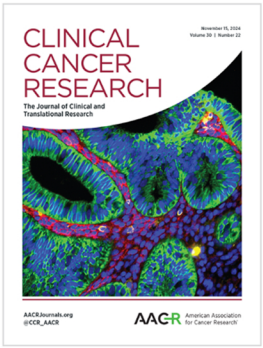

High Mechanical Conditioning by Tumor Extracellular Matrix Stiffness is a Predictive Biomarker for Antifibrotic Therapy in HER2-negative Breast Cancer

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ABSTRACT

Purpose: Tumor progression has been linked to stiffening of the extracellular matrix caused by fibrosis. Cancer cells can be mechanically conditioned by stiff extracellular matrix, exhibiting a 1,004-gene signature [mechanical conditioning (MeCo) score]. Nintedanib has demonstrated antifibrotic activity in idiopathic pulmonary fibrosis. This study explores nintedanib's antifibrotic effect on breast cancer outcomes.

Experimental Design: We present long-term follow-up and analysis of a neoadjuvant randomized phase II trial in early HER2-negative breast cancer. Patients ($N = 130$) underwent a baseline biopsy and received 12 paclitaxel courses alone (control arm) or in combination with nintedanib (experimental arm). The tumor MeCo score was determined by RNA sequencing. The primary aim was to assess nintedanib's impact on event-free survival based on MeCo scores.

Results: Follow-up data were retrieved from 111 patients; 75 baseline and 24 post-run-in phase samples were sequenced. After median follow-up of 9.67 years, median event-free survival was not statistically different between arms ($P = 0.37$). However, in the control arm, high- versus low-MeCo patients had a statistically higher relapse risk: HR = 0.21; $P = 0.0075$. This risk was corrected by nintedanib in the experimental arm: HR = 0.37; $P = 0.16$. Nintedanib demonstrated pharmacodynamic engagement, reducing the MeCo score by 25% during the run-in phase ($P < 0.01$). Patients with low MeCo after run-in had the best long-term prognosis (HR = 0.087; $P = 0.03$).

Conclusions: High MeCo is predictive of poor outcomes in HER2-negative early breast cancer, although this risk can be mitigated by nintedanib, which is able to specifically reduce MeCo.

Introduction

Early HER2-negative breast cancer continues to pose significant clinical challenges. Despite state-of-the-art care, long-term distant relapse rates for early hormone receptor–positive breast cancer (HRPBC) and triple-negative breast cancer (TNBC) are distressingly high, ranging from 20% (1–3) to 40% (4, 5), respectively. Recent therapeutic advancements have improved event-free survival (EFS) rates, especially for those at high risk. For instance, the addition of CDK4/6 inhibitors, abemaciclib for 2 years and ribociclib for 3 years, has led to a 33% (6) and 25% (7) reduction in relapse risk among high-risk and average-risk patients with early HRPBC, respectively, when combined with standard endocrine therapy. Similarly, integrating the anti-PD1 antibody pembrolizumab with a platinum-taxane neoadjuvant regimen has lowered the relative relapse risk in early TNBC (stage II or higher) by 37% (8). However, the follow-up duration for these new treatments is relatively short compared with historical data, leaving the absolute long-term risk reduction uncertain.

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Previous presentations of the study: A partial version of this manuscript, including only the relationship between MeCo score and pCR, was presented at the 2023 SABCS meeting.

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Translational Relevance

Our findings suggest a role for the mechanical conditioning (MeCo) score as a predictive biomarker in early HER2-negative breast cancer, identifying patients who can benefit from targeted antifibrotic therapy with nintedanib. High MeCo scores, indicative of extracellular matrix stiffness response, are associated with poor prognosis yet respond favorably to nintedanib, which reduces MeCo and improves event-free survival. This biomarker-guided approach could support therapeutic escalation with nintedanib—a 3-month regimen of low toxicity and cost. By offering a specific therapy targeting the biological feature identified by the MeCo score, nintedanib could provide a rational therapeutic option for high-risk groups. This strategy defines a pathway for a more personalized and cost-effective treatment paradigm in breast oncology, and it represents the first successful clinical application targeting tumor fibrosis in oncology.

Escalating treatments, although effective in lowering relapse risks, are fraught with issues such as significant toxicity and financial burden. Toxic effects from CDK4/6 inhibitors can substantially diminish patient quality of life (9), whereas those from immunotherapy may persist for an extended period or even become life-threatening (10, 11). Ongoing clinical trials are exploring additional treatment escalation strategies, such as introducing immunotherapy for early HRPBC or employing antibody–drug conjugates in early TNBC. Positive results from these trials, even if marginal, could lead to a situation in which patients with early TNBC are prescribed up to six highly toxic drugs due to the lack of effective biomarkers. Analysis of past trials indicates the need to treat at least 30 patients with HRPBC for 3 years with ribociclib to prevent only a single distant relapse. For patients with TNBC achieving pathologic complete response (pCR) with neoadjuvant therapy, adjuvant pembrolizumab treatment is required for 50 patients to avert one event. These figures highlight the critical need for predictive biomarkers to inform more rational treatment escalation or de-escalation strategies.

Successful treatment de-escalation strategies have leveraged molecular stratification tools in the past. For example, patients with HRPBC categorized by their tumor gene expression profiles as low- or high-risk are typically offered endocrine therapy alone or a combination of chemotherapy and endocrine therapy, respectively (12–14). However, these tools are not without their limitations—they lack specificity. Although they can identify less aggressive tumors manageable with endocrine therapy alone, they fail to offer a targeted treatment mechanism for the more aggressive tumors identified in the "aggressive cluster." As such, patients are often subjected to general cytotoxic chemotherapy, which, without additional biomarkers, may be hit-or-miss in preventing relapse. A specific molecular stratification tool could represent a significant breakthrough, enabling the provision of highly effective targeted therapies tailored to the molecular characteristics of high-risk patients.

Extracellular matrix (ECM) stiffening, which occurs as tissues lose normal elasticity and become fibrotic, is implicated in tumor progression (15). Exposure to stiff ECM within the primary tumor can activate mechanotransduction pathways in tumor cells, initiating metastasis (16). Recent evidence suggests that some tumor cells in a fibrotic microenvironment can develop a prometastatic phenotype, which can be retained even after disseminating to softer tissues like the bone marrow (17). This phenomenon is defined as mechanical conditioning (MeCo) and is quantified by the expression of a set of 1,004 genes constituting a MeCo score (17), which correlates with breast cancer relapse rates (17).

There is limited clinical research into targeting stiff tumor ECM with antifibrotics, and whether their downstream effects can reverse MeCo is currently unknown. Nintedanib is a multi-tyrosine kinase inhibitor with strongest activity against VEGFR1–3, PDGFRB, FGFR1–3, RET, SRC, and FLT-3 (18), initially developed as an antiangiogenic agent in oncology. Previously, our randomized phase II study showed that adding nintedanib to paclitaxel therapy increased pCR rates in neoadjuvant HER2-negative breast cancer (19). Nintedanib's antiangiogenic efficacy in other cancers was underwhelming, leading to discontinued development in oncology (20–22), except for an approved indication in second-line lung cancer in Europe (23). However, its antifibrotic action elicited through FGFR1–3 inhibition made it gain approval in idiopathic lung fibrosis (24). These antifibrotic properties, in combination with a candidate predictive biomarker to enrich for responders and monitor its effects longitudinally, prompted us to re-examine its use in breast cancer.

Our analysis revealed that high MeCo scores were associated with lower pCR and increased relapse risk in patients receiving paclitaxel monotherapy, and combination treatment with nintedanib and paclitaxel was uniquely beneficial in improving EFS only for patients with high MeCo scores at baseline. Crucially, nintedanib's ability to reverse MeCo was demonstrated in serial biopsies, and patients who were downgraded from high to low MeCo after 2 weeks of nintedanib treatment experienced the most favorable outcomes.

Materials and Methods

Patients and tumor samples

This study reports the long-term follow-up of the CNIO-BR-03-GEICAM/2010-10 clinical trial (registered at www.clinicaltrials.gov as NCT01484080). Whereas the study included proteomic/genomic/transcriptomic studies in the obtained tumor biopsies, it did not consider follow-up beyond the primary endpoint (recording of pCR after neoadjuvant treatment). Thus, a new study was designed with the objective of retrieving the long-term follow-up from the patients' medical records. All patients enrolled in the CNIO-BR-03-GEICAM/2010-10 clinical trial were candidates for this study. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice standards. The study was evaluated and approved by the Institutional Ethics Board of Hospital 12 de Octubre (Ref.#: 24/031); informed consent signature was waived by the Board for two reasons: (i) due to the long-term follow-up, it is likely that some patients have passed away due to illness or age, and the researchers deemed it inappropriate to contact family members for consent; (ii) the study concluded more than 10 years ago, and many surviving and cured patients have been referred to primary care for continuing care, making it unrealistic to obtain consent through reasonable means.

The clinical trial design is outlined in **Fig. 1A**. Inclusion and exclusion criteria and attrition are presented in **Fig. 1B**. Subject demographics are presented in **Table 1**. Briefly, 130 patients with early HER2-negative breast cancer were randomized 1:1 to each of the two study arms. Patients randomized to the control arm received 12 weekly courses of intravenous paclitaxel (80 mg/m^2), whereas those randomized to the experimental arm received 2 weeks of single-agent nintedanib (150 mg bid – run-in part) followed by 12 weeks of paclitaxel plus nintedanib (150 mg bid). A fresh tumor biopsy was

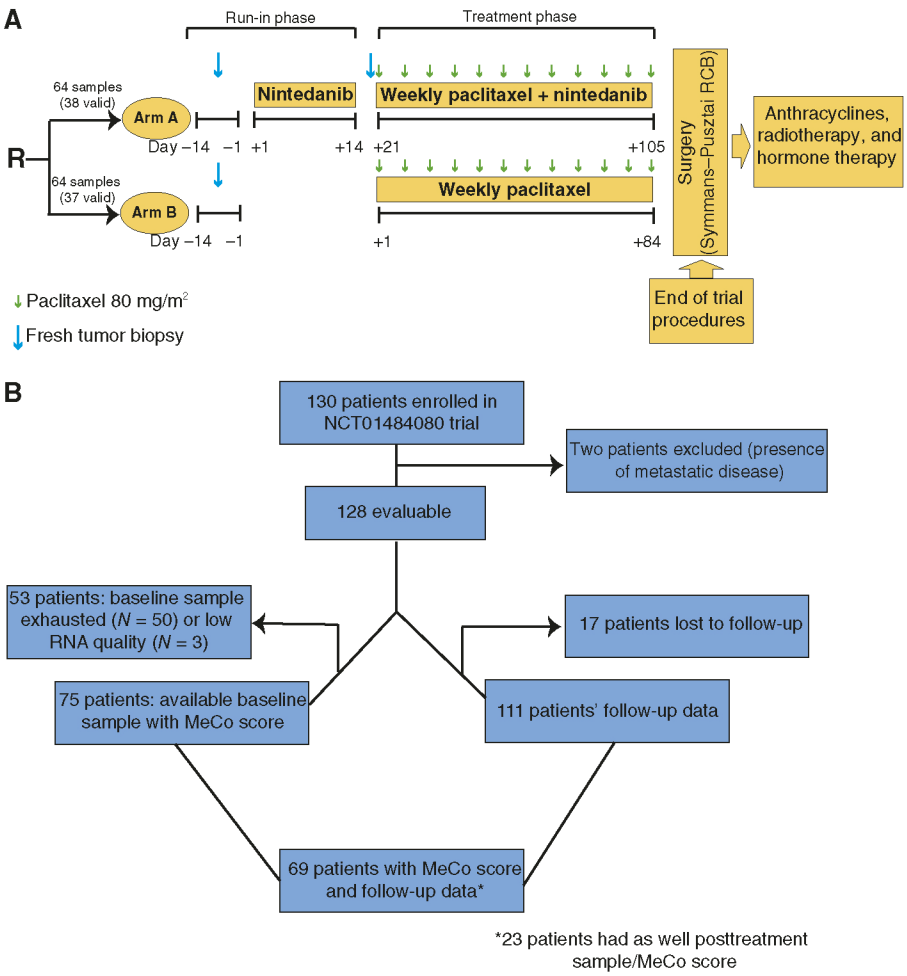

Figure 1.

A, Clinical trial design. Patients with operable HER2-negative breast cancer were randomized into two arms, in a 1:1 ratio. Patients in the experimental arm, arm A, were treated with weekly paclitaxel (85 mg/m^2) for 12 weeks plus daily oral nintedanib at 150 mg bid. The standard arm, arm B, consisted of weekly paclitaxel alone. All patients underwent a baseline fresh tumor biopsy. In addition, patients from the experimental arm also underwent a run-in phase of nintedanib monotherapy, lasting 2 weeks. Immediately after these 2 weeks, the patients underwent a second biopsy and then continued to the combination phase. After a minimum of 5 weeks after completing neoadjuvant chemotherapy/combination therapy, patients were operated, and pCR was determined. The clinical trial ended with the assessment of pCR; however, patients were offered further chemotherapy, radiotherapy, and/or hormonal therapy in the adjuvant setting, according to the achievement or not of complete pathologic response and physicians' criteria. For each treatment arm, the number of baseline successfully profiled samples is indicated. For post-nintedanib samples, 24 were successfully profiled. **B,** Consolidated Standards of Reporting Trials diagram. The original study considered a baseline biopsy for all patients and an on-treatment biopsy for the patients allocated to the control arm. Multiple tissue cores were obtained on each procedure. However, several other correlative studies planned within this trial exhausted a significant number of samples. At the time the present study was planned, only 78 patients had a remaining tumor core; of those, the great majority (96%) had adequate RNA quantity and quality. Thus, the relative lack of samples was not due to poor sample quality or preservation, but commitment to other previously reported studies.

harvested from all patients before treatment. Patients in the experimental arm also underwent a second biopsy after run-in phase.

RNA extraction and sequencing

Tumor purity was verified by a pathologist before processing, including only samples with >80% tumor content. For RNA extraction, snap-frozen tumor pieces (~50 mm^3) were homogenized in TRIzol reagent solution (Invitrogen, #AM9738) according to manufacturer's instructions using Precellys 24 tissue homogenizer (Bertin Technologies). Briefly, after homogenization, chloroform was added, and the resulting mixture was centrifuged at 12,000 *g* for 10 minutes at 4°C. The aqueous phase was transferred to a clean tube, and 70% ethanol was added. Then, total volume was filtered through the RNeasy column (Qiagen, #74104), and RNA extraction was performed following protocol instructions. RNA quality was determined by Agilent's 2100 Bioanalyzer lab-chip technology. Total RNA samples (~400 ng) were converted into cDNA sequencing libraries with the "QuantSeq 3′mRNA-Seq V2 Library Prep Kit (FWD) for Illumina" (Lexogen, #191). Briefly, library generation is initiated by reverse transcription with oligodT priming, followed by a random-primed second strand synthesis. A Unique Molecular Identifier (UMI) Second Strand Synthesis module was used, in which random primers featuring a 6 nt

Table 1. Clinical and demographic characteristics of the patients (whole trial and subset analyzed in this study).

	Complete dataset – evaluable patients			Available follow-up and MeCo score		
	Arm A	Arm B	All patients	Arm A	Arm B	All patients
	n = 64	*n* = 64	*N* = 128[a]	*n* = 36	*n* = 33	*N* = 69
Characteristic	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
Age (median, range)	47.2 (31.2–81.4)	48.2 (30.6–72.3)	47.6 (30.6–81.4)	47.3 (31.1–79.2)	49 (30.6–68)	47.3 (30.6–79.2)
Menopausal status						
Premenopausal	41 (64.1%)	35 (54.7%)	76 (59.4%)	22 (61.1%)	19 (57.6%)	41 (59.4%)
Postmenopausal	23 (35.9%)	29 (45.3%)	52 (40.6%)	14 (38.9%)	14 (42.4%)	28 (40.6%)
ECOG PS (0/1)	63 (98.5%)/1 (1.5%)	63 (98.5%)/1 (1.5%)	126 (98.5%)/2 (1.5%)	35(97.2%)/1 (2.8%)	33(100%) /0 (0%)	68(98.6%)/1 (1.4%)
cT						
T1	0 (0%)	3 (4.7%)	3 (2.3%)	0 (0%)	2 (6%)	2 (2.9%)
T2	45 (70.3%)	47 (73.4%)	92 (71.9%)	22 (62.9%)	22 (66.8%)	44 (64.7%)
T3	16 (25.0%)	13 (20.3%)	29 (22.7%)	11 (31.4%)	8 (24.2%)	19 (27.9%)
T4	3 (4.7%)	1 (1.6%)	4 (3.1%)	2 (5.7%)	1 (3%)	3 (4.4%)
cN						
N0	32 (50%)	29 (45.3%)	61 (47.7%)	16 (44.4%)	17 (51.5%)	33 (47.8%)
N1	28 (43.8%)	31 (48.4%)	59 (46.1%)	19 (52.8%)	13 (39.4%)	31 (46.4%)
N2	3 (4.7%)	4 (6.3%)	7 (5.5%)	1 (2.8%)	3 (9.1%)	4 (5.8%)
N3	1 (1.6%)	0 (0%)	1 (0.8%)	0 (0%)	0 (0%)	0 (0%)
Hormonal receptors						
HR^+	51 (79.7%)	49 (76.6%)	100 (78.1%)	30 (83.3%)	29 (87.9%)	59 (85.5%)
ER^+PR^+	43 (67.2%)	43 (67.2%)	86 (67.2%)	28 (77.8%)	26 (78.8%)	54 (78.3%)
ER^+PR^-	8 (12.5%)	6 (9.4%)	14 (10.9%)	2 (5.6%)	3 (9.1%)	5 (7.2%)
HR^-	13 (20.3%)	15 (23.4%)	28 (21.9%)	6 (16.7%)	4 (12.1%)	10 (14.5%)
Histologic subtype						
Ductal	54 (84.4%)	54 (84.4%)	108 (84.3%)	31 (86.1%)	28 (84.8%)	59 (85.5%)
Lobular	5 (7.8%)	7 (10.9%)	12 (9.4%)	3 (8.3%)	3 (9.1%)	6 (8.7%)
Other	5 (7.8%)	3 (4.7%)	8 (6.3%)	2 (5.6%)	2 (6.1%)	4 (5.8%)
Grade						
G1	10 (15.6%)	7(10.9%)	17 (13.3%)	5 (14.3%)	8 (24.2%)	13 (19.1%)
G2	38 (59.4%)	36 (56.3%)	74 (57.8%)	23 (65.7%)	18 (54.6%)	41 (60.3%)
G3	16 (25.0%)	21 (32.8%)	37 (28.9%)	7 (20%)	7 (21.2%)	14 (20.6%)
Ki67 (HR^+ only)						
≤14%	17 (26.5%)	14 (21.9%)	31 (24.2%)	8 (22.2%)	9 (27.3%)	17 (24.6%)
>14%	46 (71.9%)	49 (76.6%)	69 (74.2%)	28 (77.8%)	24(72.7%)	52 (75.4%)
N/A	1 (1.6%)	1 (1.6%)	2 (1.6%)	0 (0%)	0 (0%)	0 (0%)

Abbreviation: ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hormone receptor; ER, estrogen receptor; PR, progesterone receptor. Each characteristic was compared between the complete dataset and the analysis subset. None of the comparisons were statistically significant.
[a]Although the trial originally accrued 130 patients, two (one from each arm) were excluded from the final analysis as they were demonstrated to be M1 after starting cycle 1.

long UMI tag are used (to be addressed for bias correction at data analysis). Primers from both steps contain Illumina-adapter sequences. Libraries were completed by PCR and sequenced on an Illumina NextSeq 550 instrument (with v2.5 reagent kits) by following manufacturer's protocols. Raw images generated by the sequencer are submitted to analysis, including per-cycle basecalling and quality score assignment with Illumina's Real Time Analysis integrated primary analysis software (RTA v2). Conversion of BCL (base calls) binary files to FASTQ format is subsequently performed with bcl2fastq2 from within Local Run Manager (Illumina).

RNA sequencing analysis

RNA sequencing reads underwent adapter trimming, and low-quality reads were removed using Fastp default settings. RNA sequencing reads were aligned to the GENCODE GRCh38 v43 reference genome using Hisat2. UMI-tools was used to remove duplicated reads based on the read's UMI. HTSeq count was used to quantify aligned reads. MeCo scores were computed for each patient as previously published (17). Variance-stabilizing transformation was performed using the DeSeq2 variance-stabilizing transformation method to normalize the data. The median of the whole patient population was set as the cutoff for classifying patients as high MeCo (above median) and low MeCo (below median). Crucially, patient classification was specified before collection of long-term follow-up data and EFS analysis.

Furthermore, comparison of MeCo scores at the single-gene level before and after treatment was performed using unsupervised clustering analysis of the full MeCo gene list in 24 patients from the experimental arm with samples collected before and after treatment.

In addition, intrinsic subtypes and the 21-gene recurrence scores were determined for each patient using the "genefu" package (25). Intrinsic subtypes were classified as basal, luminal A, luminal B, and normal-like. Furthermore, using the 21-gene scores, patients were classified based on 21-gene recurrence scores as follows: in women older than 50, 0 to 25 is low and 26 to 100 is high; in women younger than 50, 0 to 15 is low, 16 to 25 is medium, and 26 to 100 is high. Only one patient was classified as medium (with a score of 18) and was eventually grouped with the low cluster for the EFS analysis.

Statistical methods

This biomarker study adhered to the guides proposed elsewhere by Simon and colleagues (26) including adequate amounts of archived tissue; randomized design; representativity of the evaluated patients in the trial; analytic and pre-analytic assay validation (17); and prespecification of the biomarker evaluation plan. Because this was a pilot study, a formal power calculation was not required. The study dataset and the original trial patients' characteristics were compared as follows: age was compared with the Mann–Whitney Wilcoxon test. Menopausal status, Eastern Cooperative Oncology Group performance status, hormonal receptor status, grade, and Ki67 were compared with the χ^2 test. Finally, nodal status, tumor size, and histologic subtype were compared with the Fisher test. MeCo score distribution by intrinsic subtypes was compared with one-way ANOVA with Tukey multiple comparison test. The comparison of MeCo score from baseline to post-run-in phase in paired patient samples was performed with a paired *t* test.

pCR rates were compared between high- and low-MeCo groups using Fisher exact test. High- and low-MeCo groups were established by a prespecified cutoff value: the median MeCo score of all samples. Kaplan–Meier curves were calculated from the randomization date up until the last follow-up visit, and groups were compared with the log-rank and Gehan–Breslow–Wilcoxon (GBW) tests. The primary endpoint of the study was EFS.

Data availability

Gene expression data presented in this study have been deposited on a public repository (GSE255359).

The data generated in this study are also available upon request and Ethics Board approval. Requests should be addressed to MQF and GM.

Results

Patients' characteristics

Long-term follow-up data were obtained for 111 of the 130 patients initially randomized. Baseline samples were available for 76 patients. Of the 65 patients assigned to the experimental arm, post-run-in samples were also available for 24. A complete set of follow-up data and successful baseline MeCo score determination were available for 69 patients. Additionally, the MeCo score was successfully determined in the post-run-in samples for 24 patients in the experimental arm (see Consolidated Standards of Reporting Trials diagram; **Fig. 1B**). The clinical and demographic characteristics of both the original 130 patients and the subset of 69 with full data are summarized in **Table 1**. The analysis subset is representative of the entire trial cohort, as no statistically significant differences were observed in the characteristics detailed in **Table 1**.

MeCo gene expression score: pCR rates and changes in response to antifibrotic treatment with nintedanib

The median MeCo score in the patient population was established at 0.33 (range: 0.05–0.62). The distribution of the MeCo score is depicted in **Fig. 2A**. It seems that more aggressive intrinsic subtypes correspond with higher MeCo scores; namely, luminal A tumors have significantly lower MeCo scores than luminal B and basal tumors. Given the association between higher MeCo scores and aggressive tumor behavior, we evaluated pCR rates (defined as RCB = 0) based on MeCo scores (prespecified at the median into "high" and "low"). The pCR rates by MeCo score and treatment arm are shown in **Fig. 2B**. Although these results are nonstatistically significant, a trend toward lower pCR rates with higher MeCo scores is suggested in the control arm; however, patients in the experimental arm had similar pCR rates regardless of the score.

In the complete trial population (19), the pCR rates were significantly higher in the nintedanib arm, prompting an investigation into whether nintedanib's effect in the high-MeCo subgroup was a result of specific antifibrotic actions or nonspecific antitumor effects. We analyzed changes in expression levels of genes comprising the MeCo signature, pre- and post-nintedanib exposure, during the run-in phase. If the trend toward pCR improvement was due to a reduction in MeCo, we would expect a reduction in the MeCo score following nintedanib treatment.

The heatmap in **Fig. 3A** illustrates the changes in the MeCo gene set expression levels in the 24 paired samples from before to after the run-in phase. This analysis does not reveal a specific subset of genes consistently different in response to antifibrotic therapy despite the global reduction in the score. **Figure 3B** shows

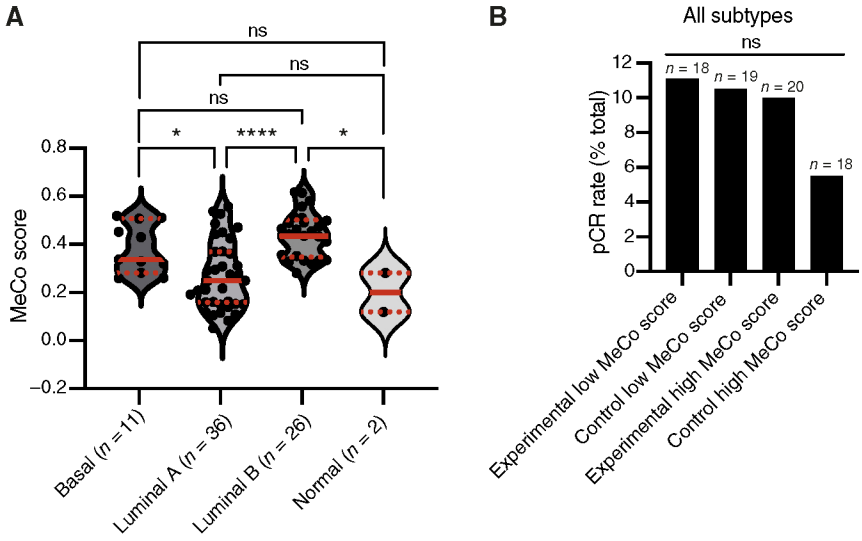

Figure 2.

MeCo gene expression score distribution by intrinsic subtype and relationship with pCR. **A,** MeCo score distribution according to the intrinsic subtype. The red lines represent the median MeCo score for each subtype. **B,** Percentage of patients achieving pCR (RCB = 0) according to reception, or not, of nintedanib, and MeCo score. *, $P < 0.05$; ****, $P < 0.0001$. ns, nonstatistically significant; LumA, luminal A; LumB, luminal B.

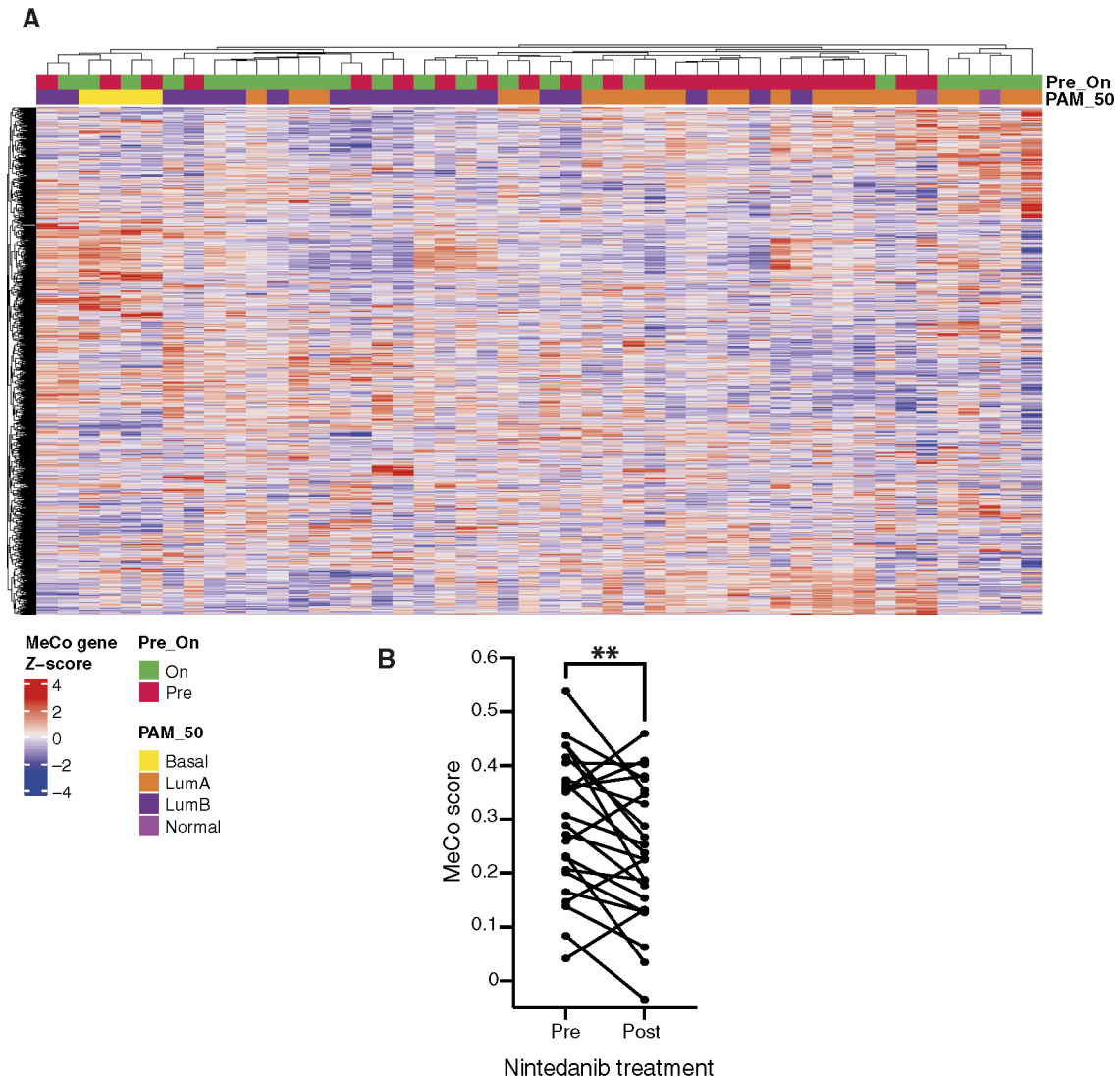

Figure 3.

Evolution of MeCo expression score in response to antifibrotic treatment with nintedanib. **A,** Normalized MeCo cluster gene expression levels in 24 sample pairs (pre–/post–run-in nintedanib monotherapy phase); the intrinsic subtype by PAM50 is shown as well. No gene subsets among the MeCo cluster show specific enrichment. **B,** the evolution of MeCo score from baseline to post-nintedanib run-in phase in the experimental arm is shown here at the individual level. $N = 24$ patients with paired tumor tissue. **, $P < 0.01$.

that the median MeCo score was reduced by 25% in response to the 2 weeks of treatment with nintedanib during the run-in phase. The median score changed from 0.45 (pre-) to 0.34 (posttreatment; 0.11 absolute difference), indicating nintedanib's effective antifibrotic action during the run-in phase. This supports the model that although the subset of genes within the MeCo score that change in response to nintedanib varies among patients, MeCo scores are significantly decreased in these patients after the run-in phase.

EFS: influence of MeCo gene expression score and therapeutic effect of nintedanib

Although a positive effect of nintedanib on the primary outcome (pCR) was previously reported (19), long-term follow-up was not part of the original trial design, leaving the impact on EFS unknown. The Kaplan–Meier curves in **Fig. 4A** illustrate a comparison between standard treatment and nintedanib, without biomarker stratification, revealing a trend toward improved EFS in the nintedanib arm [HR = 0.52; 95% confidence interval (CI), 0.21–1.2] but with only marginal statistical significance (log-rank test $P = 0.071$). Neither median EFS was reached, and average estimated EFS was 3,535 days (experimental arm) versus 3,153 days (control arm). An accumulated apparent early relapse during follow-up prompted further examination using the Gehan–Breslow–Wilcoxon test, confirming the absence of statistical significance ($P = 0.14$).

Considering that ECM stiffness effects and their association with distant metastasis incidence persist over time (17), one might expect

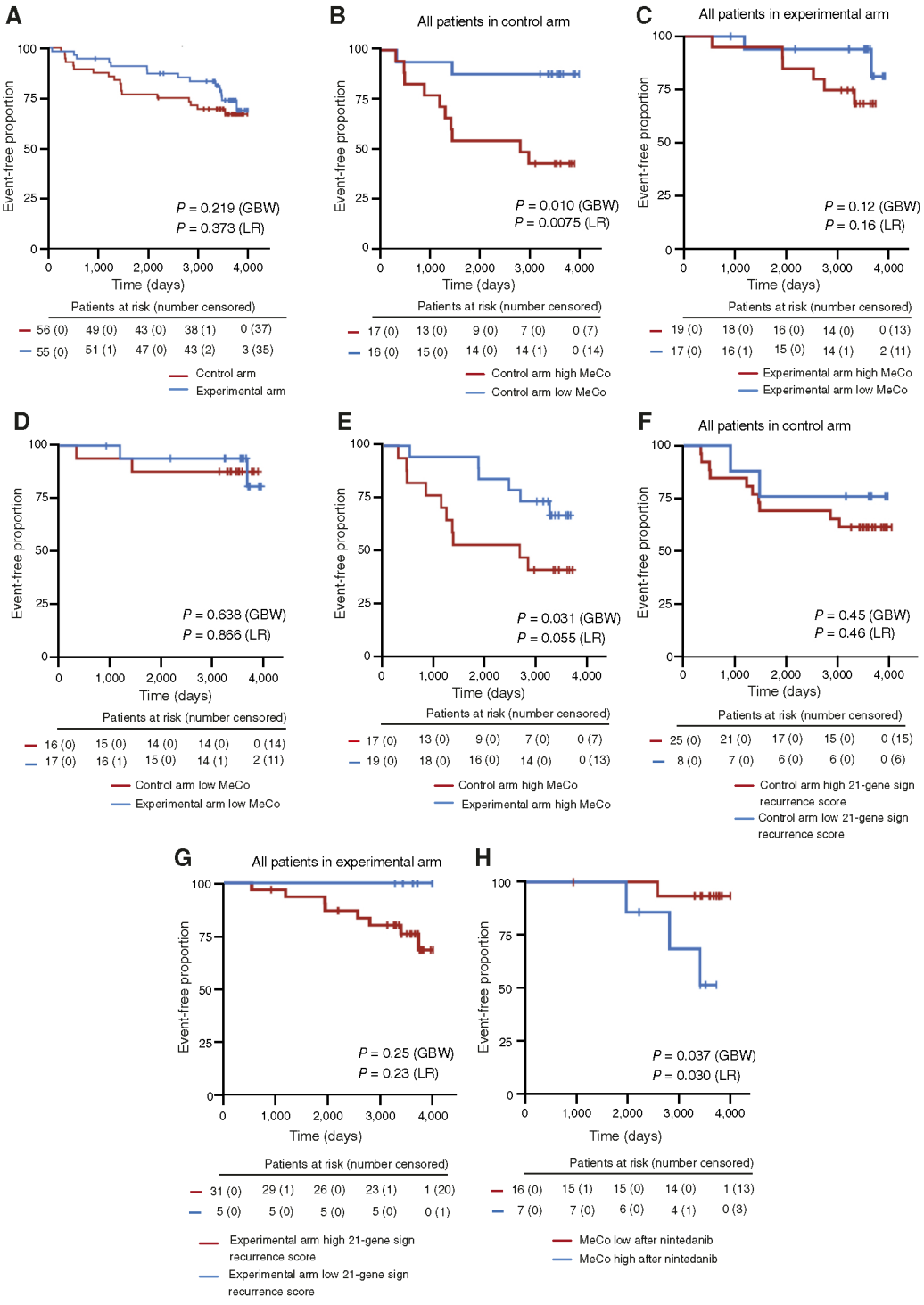

Figure 4.

EFS with or without nintedanib and specific effects according to the MeCo gene expression score. **A,** Kaplan–Meier curve comparing the EFS between the standard experimental treatment arms ($N = 111$ patients). **B,** EFS of patients with high vs. low MeCo score among patients randomized to the control arm ($N = 33$ patients). **C,** EFS of patients with high vs. low MeCo score among patients randomized to the experimental arm ($N = 36$ patients). **D,** Kaplan–Meier curve comparing nintedanib vs. control in low-MeCo patients (median EFS not-reached vs. not-reached). **E,** Kaplan–Meier curve comparing nintedanib vs. control in high-MeCo patients (median EFS 2,806 days vs. not reached). **F,** EFS of patients with high vs. low 21-gene recurrence score among patients randomized to the control arm ($N = 33$ patients). **G,** EFS of patients with high vs. low 21-gene recurrence score among patients randomized to the experimental arm ($N = 36$ patients). **H,** Kaplan–Meier curve comparing the EFS time between patients achieving a low MeCo gene expression score after the run-in phase vs. those remaining high ($N = 23$ patients).

that "correcting" a high MeCo score, as seen during the run-in phase, would not only improve pCR rates but also long-term outcomes. Data from **Figs. 2** and **3** indicate a selective positive biological effect of nintedanib in the high MeCo gene expression cluster, suggesting that benefits on EFS might be specific to high-MeCo patients, and non-detectable in the whole trial population. Thus, we compared the effect of having a high or a low MeCo score across treatment arms, obtaining the following data:

In the control arm, high-MeCo patients had significantly worse outcomes than low-MeCo counterparts (3.3 years shorter average EFS; **Fig. 4B**: median EFS time not reached vs. 2,806 days and estimated average EFS time 3,591 vs. 2,390 days, in low vs. high MeCo; HR: 0.21; 95% CI, 0.067–0.66). In contrast, in the experimental arm, high-MeCo patients had comparable EFS to low-MeCo patients when treated with nintedanib (**Fig. 4C**: median EFS not reached vs. not reached, and estimated average EFS 3,825 vs. 3,329 days; HR : 0.37; 95% CI, 0.091–1.4), suggesting nintedanib's mitigating effect on the adverse outcomes associated with high MeCo. Furthermore, in the low-MeCo group, all patients had a favorable outcome, and nintedanib did not lead to statistically significantly better outcomes (**Fig. 4D**; median EFS not reached vs. not reached and estimated average EFS 3,825 vs. 3,591 days for the experimental and control arm, respectively; HR: 0.84; 95% CI, 0.11–6). Accordingly, nintedanib treatment improved the outcome compared with the control arm in high MeCo, with >2.5 years longer average EFS (**Fig. 4E**; median EFS not reached vs. not reached, and estimated average EFS 3,329 vs. 2,390 days for the experimental and control arm, respectively; HR: 0.38; 95% CI, 0.14–1.02).

MeCo as a predictive biomarker for nintedanib

Hypothetically, the adverse disease course observed in patients with high MeCo scores could be reflective of inherently aggressive tumor characteristics rather than being directly linked to tumor ECM stiffness. If that were the case, nintedanib might be thought to rescue some cases from relapse through general antitumor activity rather than through its antifibrotic effects. To rule out this hypothesis, we examined the impact of the 21-gene recurrence score, a widely recognized platform for stratifying relapse risk in early breast cancer. If the effects of nintedanib were nonspecific, we would expect to observe a protection from relapse in 21-gene high-risk patients as well. The concordance between this score and the MeCo score is detailed in Supplementary Table S1.

High-risk patients according to the 21-gene score exhibited a trend toward poorer long-term prognosis, but the differences were not statistically significant, irrespective of treatment received. In the control arm, high-risk patients had a slightly lower estimated average EFS time than low-risk patients (average EFS time of 2,900 days compared with 3,216 for patients with low-risk recurrence score; median EFS times not reached; HR: 0.61; 95% CI, 0.17–2.2; **Fig. 4F**). Nintedanib did not seem to rescue high-risk patients: similar trends were observed in the experimental arm in EFS between high- and low-risk patients as per the 21-gene score (**Fig. 4G**; median EFS times not reached; estimated average EFS 3,536 vs. 4,020 days in high-risk vs. low-risk, respectively; HR: 0.31; 95% CI, 0.044–2.1). Next, because of the preferred use of the 21-gene score in luminal tumors, we restricted the analysis to patients with luminal tumors. The results, shown in Supplementary Fig. S1, overlap with those observed in the whole study population. When patients were compared according to their MeCo score, luminal patients from the control arm experienced statistically significantly worse outcomes in case they were high MeCo (Supplementary Fig. S1A), but the differences were mitigated in the experimental arm (Supplementary Fig. S1B). However, when patients were split according to the 21-gene risk score, the differences in EFS were not different across trial arms (Supplementary Fig. S1C and S1D). Data in basal-like tumor did not yield statistically significant results due to the limited sample size (Supplementary Fig. S2).

Lastly, we explored whether nintedanib's effects were uniformly observed among high-MeCo patients or if a specific subgroup benefited more distinctly. Comparing patients with a MeCo score downgrade after nintedanib run-in ($N = 16$) to those without downgrade ($N = 7$) suggested that patients who demonstrated a direct antifibrotic response to nintedanib in a brief window-of-opportunity showed markedly better prognoses. Although these observations are based on a small sample size and are thus preliminary, they point toward a particularly positive outcome for fast responders to nintedanib who downgraded MeCo scores from high to low after 2 weeks (**Fig. 4H**; median EFS times not reached; estimated average EFS: 3,924 vs. 3,278 days in patients with high-downgraded-to-low MeCo post-run-in vs. high MeCo post-run-in, respectively; HR: 0.087; 95% CI, 0.0095–0.79).

Discussion

Tumor fibrosis has been widely associated with tumor progression features across different malignancies (27–30). However, to date, the success of targeting this tumor characteristic has been limited. This may be in part due to the minimal number of antifibrotic therapies that have succeeded in clinical development. Nintedanib, an agent initially developed as antiangiogenic, exerts a powerful antifibrotic activity through the blockade of the FGFR family, becoming the first disease-modifying therapy approved for idiopathic lung fibrosis (24). Although its efficacy as a targeted agent has been evaluated in numerous clinical trials in oncology, it has not been formally tested as an antifibrotic drug. Two potential limitations for developing antifibrotic agents in oncology are the difficulty of showing pharmacodynamic engagement and distinguishing of the selective therapeutic effects derived from fibrosis modulation from other direct antitumor effect. Moreover, fibrosis-mediated MeCo is not expected to be induced to the same extent in all tumors with increased stiffness; this variability is evident from previously reported data showing that breast cancer cell lines and patient-derived xenografts exhibit a range of mechanical responsiveness (17). Therefore, antifibrotics may not be equally efficacious in all breast tumors. Recently, a transcriptomics score indicative of pathologic tumor stiffness response, MeCo score, seemed to detect the ECM stiffness-induced tumor cell gene expression program linked to adverse disease course, which was sustained in time even after leaving the primary tumor site (17). Because this transcriptomics score is a measurable marker in tumor tissue, and our past nintedanib trial had a favorable design to test several hypotheses related to its antifibrotic properties and their long-term impact, additional analysis of the randomized trial was planned and conducted.

In general, commonly used genomic scores in breast cancer associate the higher risk scores to biological characteristics such as increased cell replication or de-differentiation (31). Thus, it is not surprising that higher scores are linked to improved response to cytotoxic agents. In the case of MeCo score, higher scores are linked to increased metastatic potential or ECM stiffness, which is in turn associated with decreased perfusion – or chemotherapy delivery to the tumor microenvironment. Therefore, the fact that tumors with

higher MeCo scores have a trend toward worse pathologic response rates (**Fig. 2B**) seems to be specifically related to the biological trait captured by this transcriptomics signature. This stands in contrast to the 21-, 50-, or 70-gene signatures [other commonly used genomic risk score signatures to inform treatment for early breast cancer, which often correlate higher scores with traits like increased proliferation and associate high score results with the recommendation of including nonspecific cytotoxic agents in the treatment schedule (14, 32–37)]. In fact, the PCNA gene cluster [proliferation gene signature (38)] was removed from the contributing genes when the MeCo score was designed (17); theoretically, this would allow capturing the specific tumor cell response to matrix stiffness and link elevated scores to specific antifibrotic therapy recommendations. In this sense, we consider a very relevant proof of specificity and pharmacodynamic engagement that the 2-week nintedanib monotherapy course is able to decrease stiffness response score by 25% (**Fig. 3B**). Answering whether high- or low-MeCo patients within a particular intrinsic subtype achieve higher or lower pCR rates, and whether nintedanib is able to rescue differentially the score by subtype would require larger series. Nevertheless, the data shown in **Figs. 2B** and **3** suggest that achieving similar pCR rates in high- and low-MeCo patients in the experimental arm is linked to the biological effects of nintedanib. Similarly, the optimal cutoff for distinguishing high versus low MeCo could be further refined in larger patient series in which the sample size allows performing a ROC curve. Here, only one cutoff was tested (the median value) and was prespecified before the analysis, in order to have a sufficient number of patients on each group. Such cutoff optimization, technical development, and testing MeCo in larger number of patients are strict requirements prior to convert this research-use-only score into an approved diagnostic tool.

The most relevant results of this study are the long-term EFS effects. This clinical trial was not powered to detect EFS differences; in fact, the original follow-up planning included only up until surgery. The updated follow-up data shown here are mature (median = 9.7 years), and the comparison of the standard and experimental arms yields overall a negative trial (**Fig. 4A**). However, when patients are split by their MeCo score, the results suggest a positive therapeutic effect of nintedanib that is specific for fibrotic high MeCo tumors. In the absence of antifibrotic treatment, patients with elevated scores suffer from a very aggressive disease course (**Fig. 4B** and **E**), with a significant number of patients relapsing early during adjuvant hormonal treatment. It is important to point out that all patients in this study received chemotherapy, which place these results in stark contrast with other genomic risk platforms: usually, low-risk cases are treated with endocrine monotherapy, whereas high-risk cases are treated – and rescued – with nonspecific chemotherapy. Here, despite chemotherapy, high-MeCo patients have a very negative disease course (**Fig. 4B**); however, patients with high– or low–21-gene risk scores have similar disease courses (**Fig. 4F**), as they have been treated with chemotherapy already. High-MeCo patients, however, are specifically rescued by nintedanib (**Fig. 4C** and **E**). Twenty-one-gene high-risk patients experience a trend toward a worse outcome than patients with low-risk, but this risk is not corrected by nintedanib (**Fig. 4F** and **G**).

Currently, patients with early breast cancer with high risk of relapse are offered abemaciclib or ribociclib for improving their chances of remaining long-term disease-free; these therapies are associated to considerable costs and toxicity. The data shown in this study warrant studying nintedanib in this setting for several reasons: (i) a 3-month course was sufficient to improve long-term prognosis in this trial; (ii) nintedanib has a very mild toxicity profile; (iii) the MeCo score presents a predictive biomarker that can identify patients more likely to benefit from nintedanib, in contrast with the lack of biomarkers for other adjuvant therapies; and (iv) the prognosis of low-MeCo patients (at least in this cohort of patients treated with chemotherapy) seems to be very favorable, and future studies could address potential treatment de-escalation strategies. One additional data point that may help defining the role of antifibrotic therapy in future studies concerns patients starting therapy during the neoadjuvant setting, because the dynamic assessment of the pharmacodynamic response by measuring MeCo score just after 2 weeks of treatment is possible and here was associated with an improved disease course (**Fig. 4H**). The reason why nintedanib was not able to exert therapeutic antifibrotic effects in a significant portion of patients (~1/3) remains unclear, and it should be further investigated.

Our study has several limitations. The main ones are as follows: first, this study was not planned for measuring and/or comparing long-term EFS. The current study required a new institutional approval for following up all patients; a number of patients were lost to follow-up, decreasing the total N of the study. Regardless, because of the specific pharmacodynamic effects of nintedanib observed in the change in MeCo score in response to treatment and the "clean" randomized design (monotherapy or bi-therapy, instead of currently used regimes of up to five drugs, together with a run-in phase), this is the first study, to the best of our knowledge, to report positive and direct effects of antifibrotic therapy in breast cancer. Whether the improved EFS results from nintedanib's effect beyond the run-in phase (i.e., during the combination phase) cannot be answered with the current design and constitutes an additional limitation, as no further biopsies were planned after the run-in phase. Second, several correlative studies were planned with the trial samples in its original design to understand the mechanisms behind efficacy and resistance against antiangiogenics, and many samples were exhausted; thus, the present study had to be performed with a limited subset of samples (Consolidated Standards of Reporting Trials diagram). Although the overall number of samples in this study can be considered low, data shown in **Table 1** suggest that the patient subset analyzed here is representative of the full trial population. In addition, the magnitude of the HRs and the statistical significance observed across all the survival analysis (**Fig. 4**) considerably mitigate this limitation. The data shown in **Fig. 4H** also add biological plausibility to the hypothesis of pharmacologic specificity: those patients in which the adverse gene expression cluster is corrected after 2 weeks of nintedanib have an excellent prognosis compared with the remaining, with a >10-fold protective HR.

To the best of our knowledge, this is the first study reporting the therapeutic effects of antifibrotic treatment in breast cancer. The MeCo gene expression score is derived from a molecular signature that is specifically associated with a biological trait – tumor stiffness response – that in a previous study showed prognostic association with HER2-negative disease course, and in light of the current results could also have a predictive role when treated with nintedanib. In this context, nintedanib would be a specific treatment against tumors with high MeCo scores, which are reduced in response to it, suggesting pharmacodynamic engagement and direct antifibrotic effects. This, together with the fact that the long-term EFS outcomes are improved by nintedanib in high-MeCo patients, but not in 21-gene recurrence score–high patients, also suggests that the therapeutic effects are linked to the antifibrotic properties and not to

other nonspecific antitumor effects. A future pivotal prospective clinical trial will be performed to validate these findings and establish the clinical niche for MeCo testing and explore other potential mechanism of antifibrotic agents in cancer such as better drug delivery or immunomodulation (39).

Authors' Disclosures

M. Quintela-Fandino reports grants from MeCo Diagnostics and Boehringer Ingelheim during the conduct of the study, as well as personal fees from AstraZeneca and Pfizer outside the submitted work. B. Bermejo reports personal fees from Pfizer, AstraZeneca, Gilead, Novartis, and Eli Lilly and Company and other support from Pfizer outside the submitted work. F. Moreno reports grants from Pfizer and personal fees from Pfizer, MSD, Daiichi-Sankyo, Gilead, Novartis, Eli Lilly and Company, AstraZeneca, and Roche outside the submitted work. J.Á. Garcia-Saenz reports grants from AstraZeneca, Daiichi Sankyo, Eli Lilly and Company, Gilead, Novartis, Exact Science, and Roche and grants and personal fees from Menarini outside the submitted work. S. Pernas reports grants, personal fees, and nonfinancial support from Roche; personal fees and nonfinancial support from Pfizer, Novartis, AstraZeneca-Daiichi, and Gilead; and personal fees from Seagen and Eli Lilly and Company outside the submitted work, as well as membership on the SOLTI Breast Cancer Group board of directors. N. Martínez Jañez reports grants from Pfizer, Roche, Novartis, Eli Lilly and Company, and AstraZeneca-Daiichi outside the submitted work. E. Adrover reports other support from Eli Lilly and Company and AstraZeneca and nonfinancial support from Daiichi Sankyo outside the submitted work. E. Alba reports grants from Pfizer and AstraZeneca and personal fees from Exact Sciences, Roche, Novartis, Pfizer, Menarini, and Eli Lilly and Company outside the submitted work. A. Llombart-Cussac reports grants, personal fees, and nonfinancial support from Roche, Genentech, and AstraZeneca; grants and personal fees from Pfizer; grants from Eli Lilly and Company; grants and nonfinancial support from Gilead and Novartis; and nonfinancial support from Daiichi Sankyo during the conduct of the study, as well as grants from Agendia and nonfinancial support from Genomic Health outside the submitted work. J. Cortés reports personal fees from Roche, AstraZeneca, Seattle Genetics, Daiichi Sankyo, Eli Lilly and Company, Merck Sharp & Dohme, Leuko, Bioasis, Clovis Oncology, Boehringer Ingelheim, Ellipses, HiberCell, BioInvent, GEMoaB, Gilead, Menarini, Zymeworks, Reveal Genomics, Scorpion Therapeutics, ExpreS2ion Biotechnologies, Jazz Pharmaceuticals, AbbVie, Novartis, Eisai, Pfizer, Stemline Therapeutics, BridgeBio, Biocon, Circle Pharma, and BioNTech outside the submitted work, as well as a patent for Pharmaceutical Combinations of A PI3K Inhibitor and A Microtubule Destabilizing Agent (Javier Cortés Castán, Alejandro Piris Giménez, Violeta Serra Elizalde, WO 2014/199,294 A. issued) and a patent for HER2 as a predictor of response to dual HER2 blockade in the absence of cytotoxic therapy (Aleix Prat, Antonio Llombart, Javier Cortés, US 2019/ 0338368 A1. licensed). In addition J. Cortés reports research funding to the institution from Roche, Ariad Pharmaceuticals, AstraZeneca, Baxalta GMBH/Servier Affaires, Bayer Healthcare, Eisai, F. Hoffman-La Roche, Guardant Health, Merck Sharp & Dohme, Pfizer, Piqur Therapeutics, Iqvia, Queen Mary University of London; stock ownership in MAJ3 Capital and Leuko (relative); and travel, accommodation, and expenses from Roche, Novartis, Eisai, Pfizer, Daiichi Sankyo, AstraZeneca, Gilead, Merck Sharp & Dhome, and Stemline Therapeutics. A. Grant reports personal fees from MeCo Diagnostics during the conduct of the study, as well as a patent for MeCo score issued. A. Watson reports a patent for 17/293,578 pending to MeCo Diagnostics. G. Mouneimne reports personal fees from MeCo Diagnostics during the conduct of the study, as well as a patent for 17/293,578 issued and licensed to MeCo Diagnostics. No disclosures were reported by the other authors.

Authors' Contributions

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Note

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