosigna® had a tumor cellularity of 30% (Figure 2).

In addition, 11 tumors (31%) were classified as Low Risk with a mean risk of recurrence (ROR) at 10 years of 4%; all of them were luminal A tumors (Figure 2). Seven tumors (20%) were classified as Intermediate Risk with a mean ROR at 10 years of 8%. Finally, seventeen tumors (49%) were classified as High Risk with a mean 10-year ROR of 31%. In the Prosigna® High Risk group, 14 tumors were luminal B and three luminal A molecular subtype (Figure 2). A percentage of tumors (18%) classified as luminal A (either by immunohistochemistry or by Prosigna®) had a high ROR.

We finally compared the ROR distribution in relation to the events observed in our cohort and observed no significant difference of ROR distribution between patients with or without disease progression or death of disease (Figure 6). This result has to be taken with caution as only one patient experienced a local relapse and 12 had distant metastasis; in addition, the follow up of the patients was shorter than 10 years.

DISCUSSION

In this study we confirm that neuroendocrine breast carcinomas can belong to either the luminal A or luminal B subgroup, however, in stark contrast to common forms of luminal carcinomas, they harbour a significantly lower frequency of *PIK3CA* mutation. In addition, our clinical data highlight a worse progression free survival of patients affected by neuroendocrine carcinomas as compared to that of patients with an invasive breast carcinoma of no special type matched for age, size, grade and estrogen receptor status.

A strong association between neuroendocrine breast carcinomas and expression of hormone receptors has been repeatedly shown by previous immunohistochemical studies (29-31). At the transcriptomic level, Weigelt *et al* have described neuroendocrine carcinomas to form a separate cluster together with mucinous type A and mucinous type B tumors within the luminal subgroups (16).

Here we found an almost equal distribution of luminal A and B (52 and 48%, respectively) tumors by Prosigna® classification. Few studies have investigated the luminal A/B distinction within neuroendocrine carcinomas. In a limited cohort of six samples, by gene expression profiling analysis Weigelt *et al* have reported neuroendocrine carcinomas to pertain to the luminal A more frequently than to the luminal B subtype (32). In a larger series Bogina *et al* (13) have recently reported that 58% of the analyzed neuroendocrine carcinomas were of

luminal B-like subtype, with the molecular classes being determined by immunohistochemistry as a surrogate for gene expression.

As all tumors but one were luminal, we next gained insight complete luminal differentiation of these tumors. We showed that neuroendocrine carcinomas are usually GATA3, FOXA1 and CK8/18 positive. FOXA1 and GATA3 expression was comparable between neuroendocrine carcinomas and the matched cohort of invasive carcinomas of no special type; this is not surprising as GATA3 and FOXA1 are usually expressed in luminal breast carcinomas (33). Since GATA3 and FOXA1 expression is lower in poorly differentiated and high grade cancers (34, 35) we investigated possible differences among the categories of neuroendocrine carcinomas: there were no significant associations between the differentiation of the neuroendocrine tumor, histologic grade and FOXA1 or GATA3 expression.

We also report on the expression of androgen receptor in neuroendocrine carcinomas, which was found in 15% of our series and this was significantly lower than in invasive carcinomas of no special type (100%). Our results are in line with a previous study demonstrating androgen receptor expression in 18% of cases (8).

Taken together, the results of this immunophenotypic characterization show that breast tumors can develop simultaneously to several differentiation pathways both endocrine and exocrine. A mixed differentiation with neuroendocrine and exocrine protein production is a phenomenon quite common and known in the tumors of endocrine and non-endocrine organs (36-38).

The landscape of genetic alterations of neuroendocrine carcinomas of the breast is yet to be fully determined as molecular studies are scarce. At present one study has focused on point mutations of a small series of neuroendocrine carcinomas and showed recurrent mutations affecting *PIK3CA* and the *FGFR* family (FGFR1 and FGFR4) (17). *HRAS* and *KDR*

mutations were observed in single cases (17). A subsequent analysis has recently showed that neuroendocrine carcinomas seem to harbor a repertoire of somatic mutations distinct from that of common types of estrogen receptor-positive/HER2-negative breast carcinomas, featuring for instance lower frequencies of *TP53* and *PIK3CA* mutations (18) and a higher frequency of mutations in chromatin remodeling genes, such as *ARID1A*. Regrettably, with the Ion AmpliSeq 22 cancer gene hotspot panel used in the present study we could not investigate the prevalence of mutations in chromatin remodeling genes in our cohort, however we were able to explore the frequency of both *TP53* and *PIK3CA* mutations. It is important to note that all of the comparative analyses were performed by considering only the mutations within the regions interrogated in the Ion AmpliSeq 22 cancer gene hotspot panel used in the present study. Our targeted sequencing analysis identified a rate of *TP53* mutations (7%, 3 cases) significantly lower than that of luminal B carcinomas from TCGA (31%) and METABRIC (26%) as well as of a matched series of estrogen receptor-positive/HER2-negative invasive carcinomas of no special type from the METABRIC dataset (20%). Interestingly, two of the three cases were poorly differentiated carcinomas, thus mirroring what has been observed in neuroendocrine carcinomas of other sites, such as for instance the lung, where *TP53* are encountered in small cell lung carcinomas and seem to be absent or rare in lung carcinoids (39, 40).

We also detected *PIK3CA* mutations in 7% of cases (all well differentiated neuroendocrine carcinomas), a rate that is significantly lower than that of estrogen receptor positive, luminal A and luminal B carcinomas calculated from TCGA (23) and METABRIC (41) datasets. Furthermore, the prevalence of *PIK3CA* mutations in neuroendocrine carcinomas was found to be significantly lower than a series of invasive carcinomas of no special type matched for estrogen receptor, HER2 status and histologic grade extracted from METABRIC (38%). As stressed above, it is crucial to highlight that when we compared the frequency of *PIK3CA*

mutations we restricted the analysis to the regions assessed by the Ion AmpliSeq 22 cancer gene hotspot panel. Still, one could argue that we cannot exclude the possibility of false negative results in our sequencing analysis due to a cellularity as low as 30% for five samples. Nevertheless, Marchiò *et al* (18) have recently reported similar findings in a series of microdissected neuroendocrine carcinomas. Taken together, these independent observations support the concept that neuroendocrine carcinomas may represent a particular entity within the luminal cancer spectrum, similar to mucinous carcinomas of the breast, for which a low frequency of *PIK3CA* mutations has been reported in other studies (42). Interestingly, neuroendocrine carcinomas of the breast have been shown to share similar transcriptomic profiles with type B mucinous carcinomas (32). Altogether, these observations may suggest a putative common initiating cancer cell for these two entities or common etiologies.

Of note, several studies in the literature show a significant association between the presence of *PIK3CA* mutation and favorable clinicopathological characteristics such as small tumor size or low grade (43, 44). As a matter of fact, our clinical data support a worse prognosis in patients affected by neuroendocrine carcinomas as compared to that of patients with invasive breast carcinomas of no special type matched for age, size, grade and estrogen receptor status. This was confirmed also in multivariate progression free survival analysis taking into account lymph node status and therapy. A large proportion of our cohort (49%) showed a high risk of recurrence by Prosigna®, with a mean 10-year risk of recurrence of 31%. Even in the luminal A subgroup, 18% of cases had a high risk of recurrence.

We finally investigated the impact of histologic subtyping of neuroendocrine breast carcinomas according to the 2012 WHO classification on long term outcomes. Some have showed a worse prognosis of the poorly differentiated subgroup (45, 46). In our series, although overall survival and progression free survival of poorly differentiated neuroendocrine carcinomas were worse compared to the other two groups, these differences

were not statistically significant. However, we were limited by the size of our cohort (respectively seven and ten cases of poorly differentiated neuroendocrine carcinoma and carcinoma with neuroendocrine differentiation). Therefore larger studies are warranted to ascertain a possible role of such classification.

In conclusion, we have shown that the rare variant of neuroendocrine breast cancer occurs predominantly in postmenopausal women, is associated with aggressive locoregional and metastatic evolution, including unusual sites (adrenal gland), with a poorer progression free survival than invasive carcinomas of no special type. At the molecular level, the subgroup of neuroendocrine carcinomas is part of the spectrum of luminal carcinomas and is characterized by low levels of *PIK3CA* mutations. This molecular feature may contribute to partly explain the unfavorable prognosis of neuroendocrine breast carcinomas. Larger molecular profiling studies of this entity with correlation with clinical data are warranted.

Currently, the clinical decision making for patients affected by a neuroendocrine carcinoma is based on proper grading and immunophenotyping of the lesions, similar to any invasive carcinomas of no special type. Nevertheless, our results also suggest that an accurate identification of this entity may be useful to better tailor patient adjuvant therapy within luminal carcinomas.

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FIGURE LEGENDS

Figure 1: **Examples of the three subtypes of neuroendocrine carcinomas.** A: well differentiated neuroendocrine carcinoma (H&E x 40); B: well differentiated neuroendocrine carcinoma (H&E x 400); C: poorly differentiated neuroendocrine carcinoma (H&E x 100); D: poorly differentiated neuroendocrine carcinoma (H&E x 200); E: solid papillary carcinoma (H&E x 100); F: mucinous carcinoma (H&E x 200).

Figure 2: Overview of the cohort of neuroendocrine carcinomas. Heatmap illustrating the histologic and immunohistochemical features, IHC- and Prosigna®- defined molecular subtype, sequencing results, cellularity of the samples and clinical information. Cases are represented in columns; parameters are depicted in rows and color-coded according to the key. AR: androgen receptor; ER: estrogen receptor; NA: not available; NGS: next generation sequencing; ROR: risk of recurrence; WT: wild type.

Figure 3: Immunohistochemistry for neuroendocrine carcinomas. A: Chromogranin-A (well differentiated neuroendocrine carcinoma); B: Synaptophysin (poorly differentiated neuroendocrine carcinoma); C: FOXA1 (mucinous carcinoma, strong nuclear stain); D: GATA 3 (poorly differentiated neuroendocrine carcinoma, strong nuclear stain).

Figure 4: Survival Curves for the whole cohort (N= 47) of neuroendocrine carcinomas. A: progression free survival of neuroendocrine carcinomas stratified by grade; B: progression free survival of neuroendocrine carcinomas matched to patients with invasive carcinomas of no special type; C: overall survival of neuroendocrine carcinomas stratified by histologic subtype (30 well differentiated neuroendocrine carcinomas, 7 poorly differentiated neuroendocrine carcinomas, and 10 carcinomas with neuroendocrine differentiation).

NEBC: neuroendocrine breast carcinoma; NST BC: breast carcinoma of no special type; PFS: progression free survival.

Figure 5: Survival analysis for a restricted cohort of neuroendocrine carcinomas. A: after excluding the patients with history of ipsilateral or contralateral invasive carcinoma of no special type as well as the patients affected by a carcinoma of histologic special type the subset of grade 2 carcinomas still showed a better progression free survival compared to that of grade 3 cases (HR= 12.39, 95% IC 1.66-92.18, *p=0.01*). B: after excluding the patients with history of ipsilateral or contralateral invasive carcinoma of no special type as well as the patients affected by a carcinoma of histologic special type patients with a neuroendocrine carcinoma still had a progression free survival significantly shorter than matched control series of patients with invasive carcinomas of no special type (HR=8.37, 95% IC 1.72-40.99, *p=0.008*). C: We did not reveal any differences progression free survival (*p=0.35*) between well differentiated, poorly differentiated neuroendocrine carcinomas and invasive carcinomas with neuroendocrine differentiation after excluding patients with history of ipsilateral or contralateral invasive carcinoma of no special type.

NEBC: neuroendocrine breast carcinoma; NST BC: breast carcinoma of no special type; PFS: progression free survival.

Figure 6: ROR distribution and patient survival. There were no significant differences in ROR distribution between patients with or without disease progression or death of disease.

Table 1: Clinical features and treatment of the cohort of 47 neuroendocrine breast carcinomas and the control cohort of invasive carcinomas of no special type (N= 94). Cases were not matched according to patient treatment procedures. Importantly, the major prognostic parameters (size, nodal status, hormone receptors, HER2, Ki67, histologic grade) were not statistically significantly different between the two cohorts.

		Neuroendocrine Carcinomas (N=47)	*(%)*	Control cohort (N=94)	*(%)*	*p value*
Age						
	mean	69	_	67		0.97
	min-max	33-91	_	33-89		
	≤50	7	*15*	13	14	
	> 50	40	*85*	81	86	
Size (cm)						0.53
	T1 (<2)	28	*60*	55	59	
	T2 (2 - 5)	16	*34*	36	38	
	T3 (>5)	2	*4*	1	2	
	T4	1	*2*	1	1	
LN status						0.2
	N0	22	*47*	44	68	
	N1	17	*36*	21	32	
	N2	1	*2*	0	0	
	N3	0	*0*	0	0	
	not known	7	*15*	29		
ER						-
	< 10%	0	_	0	0	
	≥ 10 %	47	_	94	100	
PR						0.67
	< 10%	10		24	25	
	≥ 10 %	36		70	75	
Grade						0.74
	1	3	*6*	8	8	
	2	29	*62*	62	66	
	3	15	*32*	24	26	
HER2 IHC						0.25
	negative	46	*98*	61	100	
	positive	1	2	0		
	Not known	0		33		
Ki67						0.4
	mean	18	_	20		
	range	1;90	_	1;60		
Surgery						1.26e-05

	No	4	*9*	0		
	Lumpectomy	27	*59*	56	57	
	Mastectomy	9	*19*	38	40	
	Lumpectomy followed by mastectomy	6	*13*	0		
LN surgery						0.002
	No	4	*9*	0		
	Sentinel node	14	*30*	12	13	
	Sentinel node followed by axillary dissection	6	*13*	15	17	
	Axillary dissection	19	*40*	63	70	
	Not known	4	*8*	4		
Chemotherapy						0.002
	No	27	*58*	61	65	
	Adjuvant setting	13	*27*	33	35	
	Neoadjuvant setting	6	*13*	0		
	Not known	1	*2*	0		
Radiotherapy						0.0001
	Not received	9	*19*	0	0	
	Received	36	*77*	69	100	
	Not known	2	*4*	25		
Hormonal therapy						0.47
	Not received	6	*13*	18	19	
	Received	39	*83*	76	81	
	Not known	2	*4*	0		
Herceptin®						0.67
	Not received	45	*96*	93	99	
	Received	2	*4*	1	1	
Local relapse						0.67
	yes	37	*79*	89	95	
	no	1	*2*	5	5	
	Not known	9	*19*	0		
Metastasis						0.0001
	no	26	*55*	89	95	
	yes	12	*26*	5	5	
	Not known	9	*19*	0		
Deaths						0.58
	yes	33	*70*	79	85	
	no	4	*9*	14	15	
	Not known	10	*21*	1		

ER: estrogen receptor; IHC: immunohistochemistry; LN: lymph node; PR: progesterone receptor.

Table 2: Clinical and immunohistochemical characteristics of neuroendocrine carcinomas according to their differentiation.

PARAMETERS		**NEUROENDOCRINE DIFFERENTIATION**			***p value***
Morphology		Well differentiated (n= 30)	Poorly differentiated (n=7)	With neuroendocrine differentiation (n=10)	
					0.92
Size	< 20 mm	13	3	5	
	≥ 20 mm	17	4	5	
Grade	1	2	0	1	*0.32*
	2	19	3	7	
	3	9	4	2	
N	0	14	2	6	*0.99*
	1 - 3	11	1	3	
	4 - 9	1	0	0	
	> 9	0	0	0	
Immunohistochemistry					
ER	< 10%	0	0	0	-
	≥10%	30	7	10	
PR	< 10%	5	3	1	*0.13*
	≥ 10%	25	4	8	
HER2	0	20	2	7	*0.22*
	1+	6	3	3	
	2+	3	2	0	
	3+	1	0	0	
Ki67	< 14 %	13	1	6	*0,17*
	≥ 14 %	17	6	4	
Chromogranin-A	Negative	8	2	2	*0.89*
	Positive	22	5	8	
Synaptophysin	Negative	2	0	0	*0.55*
	Positive	28	7	10	
CK8/18	Negative	0	1	0	*0.06*
	Positive	30	6	10	
GATA3	Negative	1	0	0	*0.74*
	Positive	29	7	10	
FOXA1	Negative	2	0	0	*0.55*
	Positive	28	7	10	
AR	Negative	26	5	9	*0.52*
	Positive	4	2	1	
CDX2	Negative	29	7	10	*0.74*
	Positive	1	0	0	
Molecular Subgroups					
Immunohistochemistry	Luminal A	18	1	5	*0.10*
	Luminal B	14	6	3	

Prosigna®	Luminal A	13	1	4	*0.62*
	Luminal B	12	2	2	
	ROR	50 [4-96]	49 [31-67]	32 [19-64]	*0.53*

AR: Androgen Receptor; CDX2: Homeobox protein CDX-2; CK: cytokeratin; ER: estrogen receptor; FOXA1: Forkhead Box A1; GATA3: GATA binding protein 3; N: lymph nodes; PR: progesterone receptor; ROR: risk of recurrence.

Table 3: Frequency of *PIK3CA* and *TP53* mutations in our cohort of neuroendocrine carcinomas in comparison with breast carcinomas from METABRIC and TCGA datasets. As all cases from our cohort were estrogen receptor positive, comparisons were performed with luminal A, luminal B or estrogen receptor positive breast carcinomas. Since the METABRIC dataset provides information on histologic grade of included cases, we also matched 1:5 our cohort of neuroendocrine carcinomas subjected to sequencing (n= 42) with a series of invasive carcinomas of no special type (n= 215) of comparable histologic grade and estrogen receptor/HER2 status. Only hotspot mutations present in our Ion AmpliSeq Cancer Panel were taken into account for these analyses: *PIK3CA* mutations at codons 542, 545, 726, 1043, 1044 and 1047; *TP53* mutations in exons 2, 4-8 and 10, as well as splice sites for exons 5, 6, 7 (5' splice site only for exon 7), and 10. Comparisons were performed using Fisher's exact tests. p-values <0.05 were considered statistically significant (highlighted in bold).

		METABRIC								TCGA					
Mutated gene	**NEBCs (N= 42)**	**ER/HER2/ G matched IC-NSTs (N= 215)**	***p value***	**Lum A carcinomas (N= 504)**	***p value***	**Lum B carcinomas (N=378)**	***p value***	**ER+ (N=1282)**	***p value***	**Lum A carcinomas (N= 183)**	***p value***	**Lum B carcinomas (N=111)**	***p value***	**ER+ (N= 477)**	***p value***
PIK3CA	3 (7%)	81 (38%)	<0.0001	232 (46%)	**<0.0001**	102 (27%)	**0.004**	459 (36%)	**0.0001**	66 (36%)	**0.0002**	27 (24%)	**0.01**	102 (21%)	**0.02**
TP53	3 (7%)	44 (20%)	**0.04**	50 (12%)	0.5	99 (26%)	**0.006**	285 (22%)	**0.01**	25 (14%)	0.24	35 (31%)	**0.001**	84 (18%)	0.08

ER: estrogen receptor; G: histologic grade; Lum: Luminal; NEBCs: neuroendocrine breast carcinomas.

Figure 1

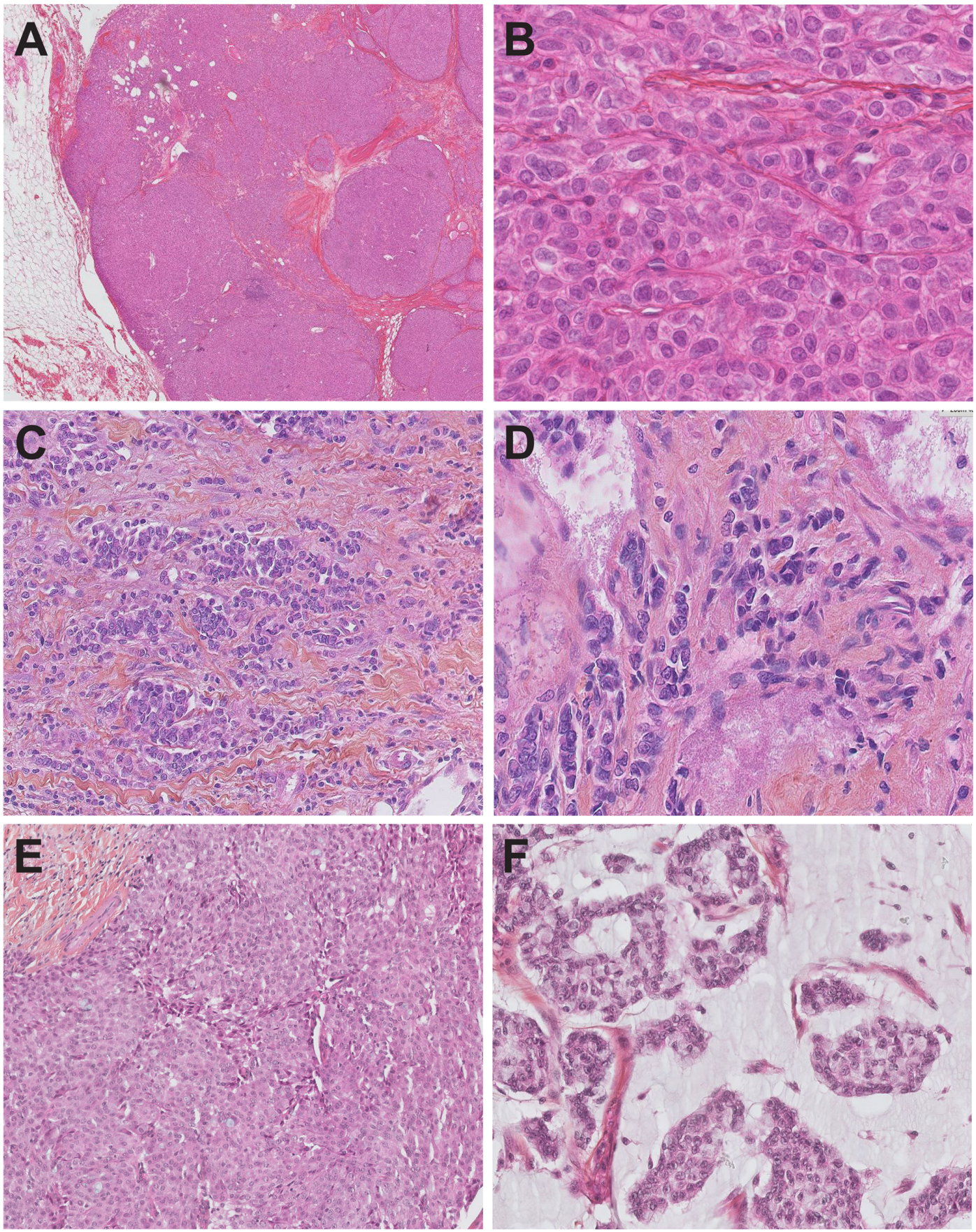
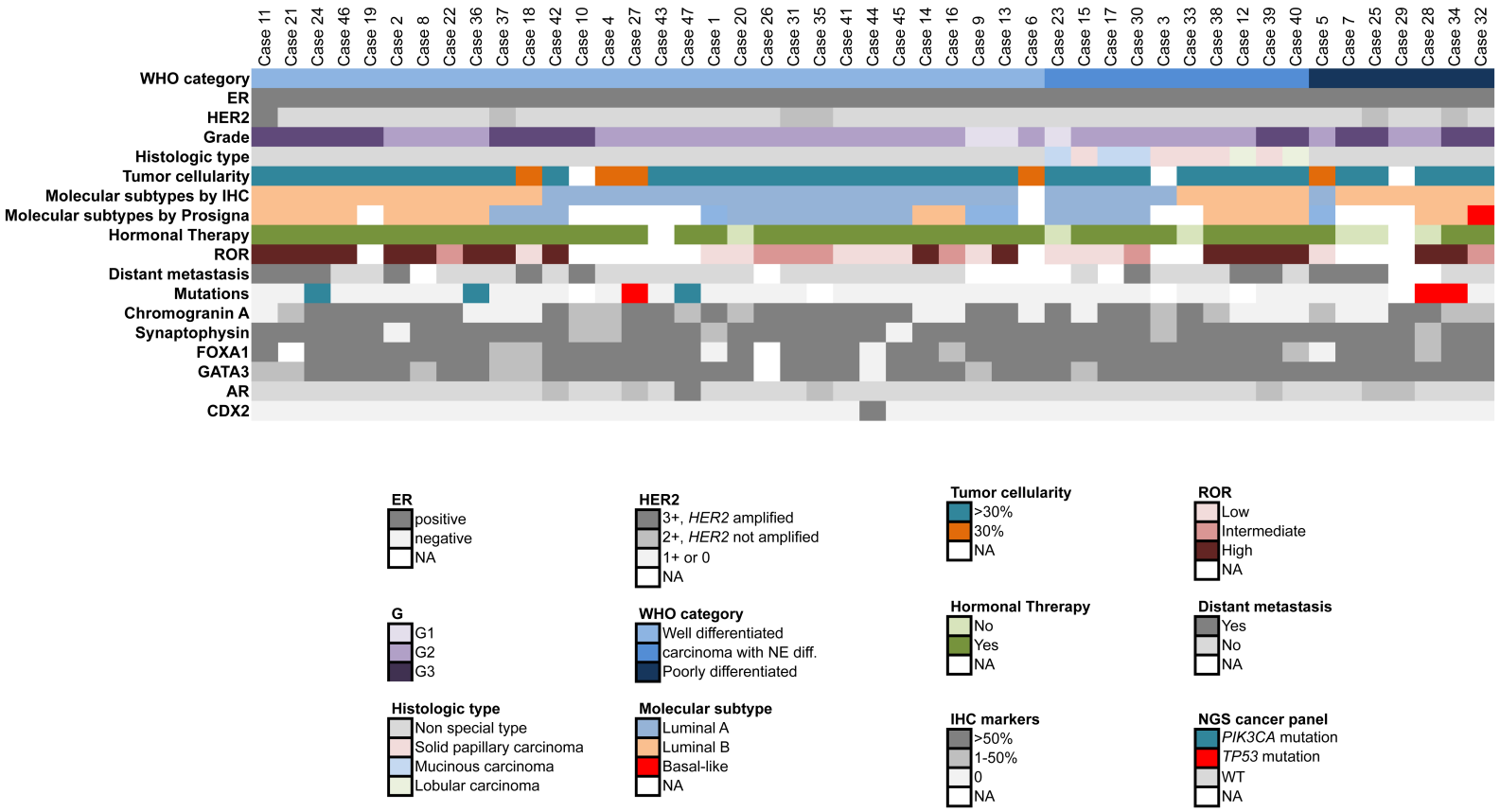


Figure 2


Case 11
Case 21
Case 24
Case 46
Case 19
Case 2
Case 8
Case 22
Case 36
Case 37
Case 18
Case 42
Case 10
Case 4
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Case 43
Case 47
Case 1
Case 20
Case 26
Case 31
Case 35
Case 41
Case 44
Case 45
Case 14
Case 16
Case 9
Case 13
Case 6
Case 23
Case 15
Case 17
Case 30
Case 3
Case 33
Case 38
Case 12
Case 39
Case 40
Case 5
Case 7
Case 25
Case 29
Case 28
Case 34
Case 32
WHO category
ER
HER2
Grade
Histologic type
Tumor cellularity
Molecular subtypes by IHC
Molecular subtypes by Prosigna
Hormonal Therapy
ROR
Distant metastasis
Mutations
Chromogranin A
Synaptophysin
FOXA1
GATA3
AR
CDX2
ER
positive
negative
NA
HER2
3+, HER2 amplified
2+, HER2 not amplified
1+ or 0
NA
Tumor cellularity
>30%
30%
NA
ROR
Low
Intermediate
High
NA
G
G1
G2
G3
WHO category
Well differentiated
carcinoma with NE diff.
Poorly differentiated
Hormonal Threrapy
No
Yes
NA
Distant metastasis
Yes
No
NA
Histologic type
Non special type
Solid papillary carcinoma
Mucinous carcinoma
Lobular carcinoma
Molecular subtype
Luminal A
Luminal B
Basal-like
NA
IHC markers
>50%
1-50%
0
NA
NGS cancer panel
PIK3CA mutation
TP53 mutation
WT
NA

Figure 3

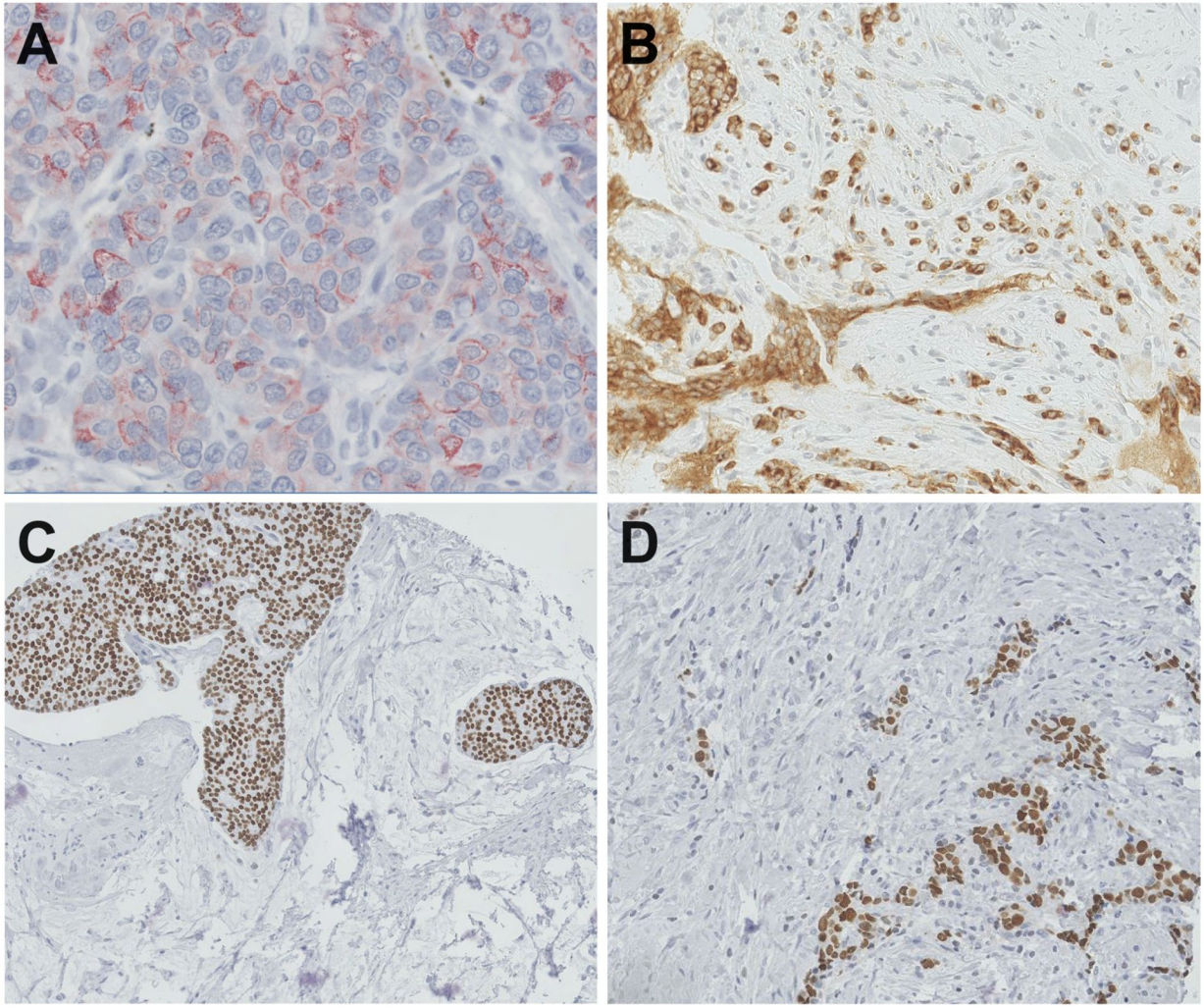


Figure 4

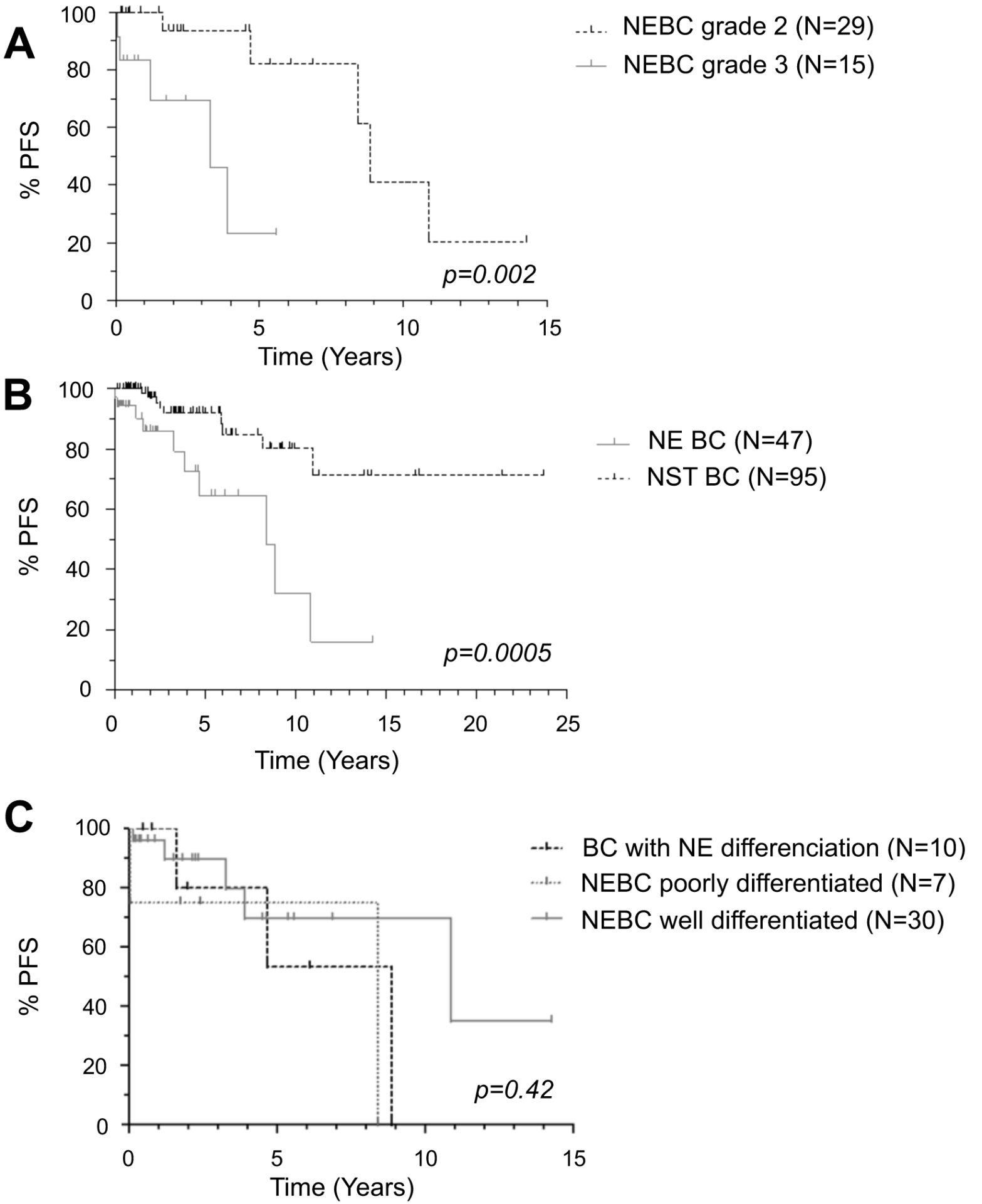
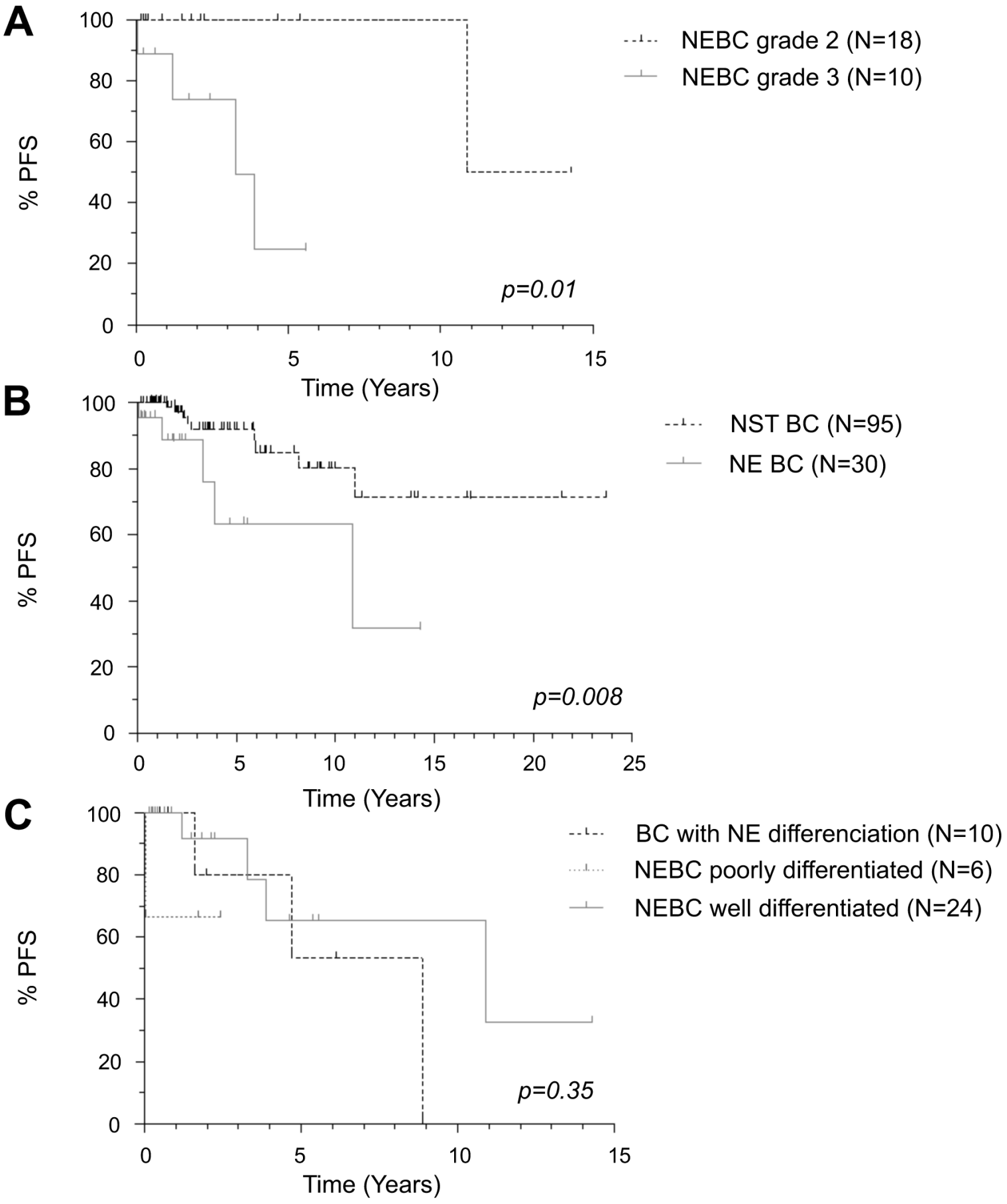

A
100
80
60
40
20
0
% PFS
0
5
10
15
Time (Years)
p=0.002
NEBC grade 2 (N=29)
NEBC grade 3 (N=15)
B
100
80
60
40
20
0
% PFS
0
5
10
15
20
25
Time (Years)
p=0.0005
NE BC (N=47)
NST BC (N=95)
C
100
80
60
40
20
0
% PFS
0
5
10
15
Time (Years)
p=0.42
BC with NE differenciation (N=10)
NEBC poorly differentiated (N=7)
NEBC well differentiated (N=30)

Figure 5


A
100
80
60
40
20
0
% PFS
0
5
10
15
Time (Years)
p=0.01
NEBC grade 2 (N=18)
NEBC grade 3 (N=10)
B
100
80
60
40
20
0
% PFS
0
5
10
15
20
25
Time (Years)
p=0.008
NST BC (N=95)
NE BC (N=30)
C
100
80
60
40
20
0
% PFS
0
5
10
15
Time (Years)
p=0.35
BC with NE differenciation (N=10)
NEBC poorly differentiated (N=6)
NEBC well differentiated (N=24)

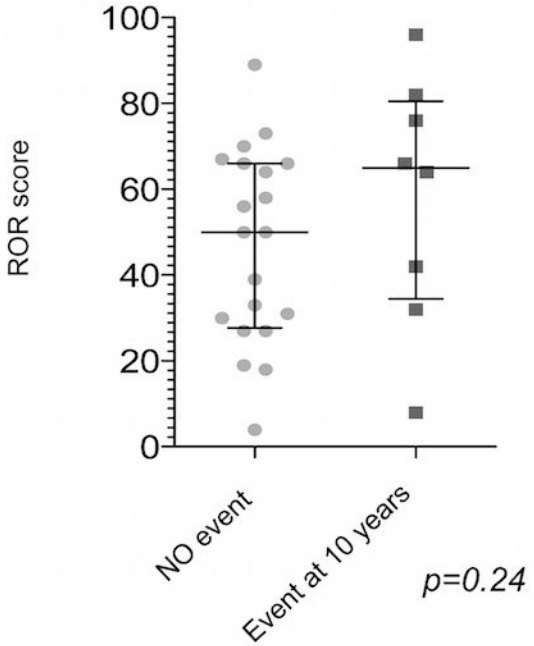



Figure 6