Title Page

Title: Basal biomarkers Nestin and INPP4B predict gemcitabine benefit in metastatic breast cancer: samples from the phase III SBG0102 clinical trial

Short title: Nestin and INPP4B markers in breast cancer

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Key-words: Nestin, INPP4B, Predictive biomarker, Basal-like breast cancer, Gemcitabine

Novelty and Impact: The current study is important in the decision-making setting for the aggressive basal-like subgroup of breast cancer that still lacks successful targeted therapies. Current immunohistochemical definitions to identify basal-like are limited to estrogen receptor negative and miss some estrogen receptor positive cases that are classified as basal-like by gene expression. This study offers an opportunity to more accurately identify basal-like patients using a practical and inexpensive immunohistochemical technology to provide them with better tailored chemotherapies.

Abbreviations used:

IHC: immunohistochemistry; INPP4B: inositol-polyphosphate-4-phosphate; OS: overall survival HER2: human epidermal growth factor receptor-2; ER: estrogen receptor; PR: progesterone receptor; Cdk5: cyclin-dependent kinase-5; PI3K: phosphoinositide 3-kinase; D: docetaxel GD: gemcitabine-docetaxel; REMARK: Reporting Recommendations for Tumor Marker Prognostic Studies; TTP: time to tumor progression; ECOG: Eastern Cooperative Oncology Group

Article Category: Tumor Markers and Signatures

Abstract

In a formal prospective-retrospective analysis of the phase III SBG0102 clinical trial randomizing metastatic breast cancer patients to gemcitabine-docetaxel or to single agent docetaxel, patients with basal-like tumors by PAM50 gene expression had significantly better overall survival in the gemcitabine arm. By immunohistochemistry (IHC), triple negative status was not predictive, but more specific biomarkers have since become available defining basal-like by nestin positivity or loss of inositol-polyphosphate-4-phosphate (INPP4B). Here, we evaluate their capacity to identify which patients benefit from gemcitabine in the metastatic setting.

Nestin and INPP4B staining and interpretation followed published methods. A prespecified statistical plan evaluated the primary hypothesis that patients with basal-like breast cancer, defined as "nestin+ or INPP4B-", would have superior overall survival on gemcitabine-docetaxel when compared to docetaxel. Interaction tests, Kaplan-Meier curves and forest plots were used to assess prognostic and predictive capacities of biomarkers relative to treatment.

Among 239 cases evaluable for this study, 36 (15%) had been classified as basal-like by PAM50. "Nestin+ or INPP4B-" was observed in 41 (17%) of the total cases and was significantly associated with PAM50 basal-like subtype. Within an estimated median follow-up of 13 years, patients assigned as IHC basal "nestin+ or INPP4B-" had significantly better overall survival on gemcitabine-docetaxel versus docetaxel monotherapy (HR=0.31, 95%CI: 0.16-0.60), whereas no differences were observed for other patients (HR=0.99), *P*-interaction<0.01.

In the metastatic setting, women with IHC basal breast cancers defined as "nestin+ or INPP4B-" have superior overall survival when randomized to gemcitabine-containing chemotherapy compared to docetaxel alone. These findings need to be validated using larger prospective-retrospective phase III clinical trials series.

Manuscript

Introduction:

Despite the high efficacy of adjuvant chemotherapy in early breast cancer [1] [2], up to a third of breast cancer patients still recur and die [3]. Currently, there is no consensus standard of care for chemotherapy regimens in metastatic breast cancer patients, who get agents that are recommended for the whole breast cancer population without regard to intrinsic molecular subtype [4]. Molecular distinctions among different breast cancer intrinsic subtypes were not prospectively incorporated into clinical trial designs until relatively recently. Gene expression assays can identify molecular subtypes and some have become recommended biomarkers for clinical use [5] [6]. Gene expression assays such as PAM50 have found that intrinsic breast cancer subtypes display distinct sensitivities to specific chemotherapies, implying that subtype could play a major role in tailoring treatment decisions for patients [7] [8] [9] [10]. Gemcitabine is a nucleoside analogue, a fluorinated deoxycytidine analogue prodrug. Once inside the cell, gemcitabine is phosphorylated into its active metabolites. These metabolites are incorporated into the growing DNA strand, terminating DNA polymerase processing and eventually DNA synthesis [11] [12] [13].

Data on gemcitabine from different trials evaluating its combination with other agents such as taxanes have displayed inconsistent results, with no definitive conclusions regarding their benefit in metastatic breast cancer [14] [15] [16] [17]. These limitations, along with gemcitabine's association with a higher incidence of hematological toxic effects [17] than is seen with other regimens such as taxanes, has made its use in the metastatic setting generally reserved for advanced lines of therapy rather than as a first line choice [4]. In general, sequential single agent chemotherapies rather than combination therapies are currently recommended for the treatment of advanced human epidermal growth factor receptor-2 (HER2) negative breast cancer [18]. As a

consequence, the addition of docetaxel to gemcitabine became less common recently, but is still given by some institutions based on physician's choice.

Preclinical data have demonstrated that tumors defective in DNA repair pathways are more sensitive to nucleoside analogues, such as gemcitabine [19]. In addition, separate lines of research have shown that BRCA1-deficient and basal-like tumors share similar molecular characteristics, including evidence that basal-like breast cancers display defects in the BRCA1 DNA repair system [20 21]. Taken together, these preclinical findings support a potentially larger role of nucleoside analogues specifically in basal-like breast cancers when compared to other subtypes.

Based on this preclinical data, our group performed a formal prospective-retrospective test of the prespecified hypothesis that basal-like breast cancers will do better on nucleoside analogue-based regimens when compared to other intrinsic breast cancer subtypes [22] using specimens from the Danish SBG0102 phase III clinical trial [15]. This trial randomized patients with advanced breast cancer to docetaxel alone, or to gemcitabine plus (lower dose) docetaxel [15]. While the original trial reported non-significant results in overall survival (OS) between gemcitabine-docetaxel doublet versus docetaxel monotherapy, analysis according to intrinsic subtype by NanoString PAM50 assay showed that basal breast cancer is the only subtype that benefitted from adding the nucleoside analogue gemcitabine to docetaxel [22]. In contrast, HER2 enriched subtype patients survived longer with higher dose docetaxel monotherapy when compared to the doublet arm, cancelling out the reverse effect in basals in the original trial [22]. As such, the overall trial result was considered negative as intrinsic subtype was not a preplanned subset analysis. Interestingly, the "triple negative" definition, characterized by negative staining

for estrogen (ER), progesterone (PR) and HER2 receptors, was also not able to identify a group that did better on gemcitabine.

The greater specificity of gene expression-based intrinsic subtyping over current immunohistochemical (IHC) "triple negative" classifications for identifying the basal-like subtype is counterbalanced, in practice, by limitations of accessibility, complexity and cost. Thus, the development of better, more specific IHC biomarkers for the basal-like subtype remains warranted. After testing many published biomarkers, an IHC panel defining basal-like breast cancers as either nestin positive or displaying loss of inositol-polyphosphate-4-phosphate (INPP4B) expression – a definition independent of ER, PR and HER2 status – was recently optimized by our group, and shown to be more strongly associated with a basal-like gene expression profile than other previously published IHC definitions [23].

Nestin is an intermediate filament, originally identified as expressed on neural stem cells but since shown to play a role in cell proliferation, cell survival and apoptosis through regulating cyclin-dependent kinase-5 (cdk5), phosphoinositide 3-kinase (PI3K), and AKT [24 25 26]. Nestin is associated with epithelial-mesenchymal transition, cell migration and invasion via the Wnt/β-catenin pathway [27]. INPP4B in contrast is a tumor suppressor protein, previously reported to be lost in the majority of basal-like breast cancers [28]. When expressed, INPP4B acts to inhibit PI3K/AKT signaling, a cell growth and survival pathway that is frequently activated in aggressive breast cancers [28].

In the current study, we apply this IHC-basal panel, defining basal-like cases as nestin positive or INPP4B negative, to breast tumors obtained from patients in the phase III SBG0102 randomized clinical trial, in order to (1) confirm its association with the basal-like subtype by

PAM50 gene expression and (2) to assess its capacity to predict benefit from a gemcitabine-containing chemotherapy regimen.

Methods:

Study Population and Design

The current study used primary tumor tissue specimens assembled from the prospective Danish SBG0102 phase III randomized clinical trial that was conducted in 12 Danish centers during the period December 2001 to October 2005 [15]. The SBG0102 trial enrolled 337 patients who were randomized to receive either docetaxel (D) alone or docetaxel in combination with gemcitabine (GD) for locally advanced or metastatic breast cancer disease. Treatment in the D arm used intravenous docetaxel 100 mg/m^2, given on day 8 every 21 days, while the GD arm included intravenous gemcitabine 1,000 mg/m^2, administrated on days 1 and 8 plus intravenous docetaxel at the reduced dose of 75mg/m^2 on day 8 every 21 days [15]. Treatment regimens were continued until progression, unacceptable toxicity, or withdrawal. Full details on the trial characteristics, eligibility criteria and protocols have been published [15]. The study was conducted in accordance with the Declaration of Helsinki and approved by the Danish National Committee on Biomedical Research Ethics for the original study protocol as well as for correlative study tissue assembly, biomarker assessments and analyses [KF-12-315632 (August 2006)/ H-KF-02-045-01 (June 2007), and Uniform Biological Material Transfer Agreement (January 2014)].

This study was designed in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria [29] that sets guidelines for use of archived trial specimens for biomarker evaluation [30]. The current study was conducted in a prospective-retrospective design [30] and aimed to assess the prespecified hypothesis that patients assigned as basal-like breast cancer, as defined by the IHC panel of nestin positivity or INPP4B negativity, will have a

greater benefit from GD versus D for the primary endpoint of OS. For this correlative study, the secondary end point was time to tumor progression (TTP).

Tissue microarrays and IHC biomarker assessment

Formalin-fixed paraffin-embedded tissue microarray blocks corresponding to 270 patients recruited in the SBG0102 trial were provided by the Danish Breast Cancer Cooperative Group Tumor Tissue Data Repository. The current study utilized a series of 11 tissue microarrays that included tissue cores of 2mm diameter, constructed from primary invasive breast tumor samples per a previously described protocol [31]. Serial 4 μm sections from these tissue microarrays were stained for nestin and INPP4B using previously published methods [23 32]. Slides for nestin staining underwent antigen retrieval with mild Cell Conditioning 1 reagent treatment (Ventana Medical Systems Inc. Tucson, AZ USA) followed by 60 minutes of primary antibody incubation with heat, and detected using a DAB Map Detection Kit (Ventana Medical Systems). The primary antibody applied for nestin was a commercial mouse monoclonal antibody at a dilution of 1:50 (Santa Cruz Biotechnology Inc., clone 10C2, Dallas, Texas USA), and was followed by an incubation of the slides with a Ventana universal secondary antibody for an additional 32 minutes following the Discovery XT semi-automated immunostainer protocol. Slides for INPP4B staining underwent antigen retrieval with mild standard Cell Conditioning 1 (Ventana Medical Systems Inc. Tucson, AZ USA) followed by 2 hours of primary antibody incubation, and detected using a DAB Map Detection Kit (Ventana Medical Systems). The staining with the primary antibody for INPP4B applied a monoclonal rabbit antibody (Epitomics, clone EPR3108Y, Burlingame, CA USA) at a 1:50 dilution and was followed by an incubation of the slides with anti-rabbit horseradish peroxidase secondary antibody for an additional 16 minutes, following the Discovery XT semi-automated immunostainer protocol. Staining and visual

scoring for additional IHC markers detecting ER, PR, HER2, cytokeratin 5 and epidermal growth factor receptor were also performed as previously described in publications from the Genetic Pathology Evaluation Center [33] [34] [35].

Nestin and INPP4B Scoring System and PAM50 Intrinsic Subtyping

Scoring of biomarkers was performed by pathologists who did not have any access to clinical or molecular data. The scoring method used prespecified published criteria for nestin and for INPP4B as previously reported by others [32] [36] and validated by our group [37] [23] [38] . Nestin expression was considered positive if cytoplasmic staining of any intensity above background was observed in ≥1% of invasive breast carcinoma cells. INPP4B negativity was defined as cytoplasmic expression in <5%. For statistical analysis, consistent with previous publications, nestin expression was binarized into negative (<1%) or positive (≥1%) categories and similarly INPP4B expression was categorized as negative (<5%) or positive (≥5%). Representative positive and negative stains for nestin and INPP4B have been shown in previous publications [23] [37].

PAM50 nCounter Intrinsic Subtyping

The full methods involved in PAM50 nCounter intrinsic subtyping for the SBG0102 clinical trial, including slide macrodissection, RNA extraction, NanoString nCounter processing and analysis for the study cohort were previously described and published [22]. The classification of intrinsic subtypes according to PAM50 was conducted in a blinded way, using barcoded identification and without access to pathological data or clinical outcome information from the SBG0102 trial.

Statistical Analysis

This study's statistical analysis plan was prespecified by the Vancouver group and then independently executed by the Danish Breast Cancer Cooperative Group central statistical office. Chi-square or Fisher's exact testing were used to assess the associations between biomarker IHC expression, clinicopathological characteristics and intrinsic molecular subtypes. The associations between clinicopathological characteristics and PAM50 subtype calls have been previously published [22]. The performance characteristics of IHC nestin/INPP4B against a PAM50 gene profiling gold standard were assessed by Cohen's Kappa Coefficient test (k). Kaplan-Meier curves were used to display survival outcomes according to biomarker expression status. The primary outcome was OS, defined as time from randomization to date of death with censoring for surviving patients at last update from the Danish Civil Registration System (31-12-2016). Time to tumor progression was defined as a secondary endpoint and measured from random assignment to date of documented progression with censoring or death at last follow-up. Stratified log-rank testing was used to assess differences in clinical outcomes between biomarker-defined patient groups. Cox regression was used to assess the hazard ratio in univariate and multivariate survival analyses according to IHC biomarker expression status. *P*-values for tests of proportional hazard were calculated using Schoenfeld residuals models. Predictive effects for biomarker stratification according to prespecified cut-points were evaluated using the Wald test of interaction between marker expression groups and treatment arm. Results were displayed using forest plots. All tests performed were 2-sided at a significance level of 0.05. Statistical analyses were conducted using the SAS System (version v9.4).

Results:

Two hundred and seventy archival tumor tissue samples were available from the 337 patients enrolled in the SBG0102 trial and included in this translational study. Details regarding the study workflow are displayed in a CONSORT flow diagram (Supplementary Fig. 1). Biomarker data including IHC results for either nestin or INPP4B biomarkers were obtained for 252 cases, among which 239 had available staining for both nestin and INPP4B (Supplementary Table 1). The majority of the included cohort (93%, n=222) consisted of patients with metastatic disease, and there were no imbalances in clinical characteristics observed between the included versus the excluded study populations.

Identification of intrinsic PAM50 subtype by IHC biomarker expression

Classification of the study cohort by gene expression into different PAM50 intrinsic subtypes was performed using the nCounter PAM50 assay as published previously [22]. In the current study, IHC-basal cases (defined by positive staining for nestin or loss of INPP4B) was observed in 17% (n=41) of patients and was strongly associated with PAM50 basal-like subtype ($p<0.01$), whereas "negative staining of nestin and positivity for INPP4B" was significantly associated with non basal-like PAM50 subtypes (Table 1). The performance characteristics and diagnostic accuracy of the nestin/INPP4B IHC panel to identify intrinsic subtype showed moderate agreement by Cohen's Kappa Coefficient test (k)=0.5 against the PAM50 assay as a gold-standard.

The evaluation of EGFR+ or CK5+ was found to be significantly associated with basal-like PAM50 gene expression in the context of triple negative cases only (i.e. the "core basal" group), while no such association was observed in non triple negative cases (Supplementary table 2).

Clinicopathological characteristics and IHC biomarker expression

Patient and baseline characteristics according to treatment allocation in the original trial were previously reported [22] and are displayed for the N=239 biomarker study set in Supplementary Table 3. "Nestin+ or INPP4B-" status was significantly associated with poor pathological characteristics including ER negativity, high Ki67 and alternative IHC definitions of the basal-like subtype: "triple negative" and "core basal" (a definition that adds positivity of either epidermal growth factor receptor or cytokeratin 5/6 to triple negativity) (Table 2). The distribution of clinicopathological characteristics according to IHC-basal (nestin+ or INPP4B-) versus IHC non-basal (nestin- and INPP4B+) study populations is shown in Table 2.

Prognostic value of the IHC-basal nestin/INPP4B panel

Within a median of 13 years of follow-up from the time of randomization in this population of patients with advanced breast cancer, univariate prognostic analyses showed that patients assigned as IHC-basal by virtue of their primary tumors being "nestin+ or INPP4B-" demonstrated a trend towards a lower OS when compared to non-basal cases defined as "nestin- and INPP4B+" (Fig. 1). These results were significant in a multivariate analysis including performance status, stage of disease, type of disease (visceral vs. non-visceral), metastatic site and treatment arm (HR=1.43, 95%CI: 1.00-2.05) (*P*=0.05) (Table 3).

When assessing nestin as a single marker, univariate analysis revealed significantly inferior outcomes for OS and TTP among nestin+ cases (Supplementary Table 4). This significant association was conserved in multivariate analysis only for the pre-specified primary outcome of OS. However, the Schoenfeld residuals model showed signs of the hazard ratio being non-proportional for OS and thus a remodeling was performed to avoid violating proportional hazard assumptions. In a time-dependent stratified analysis, the prognostic association was found to be

restricted to the first 1.5 years of follow up (HR for nestin+ vs. nestin- before 1.5 years=2.56, 95%CI: 1.57-4.19) (P<0.01), while no significant effect on OS was observed after 1.5 years of follow-up (Supplementary Table 4). Univariate and multivariate analyses evaluating INPP4B as a single marker did not reveal prognostic significance in this study set.

Predictive value of the IHC-basal nestin/INPP4B panel for gemcitabine benefit

Univariate analysis showed a significantly improved OS among patients assigned as IHC-basal (nestin+ or INPP4B-) who were treated with the gemcitabine-docetaxel doublet when compared to the docetaxel higher dose monotherapy arm. However, this difference was not observed among patients classified as non-basal by virtue of being nestin- and INPP4B+ (Fig. 2). These findings were further confirmed in a multivariate analysis with a test of heterogeneity, indicating that IHC-basal (nestin+ or INPP4B-) predicts superior benefit from the addition of gemcitabine (HR=0.31, 95%CI: 0.16-0.60) whereas there was no such difference in outcome for the other patients (HR=0.99, 95%CI: 0.73-1.34) with a highly significant test of interaction (P<0.01) (Fig. 3). Secondary analyses assessing the predictive capacity of nestin and INPP4B as separate predictive markers demonstrated a significantly higher OS from GD vs. D among patients with INPP4B- tumors or with nestin+ tumors (Fig. 3). Analyses for the TTP endpoint, prespecified as a secondary outcome in this nestin/INPP4B biomarker analysis, did not show a significant predictive effect. For context, “triple negative” IHC status showed no statistically significant treatment effect on OS in the SBG0102 trial [22]. However, an exploratory analysis in the current study revealed that “core basal” status significantly predicted gemcitabine benefit (HR=0.30, 95%CI: 0.14-0.62) when compared to “non-core basal cases” (HR=0.98, 95%CI: 0.73-1.32) (P-interaction<0.01).

Discussion:

The current study shows that a basal-like phenotype, as defined by positive expression of nestin or negative expression of INPP4B, in the primary tumor from patients in the SBG0102 trial is associated with a significant benefit from the addition of the nucleoside analogue gemcitabine to docetaxel, when compared to the (higher-dose) single agent docetaxel control arm. The magnitude of gemcitabine benefit was evident for this correlative study's primary endpoint of OS but not for TTP, likely reflecting the value of long-term follow up information (median 13 years).

Currently, there is a limited arsenal of anticancer drugs that have been proven to benefit survival in patients with metastatic basal-like breast cancers. Treatment strategies were not designed to target specific molecular subtypes in the metastatic setting, and when patients with molecularly disparate tumors are lumped together, significant benefits are not identified [15]. In a previous study evaluating PAM50 gene expression subtypes on cases from SBG0102, we provided potentially important evidence for a higher susceptibility of basal-like cancers to nucleoside analogues (i.e. gemcitabine), supporting that gene expression-based intrinsic subtyping could predict a survival benefit from gemcitabine [22]. However, as the commonly-used "triple negative" definition did not identify a group who benefited, findings highlighted a need for better IHC biomarkers to identify the basal-like molecular subtype in an easily applicable fashion, capable of predicting gemcitabine benefit.

In the current study we provide evidence that the recently-optimized IHC marker panel defining basal by nestin positivity or INPP4B negativity [23] does predict a significant improvement in OS in metastatic breast cancer patients treated with nucleoside analogues (gemcitabine). IHC "triple negative" (ER-/PR-/HER2-) status was previously found to be a poor

surrogate for the basal-like gene expression subtype [23] and lacks predictive significance for gemcitabine benefit [22]. Commonly used basal definitions in current clinical practice such as "triple negative" and "core basal" are by definition limited to ER- cases only, despite multiple gene expression studies showing that triple negative status identifies a heterogeneous group [39] [40] [41] that does not entirely overlap with the basal-like expression subtype [42], and that some breast cancers that are ER+ by IHC actually classify as basal using gene expression methods [43] [44]. Although the "core basal" definition significantly predicted gemcitabine benefit in the current dataset, it requires assessment of five rather than two biomarkers, and is not able to identify ER+ cases assigned as basal-like by PAM50 that could benefit from the addition of gemcitabine. Taken together, these findings demonstrate the superior predictive utility of nestin/INPP4B panel over "triple negative" and "core basal" panels, highlighting the need to revisit current IHC methods to identify basal-like cases so as to better tailor treatment decisions. Specifically, cases that are currently assigned as ER+, but yet possess biological and molecular characteristics of the basal-like gene expression subtype will often be treated using endocrine therapies based on this sole ER biomarker result, instead of more potentially effective chemotherapies that would be active in basal tumors, such as gemcitabine.

Our current study on phase III clinical trial specimens supports the capacity of IHC biomarkers of the basal-like subtype to predict improved survival with gemcitabine chemotherapy. Results were positive by interaction test, executed independently at a clinical trial group statistical office for the primary hypothesis prespecified in the statistical plan, using predefined, externally-validated biomarker cutoffs, assessed by pathologists blinded to both molecular and outcome data. The rigorous design of this prospective-retrospective correlative study adheres to the REMARK reporting recommendations for tumor markers studies [29] and to

guidelines for the use of archived specimens in evaluation of predictive biomarkers [30], and helps to establish the clinical validity of IHC-assessed nestin and INPP4B biomarkers to predict the survival benefit realized by treating basal-like cancers with chemotherapies using nucleoside analogues. The use of phase III randomized clinical trial specimens with this study design and its reported results provide a level 2 evidence; confirmation in a second, similar and larger prospective-retrospective clinical trial series would be required to reach level 1B evidence [30]. Alternatively, the nestin/INPP4B basal IHC panel could be implemented as a stratifying marker in a prospective study design assessing nucleoside analogue chemotherapy in breast cancer.

Despite the prespecifed study design and analysis plan that we used and the positive result for our primary hypothesis, our study has several limitations. Firstly, the breakdown into molecularly-distinct subtypes in each treatment arm resulted in a smaller sample size that limited the statistical power. Accordingly, the current study is not sufficiently powered to provide definitive conclusions and findings need to be validated in a larger cohort. Secondly, our study primary hypothesis on the predictive capacity of nestin/INPP4B IHC panel and gemcitabine benefit was presaged by previous significant findings observed when using the basal-like PAM50 subtype classification by gene expression. Thirdly, the overall agreement reported for the IHC nestin/INPP4B basal panel against the PAM50 as a reference standard was moderate (k)=0.5, whereas it had been substantial (k)=0.7 in the original study that established the analytical validity of nestin/INPP4B panel [23]. This difference might be explained by the fact that the metastatic cases accrued to SBG0102, in comparison with the early breast cancers used to establish the IHC panel, are more heterogeneous and display more aggressive biological characteristics that might lead to differences in the expression patterns of nestin and INPP4B in relation to other patterns defining the intrinsic subtypes.

In current clinical practice, the use of nucleoside analogues is very limited (particularly in early stage breast cancer). However, the results we present support a hypothesis that nucleoside analogues are particularly effective in basal breast cancer cases. In line with this hypothesis, subset analyses in the phase III FinXX [45] [46] and CREATE-X [47] trials evaluating the nucleoside analogue capecitabine as an adjuvant treatment for early stage breast cancer reported that the triple negative subgroup most strongly benefitted from capecitabine. Moreover, a recent meta-analysis assessing capecitabine in early breast cancer [48] reported that, while no overall significant benefit from capecitabine was present in most of the original trials, capecitabine appears to improve survival outcomes in the subset of triple negative breast cancers. Overall these findings, in conjunction with the current study on SBG0102, suggest that an IHC panel that more closely approximates the basal breast cancer intrinsic subtype (nestin/INPP4B) could be an even stronger predictor for nucleoside analogue benefit, and could provide an accessible and inexpensive method to identify the best clinical situations for the use of nucleoside analogue chemotherapy in the treatment of breast cancer or possibly even new immunotherapy strategies thought to be of greatest relevance, in breast cancer, to subsets of basal/triple negative cases.

Conclusions:

The current study presents data from high quality phase III randomized clinical trial series, including both ER+ and ER- cases, and finds that advanced breast cancers that are immunohistochemically basal-like by virtue of being nestin positive or INPP4B negative gain a significant survival benefit from nucleoside analogue-containing chemotherapy. This study provides a level 2 evidence for the clinical validity of this easily-applicable IHC panel to identify patients with the basal-like molecular subtype who benefit from nucleoside analogue

chemotherapy. Findings need to be validated using larger prospective-retrospective phase III clinical trials series to achieve level 1B evidence.

Acknowledgement:

This work was supported by the Canadian Breast Cancer Foundation and Canadian Cancer Society Research Institute, the Danish Cancer Research Foundation and the Research Council of Herlev University Hospital, Denmark. K.Asleh was supported by the 2017 San Antonio Breast Cancer Symposium (Basic Science AACR Scholar-In-Training Award) for this study, the Laurel-Watters Breast Cancer Fellowship and the University of British Columbia – (Cordula and Gunter Paetzold Affiliated Fellowship).

Conflict of Interest:

Torsten O. Nielsen played a role in the development of the PAM50 gene expression classifier, which has been licensed to NanoString technologies (who did not fund this work). None of the remaining authors have any financial or non-financial conflict of interest to declare for this work.

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Tables:

Table 1: The association between nestin and INPP4B immunohistochemical biomarker expression and intrinsic breast cancer subtype by PAM50 gene expression profile

	"Nestin+ or INPP4B-" n=41	**"Nestin- and INPP4B+" n=198**	***P*- value**
PAM50 subtype			<0.01
Luminal A	5 (12%)	63 (32%)	
Luminal B	9 (22%)	79 (40%)	
Her2-Enriched	7 (17%)	37 (19%)	
Basal-like	20 (49%)	16 (8%)	
Unknown	0 (0%)	3 (1%)	

Table 2: The distribution of clinicopathological characteristics according to basal "nestin+ or INPP4B-" versus non-basal "nestin- and INPP4B+" study populations

Characteristic	"Nestin+ or INPP4B-" (IHC-basal) n=41	"Nestin- and INPP4B+" (IHC non-basal) n=198	*P*-value
ECOG Performance status			0.79
0,1, and unknown	37 (90%)	174 (88%)	
2	4 (10%)	24 (12%)	
Stage of Disease			0.01
Locally advanced	7 (17%)	10 (5%)	
Metastatic	34 (83%)	188 (95%)	
Number of metastatic sites			0.62
1	12 (29%)	58 (29%)	
2	17 (42%)	68 (35%)	
≥3	12 (29%)	72 (36%)	
Type of metastatic site[a]			0.49
Visceral	20 (49%)	85 (43%)	
Lung	14 (34%)	56 (28%)	0.45
Liver	11 (27%)	76 (38%)	0.16
Non-visceral	21 (51%)	113 (57%)	
Receptor status			<0.01
Negative	23 (56%)	39 (20%)	

Positive	19 (44%)	158 (80%)	
Unknown	0 (0%)	1 (1%)	
Her2 status			0.41
Positive	3 (7%)	29 (15%)	
Negative	37 (90%)	162 (82%)	
Unknown	1 (2%)	7 (4%)	
KI67			0.02
<14%	11 (27%)	97 (49%)	
≥14%	30 (73%)	99 (50%)	
Unknown	0 (0%)	2 (1%)	
Prior chemotherapy			
(Neo) adjuvant[b]	27 (66%)	88 (44%)	0.01
Anthracycline	14 (34%)	48 (24%)	
Non-anthracycline	13 (32%)	40 (20%)	
Advanced stage[c]	14 (34%)	83 (42%)	0.36
Anthracycline	13 (32%)	69 (35%)	
Non-anthracycline	1 (2%)	14 (7%)	
Triple negative status			<0.01
Triple negative	22 (54%)	17 (8%)	
Non-triple negative	19 (46%)	180 (91%)	
Unknown	0 (0%)	1 (1%)	
Core basal status[d]			<0.01
Core basal	21 (51%)	12 (6%)	

Non-core basal	20 (49%)	185 (93%)	
Unknown	0 (0%)	1 (1%)	

IHC: immunohistochemistry; ECOG: Eastern Cooperative Oncology Group; Her2: Human epidermal growth factor receptor-2; INPP4B: inositol polyphosphate-4-phosphate. [a]Comparing visceral vs. non-visceral. *P*-value for lung: comparing metastasis in lung vs. no metastasis in lung. *P*-value for liver: comparing metastasis in liver vs. no metastasis in liver. [b]Comparing number of patients receiving (Neo) adjuvant chemotherapy vs. those that did not. [c]Comparing patients who received a 1st line of chemotherapy for advanced stage prior to randomization in the trial vs. other patients. [d]Core basal status: a definition that adds positivity of either epidermal growth factor receptor or cytokeratin 5/6 to triple negative.

Table 3: Multivariate analysis with hazard model for OS and TTP assessing the combination of nestin and INPP4B in the study cohort

	Multivariate Analysis for OS		Multivariate Analysis for TTP	
Variable	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Nestin+ or INPP4B- vs. Nestin- and INPP4B+	1.43 (1.00 – 2.05)	0.05	1.11 (0.71 - 1.73)	0.64
Gemcitabine-docetaxel (GD) vs. docetaxel (D)	0.81 (0.61 - 1.07)	0.14	0.49 (0.34 - 0.69)	<0.01
Type of disease (visceral vs. non-visceral)	1.37 (0.99 - 1.89)	0.06	1.85 (1.21 - 2.82)	<0.01
Stage of disease (locally-advanced vs. metastatic)	0.87 (0.50 - 1.51)	0.62	3.12 (1.59 - 6.14)	<0.01
Performance status (2 vs. 0,1)	1.80 (1.17 - 2.75)	<0.01	0.97 (0.57 - 1.66)	0.91
Number of metastatic sites (>2 vs. 1,2)	1.33 (0.99 - 1.79)	0.06	1.20 (0.82 - 1.74)	0.35

OS: overall survival; TTP: time to tumor progression; INPP4B: inositol polyphosphate-4-phosphate.

Figure legends:

Figure 1. Kaplan-Meier curves for overall survival (A) and Time to tumor progression (B) stratified based on nestin and INPP4B tests results on tissue specimens from the SBG0102 trial. “Nestin+ or INPP4B-" defines IHC basal-like cases (blue). “Nestin- and INPP4B+” defines non-basal cases (red).

Figure 2. Kaplan-Meier curves of nestin and INPP4B biomarkers expression in tissue specimens from the SBG0102 trial populations treated with docetaxel (D, solid lines) vs. Gemcitabine-docetaxel (GD, dotted lines). “Nestin+ or INPP4B-" defines IHC basal-like cases (blue). “Nestin- and INPP4B+” defines non-basal cases (red).

Figure 3. Forest plot for overall survival (OS) according to nestin and INPP4B expression status in tissue specimens from the SBG0102 trial populations treated with docetaxel (D) vs. Gemcitabine-docetaxel (GD). “Nestin+ or INPP4B-" defines IHC basal-like cases. “Nestin- and INPP4B+” defines non-basal cases. The displayed *P*-values indicate the results of test of heterogeneity.

Supplementary Figure 1. CONSORT flow diagram for cases included in the study.

Figure 1

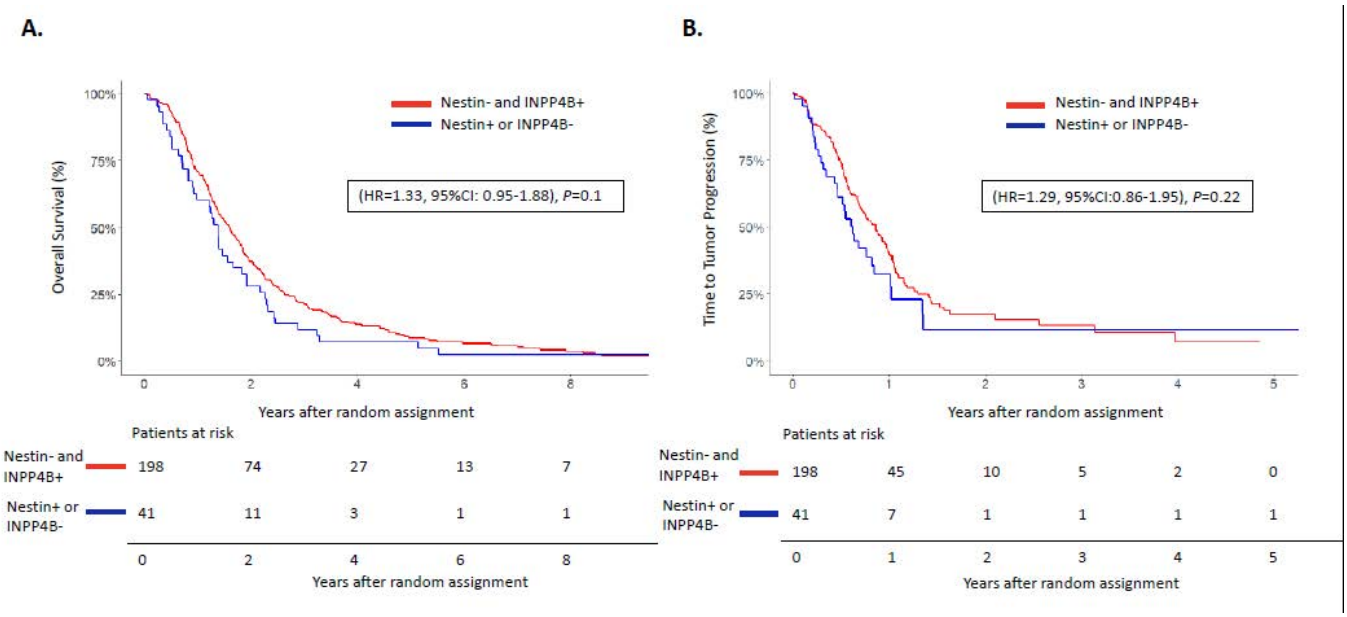

A.
Overall Survival (%)
100%
75%
50%
25%
0%
0
2
4
6
8
Nestin- and INPP4B+
Nestin+ or INPP4B-
(HR=1.33, 95%CI: 0.95-1.88), P=0.1
Years after random assignment
Patients at risk
Nestin- and INPP4B+ 198 74 27 13 7
Nestin+ or INPP4B- 41 11 3 1 1
0 2 4 6 8
Years after random assignment
B.
Time to Tumor Progression (%)
100%
75%
50%
25%
0%
0
1
2
3
4
5
Nestin- and INPP4B+
Nestin+ or INPP4B-
(HR=1.29, 95%CI:0.86-1.95), P=0.22
Years after random assignment
Patients at risk
Nestin- and INPP4B+ 198 45 10 5 2 0
Nestin+ or INPP4B- 41 7 1 1 1 1
0 1 2 3 4 5
Years after random assignment

Figure 2

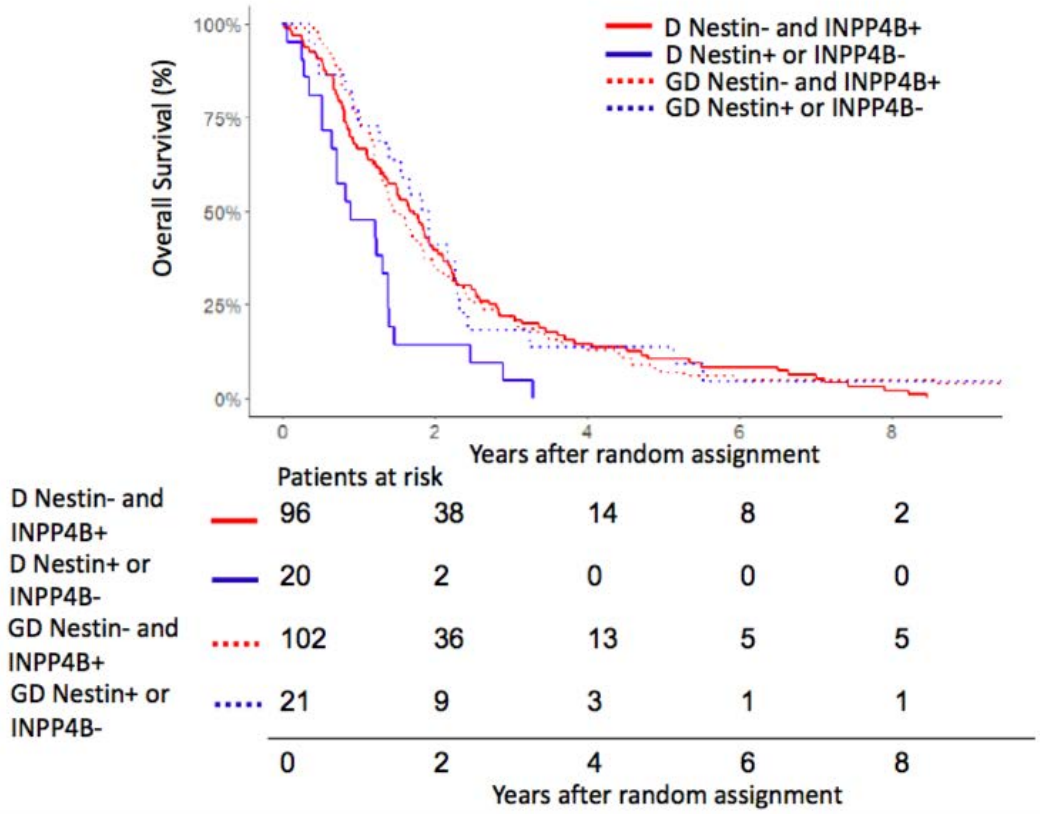
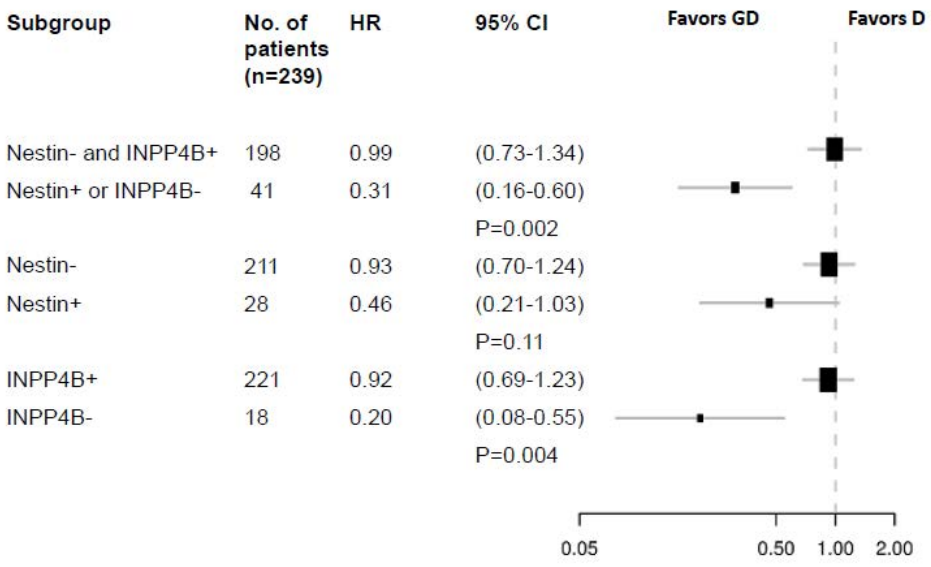

D Nestin- and INPP4B+
D Nestin+ or INPP4B-
GD Nestin- and INPP4B+
GD Nestin+ or INPP4B-
Overall Survival (%)
100%
75%
50%
25%
0%
0
2
4
6
8
Years after random assignment
Patients at risk
D Nestin- and INPP4B+ 96 38 14 8 2
D Nestin+ or INPP4B- 20 2 0 0 0
GD Nestin- and INPP4B+ 102 36 13 5 5
GD Nestin+ or INPP4B- 21 9 3 1 1
0 2 4 6 8
Years after random assignment

Figure 3


Subgroup
No. of patients (n=239)
HR
95% CI
Favors GD
Favors D
Nestin- and INPP4B+ 198 0.99 (0.73-1.34)
Nestin+ or INPP4B- 41 0.31 (0.16-0.60)
P=0.002
Nestin- 211 0.93 (0.70-1.24)
Nestin+ 28 0.46 (0.21-1.03)
P=0.11
INPP4B+ 221 0.92 (0.69-1.23)
INPP4B- 18 0.20 (0.08-0.55)
P=0.004
0.05
0.50
1.00
2.00

Supplementary Figure 1