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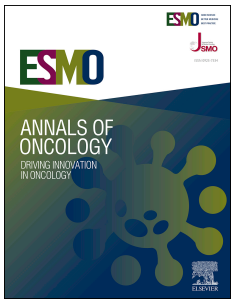

TNBC-DX genomic test in early-stage triple-negative breast cancer treated with neoadjuvant taxane-based therapy

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TNBC-DX genomic test in early-stage triple-negative breast cancer treated with neoadjuvant taxane-based therapy

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Abstract

Background: Identification of biomarkers to optimize treatment strategies for early-stage triple-negative breast cancer (TNBC) is crucial. This study presents the development and validation of TNBC-DX, a novel test aimed at predicting both short- and long-term outcomes in early-stage TNBC.

Methods: Information from 1,259 patients with early-stage TNBC (SCAN-B, CALGB-40603, and BrighTNess) were used to establish the TNBC-DX scores. Independent validation of TNBC-DX was carried out in 3 studies: i) WSG-ADAPT-TN, ii) MMJ-CAR-2014-01, and iii) NeoPACT, including 527 patients with stage I-III TNBC undergoing neoadjuvant chemotherapy. In WSG-ADAPT-TN, patients were randomized to receive nab-paclitaxel plus gemcitabine or carboplatin. In MMJ-CAR-2014-01, patients received carboplatin plus docetaxel. In NeoPACT, patients received carboplatin plus docetaxel and pembrolizumab. The objective of this study was to evaluate the association between TNBC-DX and efficacy outcomes (pCR, distant disease-free survival [DDFS] or event-free survival [EFS], and overall survival [OS]) in the validation cohorts.

Results: TNBC-DX test was created incorporating 10-gene core immune gene module, 4-gene tumor cell proliferation signature, tumor size, and nodal staging. In the 2 independent validation cohorts without pembrolizumab, the TNBC-DX pCR score was significantly associated with pCR after adjustment for clinicopathological variables and treatment regimen (odds ratio per 10-units increment=1.34, 95% CI 1.20-1.52, p<0.001). pCR rates for the TNBC-DX pCR-high, -medium, and -low categories were 56.3%, 53.6%, and 22.5% respectively (odds ratio for pCR-high vs pCR-low=3.48 [95% CI 1.72-7.15], p<0.001). Additionally, the TNBC-DX risk score was significantly associated with DDFS (hazard ratio [HR] high-risk vs low-risk=0.24, 95% CI 0.15-0.41, p<0.001) and OS (HR=0.19, 95% CI 0.11-0.35, p<0.001). In the validation cohort with pembrolizumab, the TNBC-DX scores were significantly associated with pCR, EFS, and OS.

Conclusions: TNBC-DX predicts pCR to neoadjuvant taxane-carboplatin in stage I-III TNBC and helps to forecast the patient´s long-term survival in the absence of neoadjuvant anthracycline/cyclophosphamide, and independent of pembrolizumab use.

Introduction

TNBC presents a significant treatment challenge due to its aggressive nature and limited targeted therapy options[1,2]. Systemic multiagent chemotherapy improves long-term outcomes and is recommended for stage I–III TNBC disease, with most patients in the current era being treated with neoadjuvant chemotherapy[3]. Anthracyclines and cyclophosphamide (AC) have typically constituted the chemotherapy backbone of multiagent regimens in combination with taxane-based therapy (AC-T), which often includes carboplatin (AC-TCb)[3]. More recently, neoadjuvant and adjuvant pembrolizumab has been approved for the treatment of stage II-III TNBC in combination with AC-TCb[4].

Given the short-term and long-term toxicities associated with AC[4-6], interest in anthracycline-free chemotherapy regimens has been gaining momentum among patients and physicians. Indeed, several studies have evaluated the possibility to eliminate the use of anthracyclines and focus on the use of a taxane-carboplatin regimen. This combination yields pathologic complete response (pCR) rates of 45-55% in TNBC, and patients achieving a pCR with these regimens demonstrate excellent 3-year outcomes without adjuvant anthracycline[7-11]. In fact, in a randomized phase III trial, 6 cycles of adjuvant carboplatin plus paclitaxel showed superior disease-free survival compared to an anthracycline plus taxane regimen[12]. It is unclear whether the four-drug AC-TCb chemotherapy backbone is necessary for all patients receiving neoadjuvant pembrolizumab. The SCARLET phase III trial (NCT05929768) is comparing the traditional AC-TCb and pembrolizumab regimen with docetaxel-carboplatin and pembrolizumab to optimize neoadjuvant therapy.

At the same time, there is an emerging focus in early-stage TNBC to modulate the intensity of immunotherapy and other therapies —either by escalating or de-escalating – and several phase III trials are underway. The OptimICE-pCR (NCT05812807) trial is investigating if adjuvant pembrolizumab is beneficial for patients who had a pCR following preoperative chemotherapy with pembrolizumab. In contrast, SASCIA (NCT04595565) and OptimICE-RD (NCT05633654) trials are examining the benefits of escalating therapy using the anti-TROP2 antibody-drug conjugate sacituzumab govitecan with or without pembrolizumab for patients with residual disease after neoadjuvant therapy.

Until recently, percentage of tumor-infiltrating lymphocytes (TILs) has been recognized as a potential biomarker for patients with early-stage TNBC[13-15], though not yet fully established in

clinical guidelines for widespread use. Additionally, translational research in the past decade has uncovered robust genomic-based immune biomarkers[16-18], showing promising potential for clinical application. Among them, the recently developed HER2DX genomic test for early-stage HER2+ breast cancer includes a 14-gene immunoglobulin signature[19-21]. In this study, we developed and validated a new genomic test (TNBC-DX) to predict short- and long-term outcomes in patients with early-stage TNBC.

Methods

TNBC-DX Development

The standardized 27-gene HER2DX genomic test for early-stage HER2+ breast cancer[19-24] was used as a reference to develop the TNBC-DX genomic test. The HER2DX assay is based on 4 different gene signatures comprising 27 genes, including the 14-gene immunoglobulin (IGG) module (i.e. *CD27, CD79A, HLA-C, IGJ, IGKC, IGL, IGLV3-25, IL2RG, CXCL8, LAX1, NTN3, PIM2, POU2AF1 and TNFRSF17*). The other three gene signatures are a 4-gene tumor cell proliferation signature (*EXO1, ASPM, NEK2, and KIF23*), a 5-gene luminal differentiation signature (*BCL2, DNAJC12, AGR3, AFF3, and ESR1*) and the 4-gene HER2 amplicon signature (*ERBB2, GRB7, STARD3, and TCAP*). Two scores are calculated for each patient: (i) HER2DX pCR score and (ii) HER2DX risk score (both from 0 to 100). Pre-established cut-offs are used to create the HER2DX pCR groups [low (0-33.3), medium (33.3-66.7) and high (66.7-100)], and to create the HER2DX risk groups [low (0-50) and high (50-100)].

Three in-silico datasets with information from 1,259 patients with early-stage TNBC (i.e., SCAN-B, CALGB-40603, and BrighTNess trials) were used to improve the model in the context of TNBC. Of note, CALGB-40603 and SCAN-B used an estrogen receptor 10% cut-off, while BrighTNess used an estrogen receptor 1% cut-off. The signatures defined in the HER2DX assay and individual genes were evaluated across the 3 studies to assess its association with efficacy outcomes. Additionally, a new 10-gene Core Immune Gene (CIG) module (i.e., *CD274, CD79A, CXCR6, IRF4, LAX1, PDCD1, PIM2, POU2AF1, SLAMF1, and TNFRSF17*), which was obtained from a previous analysis[17], was also evaluated to determine whether incorporating this information could improve the prognostic performance of the model. The development cohorts (n=1,259) were solely utilized to define the TNBC-DX test, without being used for formal validation or for quantifying its association with pCR status and survival outcomes.

TNBC-DX Validation Studies

After the development of the TNBC-DX, the model was externally validated in 527 patients across the MMJ-CAR-2014-01 (n=292), ADAPT-TN (n=126), and NeoPACT (n=109) studies. TNBC-DX was performed using RNA in the MMJ-CAR-2014-01 and ADAPT-TN cohorts and using RNA-seq data in the NeoPACT cohort.

The MMJ-CAR-2014-01 (NCT01560663)[11] is an ongoing prospective, multicenter, nonrandomized trial exploring the antitumor activity of neoadjuvant carboplatin and docetaxel in early-stage TNBC, exhibiting less than 1% expression of ER and PR. Eligible patients included females with pathologically confirmed diagnosis of primary invasive breast cancer, stage I–III. The patients were diagnosed at any of the participant academic institutions. From 2013-2019, 299 enrolled patients were treated with 6 cycles of carboplatin (AUC 6) and docetaxel (75 mg/m2) given every 21 days. Patients with non-pCR could receive adjuvant anthracycline-based therapy per investigator discretion.

The ADAPT-TN study[8,9], a phase II prospective neoadjuvant trial (WSG-ADAPT TN Trial, NCT01815242), enrolled patients diagnosed with stage I–III TNBC confirmed centrally, exhibiting less than 1% expression of ER and PR. Participants were randomized in a 1:1 ratio, with stratification based on nodal status and study center, to undergo 12 weeks of treatment. Patients were randomized to receive i) nab-paclitaxel at a dose of 125 mg/m2 administered on days 1 and 8, combined with either gemcitabine (1,000 mg/m2 on days 1 and 8, referred to as the gem arm) or ii) carboplatin (AUC 2 on days 1 and 8, referred to as the nab-pac/carbo arm). For patients without a pCR in the breast or axillary nodes during surgery, an additional four cycles of anthracycline-based chemotherapy (epirubicin at 90 mg/m2 and cyclophosphamide at 600 mg/m2 every two or three weeks) were mandated. This could also be administered pre-surgery as additional neoadjuvant chemotherapy upon confirming non-pCR status via core biopsy. At discretion of the investigator, those who had a pCR were allowed to forego further standard chemotherapy. Among patients with pCR, there were 12 instances of invasive disease-free survival (iDFS) events, including 6 distant events, with no significant iDFS risk difference between patients who did and did not receive further chemotherapy[9].

The NeoPACT (NCT03639948)[25] is an open-label multi-center phase 2 clinical trial which enrolled 115 female patients with stage I to III TNBC (including tumors with an estrogen

receptor expression up to 10%) who received neoadjuvant carboplatin (AUC 6) and docetaxel (75 mg/m2) plus pembrolizumab (200 mg) every 21 days for 6 cycles from 2018-2022. After surgery, no adjuvant pembrolizumab was indicated. Patients with non-pCR could receive adjuvant anthracycline-based therapy per investigator discretion.

Additionally, gene expression and mutation data from 153 TNBC tumors from The Cancer Genome Atlas (TCGA)[11] dataset was obtained from cBioPortal[11]. TNBCDX scores were applied on to RNA-seq data. TNBC subtype and TIME classification were obtained from Lehmann et al[11].

Clinical Endpoints

The co-primary endpoints for this analysis were i) pCR and ii) distant disease-free survival (DDFS) or event-free survival (EFS). pCR was defined as the absence of residual invasive disease in the breast and axilla with or without ductal carcinoma in situ (ypT0/isN0). Pathologic response was determined locally. DDFS was defined as the time from registration, before initiating neoadjuvant therapy, to the time to distant breast cancer recurrence, secondary invasive malignancy, or death, whichever occurs first. EFS was defined as time from diagnosis to first invasive locoregional or distant recurrence, study treatment-related death, or breast cancer−related death. Secondary endpoints include overall survival (OS), invasive DFS (iDFS), defined as the time from registration to any invasive cancer event or death, pCR status according to the chemotherapy regimen received during the neoadjuvant treatment and survival outcomes by pCR status. The TNBC-DX assay was retrospectively evaluated in a blinded manner, with results centrally analyzed and subsequently linked to clinical data.

Statistical Analysis

To validate the model, the first objective was to assess the association between the TNBC-DX pCR score (as a continuous variable and group categories) with pCR status in the independent validation cohorts without pembrolizumab. Univariable and multivariable logistic regression models were used to investigate the association for each variable with pCR in terms of odds ratios (ORs) with 95% confidence interval (95% CI). To evaluate the performance of the TNBC-DX pCR score, the area under the ROC curve (AUC) and calibration plots were calculated[26]. The second objective to validate the model was to assess the ability of the TNBC-DX risk score (as a continuous variable and group categories) to predict survival outcome (DDFS and OS) in the independent validation cohorts without pembrolizumab. The Kaplan–

Meier method was used to estimate survival outcomes. Stratified univariable and multivariable Cox proportional hazards models were used to obtain hazard ratios (HRs). The cohort type was used as a stratification factor (ADAPT-TN and MMJ-CAR-2014-01), allowing a different baseline hazard function for each study. The proportional hazards assumption was tested and inspected visually by means of Schoenfeld residuals (**Supplementary Table 1**). All variables evaluated in the univariable analysis were included in the multivariable model. Missing at random values were imputed using the chained equations method[27]. The prevalence of missing data was <5% in all variables (**Supplementary Table 2**). Following imputation, a sensitivity analysis was conducted to ensure that these imputations did not alter the obtained results. Details of the adjuvant chemotherapy used in study MMJ-CAR-2014-01 is provided in **Supplementary Table 3**. The third objective was to assess the association between the TNBC-DX pCR and risk scores (as a continuous variable and group categories) with pCR status, EFS and OS in the independent validation cohort with pembrolizumab. The median follow-up was calculated using the reverse Kaplan-Meier method. For all statistical analyses, the significance level was set at two-sided alpha of 0.05 and all analyses were carried out using R statistical software version 4.3.2.

Ethical Approval

The MMJ-CAR-2014-01, ADAPT-TN, and NeoPACT trials received approval from relevant ethics committees, and institutional review boards, adhering to the Declaration of Helsinki. Participation was contingent upon the provision of written informed consent by all patients. Material transfer agreements were established, and ethical approvals were obtained for the correlative analyses conducted. These approvals cover the use of patient samples and data for the analyses presented in this study.

Role of the Funding Source

The study was designed and performed by investigators from the West German Study Group, Gregorio Marañón General Hospital, University of Kansas, and Reveal Genomics. Reveal Genomics funded the study. All authors had full access to all data in the study and had final responsibility for the decision to submit for publication.

Results

TNBC-DX development

The TNBC-DX genomic test was created based on two different gene signatures, the 10-gene Core Immune Gene (CIG) module (i.e., *CD274, CD79A, CXCR6, IRF4, LAX1, PDCD1, PIM2, POU2AF1, SLAMF1, and TNFRSF17*) and the 4-gene tumor cell proliferation signature (*EXO1, ASPM, NEK2, and KIF23*), as well as incorporating tumor size and nodal staging.

In the development cohorts (SCAN-B, CALGB-40603, and BrighTNess trials; n=1,259), the 10-CIG module and the 4-gene tumor cell proliferation signature were consistently associated with pCR and survival outcomes. The IGG signature was also associated with efficacy outcomes. Of note, 5 of the 10 CIGs (i.e., *CD79A, LAX1, PIM2, POU2AF1, and TNFRSF17*) were part of the 14-gene IGG module. As the CIG module presented better results, the IGG signature was not included in the score. Other signatures considered for inclusion, such as the HER2 amplicon signature and luminal differentiation, were not associated with efficacy outcomes and were not incorporated into the model. Additionally, the *ERBB2* gene (i.e., the TNBC-DX ERBB2 score), was included to identify clinical HER2 status, but it did not contribute to the calculation of the pCR or risk scores. Thus, the final TNBC-DX test is based on the 10-CIG module, the HER2DX 4-gene tumor cell proliferation signature, tumor size and nodal staging. Pre-established cut-offs were used to create the TNBC-DX pCR groups [low (0-33.3), medium (33.3-66.7) and high (66.7-100)], and to create the TNBC-DX risk groups [low (0-58) and high (59-100)]. Further details on the development of TNBC-DX can be found in **Supplementary materials.**

Baseline characteristics of the validation cohorts without pembrolizumab

A total of 527 patients with stage I-III TNBC were included in the first two external cohorts to validate the performance of the TNBC-DX test in the absence of pembrolizumab (**Table 1**). In the combined cohort, median age was 52 years (range 26 to 80), clinical stage II disease represented 69.7%, and 41.9% had clinically node-positive disease. TILs (i.e., ≥10%) were observed in 56.8% of the patients. The TNBC-DX low- and high-risk categories represented 55.4% and 44.6% of the cases, respectively. The TNBC-DX pCR-low, pCR-med and pCR-high categories represented 33.0%, 33.0% and 34.0% of the cases, respectively. A significant association was observed between TNBC-DX risk groups and pCR groups, where the pCR-high group was found more prevalent in the low-risk group than in the high-risk group (79.9% vs. 20.1%). A moderate correlation was observed between both continuous scores (ρ=-0.56). Clinicopathologic characteristics according to TNBC-DX pCR and risk groups are provided in **Supplementary Table 4.**

TNBC-DX association with pCR in the absence of pembrolizumab

The pCR rate was 34.1% (95% CI 26.1-43.2%) in the ADAPT-TN cohort, 48.6% (95% CI 42.8-54.5%) in the MMJ-CAR-2014-01 cohort, and 44.3% (95% CI 39.5-49.2%) in the combined cohort. The pCR rate with taxane-carboplatin and nab-paclitaxel-gemcitabine regimens was 46.4% and 34.7%, respectively. TNBC-DX pCR score was significantly associated with pCR in the ADAPT-TN cohort (odds-ratio [OR] per 10-unit increase=1.22, 95% CI 1.06-1.41, p=0.006), in the MMJ-CAR-2014-01 cohort (OR=1.37, 95% CI 1.24-1.52, p<0.001), and in the combined series (OR=1.28, 95% CI 1.18-1.38, p<0.001; and **Figure 1A**). The pCR rate in the TNBC-DX pCR-high group was higher than that in the pCR-low group (56.3% vs 22.5%, OR=4.45, 95% CI 2.67-7.57, p<0.001). Discrimination, calibration plots and sensitivity analysis are shown in **Supplementary Figure 1-5.** In the multivariable model from the combined cohort including clinicopathological factors and treatment, the TNBC-DX pCR score remained significantly associated with pCR (OR=1.34, 95% CI 1.20-1.52, p<0.001) along with clinical nodal stage and the chemotherapy regimen. Of note, despite TILs being associated with pCR in univariate analysis, the TILs variable lost its significance in the multivariable analysis when TNBC-DX pCR score was included in the model (OR=1.03, 95% CI 0.94-1.14, p=0.48). Analysis evaluating TNBC-DX and TILs both as a continuous score and as a group category are shown in **Supplementary Table 5-6** and **Supplementary Figure 6**. The association between TNBC-DX pCR groups and pCR endpoint was consistent across the chemotherapy regimens (**Figure 1B**).

TNBC-DX association with survival in the absence of pembrolizumab

The median follow-up of the ADAPT-TN and MMJ-CAR-2014-01 cohorts was 60.2 and 50.5 months, respectively. Similar outcomes were observed between both cohorts in DDFS and OS (**Supplementary Figure 7-8**). The 5-year DDFS and OS of the combined cohort was 80.0% (95% 76.1-84.2) and 82.3% (95% 78.4-86.4), respectively. In the DDFS univariable analysis, a statistically significant association between the TNBC-DX risk score and DDFS in each individual study and in the combined cohort (HR per 10-unit increase=1.37, 95% CI 1.25-1.51, p<0.001) was observed (**Figure 2A**). The 5-year DDFS in the TNBC-DX low-risk group was higher than in the high-risk group (89.9% vs 69.4%, HR=0.24, 95% CI 0.15-0.41, p<0.001) (**Figure 2B**). In the multivariable model including all the evaluated factors, TNBC-DX risk score remained significantly associated with DDFS (HR per 10-unit increase=1.33, 95% CI 1.09-1.61, p=0.004). Of note, TILs were not associated with DDFS in the multivariable analysis

(HR=1.03, 95% CI 0.92-1.14, p=0.61). Results from analyses evaluating TNBC-DX and TILs, both as continuous scores and as categorical groups, are presented in **Supplementary Table 7-8**.

Similar results were observed when OS was evaluated (**Supplementary Figure 9**). TNBC-DX was associated with OS in each validation cohort and in the combined analysis, both as a risk score and as a risk group. The 5-year OS in the combined cohort was 93.8% in the TNBC-DX low-risk group and 70.8% in the high-risk group (HR=0.19, 95% CI 0.11-0.35, p<0.001) (**Figure 2C**). TNBC-DX risk-score remained statistically associated with OS after adjustment by clinical variables, TILs and treatment regimen (HR per 10-units increment=1.34, 95% CI 1.09-1.65; p=0.006). However, no association was observed between TILs and OS in the multivariable model (**Supplementary Figure 9**). Overall, the prognostic value of TNBC-DX, both as a continuous score and as a risk group, in identifying patients with a higher likelihood of achieving a pCR and a lower risk of disease recurrence and death was independent of clinicopathological factors and treatment characteristics (**Figure 3**). Similar results were observed when using iDFS (**Supplementary Figure 10**).

TNBC-DX risk score beyond pCR in the absence of pembrolizumab

pCR status was significantly associated with survival outcomes (**Figure 4A-B**). Among patients who had a pCR following neoadjuvant therapy in the combined cohort (n=185), the TNBC-DX low- and high-risk categories represented 41.6% and 58.4% of the cases, respectively; TNBC-DX risk score as a continuous score was not significantly associated with DDFS (HR per 10-units increment=1.18, 95% CI 0.95-1.45, p=0.14), but it was associated with OS (HR per 10-units increment=1.29, 95% CI 1.01-1.65, p=0.04) (**Supplementary Table 9**). Among patients who did achieve a pCR following neoadjuvant therapy (n=233), the TNBC-DX low- and high-risk categories represented 46.8% and 53.2% of the cases, respectively; TNBC-DX risk score as a continuous score was significantly associated with DDFS (HR per 10-units increment=1.37, 95% CI 1.22-1.53, p<0.001) and with OS (HR per 10-units increment=1.36, 95% CI 1.21 -1.53, p<0.001) (**Supplementary Table 10**). **Figure 4C-D** shows the association between pCR status, survival outcomes and TNBC-DX risk groups.

Validation of the TNBC-DX scores in the NeoPACT trial

Among the 115 patients originally recruited in the NeoPACT trial[25] (i.e., docetaxel, carboplatin and pembrolizumab), 109 (94.8%) had available TNBC-DX results. In these 109 patients

treated, the overall pCR rate was 57.8% (95% CI 48.0-67.1%). TNBC-DX pCR score as a continuous score was significantly associated with pCR in the univariable and in the multivariable analysis after adjustment for clinicopathological factors (OR per 10-unit increase=1.28, 95% CI 1.03-1.61, p=0.030) (**Supplementary Table 11**). The pCR rates in TNBC-DX pCR-high, pCR-medium and pCR-low groups were 78.4%, 66.1% and 33.3%, respectively (high vs. low: OR=7.25, 95% CI 2.55-20.62, p<0.001) (**Figure 5A**).

With a median follow-up of 31 months, the 3-year EFS and OS was 86.6% (95% 78.9-95.1) and 92.4% (95% 87.0-98.0), respectively. In the EFS univariate analysis, a statistically significant association was observed (HR per 10-unit increase=1.75, 95% CI 1.27-2.42, p=0.001) (**Supplemental Table 12**). The 3-year EFS in the TNBC-DX low-risk group was higher than in the high-risk group (93.6% vs 69.3%, HR=0.08, 95% CI 0.02-0.36, p=0.001) (**Figure 5B**). Of note, TILs were not found significantly associated with EFS (HR per 10-unit increase=0.92, 95% CI 0.45-1.14, p=0.45). Similar results were observed when OS was evaluated (**Figure 5C** and **Supplemental Table 13**). The association between TNBC-DX risk groups and survival outcomes remained consistent after accounting for pCR status (**Figure 5D-E**)**.**

Biology associated with TNBC-DX

Finally, to further explore the biology of TNBC-DX scores, we interrogated immune and proliferation gene expression, *TP53* and *PIK3CA* mutations and PAM50, TNBC subtypes and tumor microenvironment (TIME)[28] classification in TNBC tumors from TCGA[26]. TNBC-DX low-risk tumors were enriched for immune gene expression, while TNBC-DX pCR-high group had higher expression of proliferative genes (**Supplementary Figure 11**). Additionally, the Basal-like 1 TNBC subtype had significantly higher TNBC-DX pCR score (p=0.003). TNBC-DX risk scores were not significantly associated with *TP53* and *PIK3CA* mutations, PAM50, or TIME classification.

Discussion

TNBC-DX is a novel genomic test designed for patients with newly diagnosed stage I-III TNBC. Using a machine learning approach, the assay integrates tumor and nodal staging with immune and proliferation signatures and provides two scores (ranging from 0 to 100): one predicting pCR and another forecasting long-term survival outcomes. In this study, we validated both TNBC-DX scores in 527 patients treated with neoadjuvant taxane-based

chemotherapy with or without pembrolizumab across three studies with long-term patient follow-up.

Today, the conventional approach to treating stage I-III TNBC has predominantly involved multi-agent chemotherapy regimens, such as anthracycline-cyclophosphamide and taxane (AC-T or AC-TCb), complemented by pembrolizumab for stage II-III disease[1,3]. While these advancements have improved patient outcomes, they often lead to overtreatment and associated toxicities, as evidenced by real-world studies evaluating the implementation of neoadjuvant pembrolizumab[28-30]. Consequently, there is a discernible shift towards systemic therapy de-escalation, particularly through omitting anthracyclines in favor of a taxane and carboplatin combination. This strategy, which has shown promise in achieving favorable pCR and 3-year survival rates[7-11,25], needs further validation.

The potential de-escalation of immunotherapy, especially in the context of the KEYNOTE-522[4] trial findings, has also drawn significant attention[31]. The trial showcased marked improvements in pCR and event-free survival with the addition of neoadjuvant pembrolizumab plus chemotherapy, followed by adjuvant pembrolizumab for stage II-III TNBC[4,32]. However, no definitive evidence has shown that patients with a pCR following neoadjuvant therapy do not benefit from continued pembrolizumab treatment in the adjuvant setting. While this raises the possibility that continued pembrolizumab may not substantially improve outcomes after pCR[33], this hypothesis requires further validation in randomized trials such as OptimICE-pCR. This consideration is supported by findings from the randomized GeparNuevo phase II trial with durvalumab in combination with chemotherapy, where the immune checkpoint inhibitor was only administered during the neoadjuvant phase[34]. Furthermore, while the NeoPACT neoadjuvant phase II trial with docetaxel-carboplatin-pembrolizumab for 18 weeks reported pCR rates (58%) comparable to those observed in the KEYNOTE-522 regimen (64.8%)[4,25], it is important to note that differences in study populations—such as variations in node positivity, inclusion of stage I patients, and ER/PR threshold of 1% vs 10%—limit the direct comparability of these results.

The absence of established biomarkers to calibrate chemotherapy intensity and guide the omission of (neo)adjuvant pembrolizumab underscores the complexity of decision-making in patients with stage I-III TNBC. This challenge highlights the potential value of TNBC-DX as a clinical tool. For instance, in patients identified as pCR-high and/or low-risk by TNBC-DX,

clinicians could opt for less aggressive treatment regimens. An 18-week course of neoadjuvant docetaxel-carboplatin, with or without pembrolizumab, could be used instead of AC-TCb with pembrolizumab. Additionally, stratifying patients in this way would mean that those who achieve a pCR may not require further systemic therapy. This TNBC-DX-tailored approach could reduce unnecessary systemic therapies, their associated side effects, and positively impact patients' quality of life.

The pCR rates in the TNBC-DX pCR-low group range from 22-33%. In clinical stage II-III TNBC, a TNBC-DX pCR-low classification is unlikely to change the standard course of treatment, such as the use of the KEYNOTE-522 regimen. However, combining a pCR-low result with a high-risk score could help identify a subgroup with unmet needs, making them a priority for future trials focused on treatment escalation with novel therapies. In addition, in clinical stage I, where uncertainty exists between opting for primary surgery or neoadjuvant therapy, a TNBC-DX result showing both pCR-low and low-risk disease may favor the decision for primary surgery, potentially avoiding unnecessary neoadjuvant and adjuvant therapy. Further studies are required to better define the clinical utility of TNBC-DX in guiding treatment decisions in these situations.

The moderate correlation observed between the TNBC-DX risk score and pCR score reflects their distinct but complementary roles. While the proliferation component within TNBC-DX is key for predicting pCR, it also helps identify tumors with a high-risk profile for long-term outcomes, demonstrating the dual utility of the TNBC-DX test. Additionally, although the pCR rates between the medium and high pCR score groups were similar in our combined dataset without pembrolizumab, the NeoPACT trial showed a numerically higher pCR rate in the pCR-high vs medium group (78.4% vs. 61.1%). This high pCR rate approaching 80% in the pCR high group with carboplatin/docetaxel plus pembrolizumab is particularly notable. In addition, the identification of patients with pCR whose tumor is TNBC-DX high-risk underscores the complementary nature of the TNBC-DX risk score and pCR. While pCR reflects individual response to therapy, the TNBC-DX risk score captures baseline risk independent of therapy, providing a more comprehensive assessment of prognosis and helping guide further management in patients who achieve pCR but remain at high risk for recurrence.

Several ongoing trials are exploring de-escalation strategies that TNBC-DX could enhance. Notably, the OptimICE-pCR phase III trial (NCT05812807) is comparing the effect of

pembrolizumab to observation for the treatment of 1,295 patients with early-stage TNBC who achieved a pCR after preoperative chemotherapy in combination with pembrolizumab. The SCARLET phase III trial (NCT05929768) is comparing the effect of pembrolizumab in combination with neoadjuvant docetaxel-carboplatin to pembrolizumab in combination with AC and paclitaxel-carboplatin for the treatment of 2,400 patients with stage II-III TNBC. In addition, two phase II trials are exploring other treatment strategies. The ETNA trial (NCT06078384) will evaluate the survival outcomes in 354 patients with surgically resected stage I TNBC following adjuvant treatment with paclitaxel-pembrolizumab or no therapy. Finally, the ADAPT-TN-III neoadjuvant trial (NCT06081244) will evaluate 12-weeks of sacituzumab govitecan +/- pembrolizumab in clinically stage I TNBC.

Other phase III trials are underway to explore escalation strategies. The SASCIA phase III trial (NCT04595565) is comparing the effect of sacituzumab govitecan to capecitabine or platinum therapy for the treatment of 1,332 patients with early-stage HER2-negative disease, including TNBC, who did not achieve a pCR after preoperative chemotherapy. The ASCENT-05/OptimICE-RD phase III trial (NCT05633654) will evaluate the efficacy and safety of sacituzumab govitecan in combination with pembrolizumab versus pembrolizumab (± capecitabine, as per treating physician discretion) in 1,500 patients with TNBC without a pCR following neoadjuvant chemotherapy. Finally, the MK-2870-012 phase III trial (NCT06393374) will evaluate the efficacy and safety of sacituzumab tirumotecan in combination with pembrolizumab versus compared to treatment of physician's choice in 1,530 patients with TNBC without a pCR following neoadjuvant chemotherapy. These trials highlight TNBC-DX's potential to guide personalized treatments and optimize outcomes.

Beyond TNBC-DX, pre-treatment baseline TILs have been extensively investigated in early-stage TNBC. A high proportion of TILs has been shown to predict pCR to neoadjuvant chemotherapy and better survival outcomes, even in the absence of (neo)adjuvant chemotherapy[13-15,35,36]. In our study, we found that while the immune signature of TNBC-DX was moderately correlated with TILs levels, TNBC-DX scores demonstrated superior predictive power for pCR and survival outcomes compared to TILs alone. However, it is important to acknowledge that the most significant potential clinical use of TILs, particularly in small, lymphocyte-rich TNBC, is the possibility of completely forgoing adjuvant treatment—an area where TNBC-DX data is not currently available. Another added value of TNBC-DX scores over TILs is their potential for standardization, offering more consistent and

reproducible measurements across different laboratories. The superiority of gene expression over TILs for predicting patient outcomes has also been observed in early-stage HER2+ breast cancer[37].

The use of other biomarkers in early-stage TNBC, such as dynamics of circulating tumor DNA (ctDNA) during neoadjuvant therapy, is also being actively investigated[38,39]. TNBC-DX differs from ctDNA in several critical ways, primarily in its timing and application. Thus, ctDNA could eventually complement TNBC-DX by offering additional, real-time information that can guide adjustments in therapy mid-course or after surgery, ensuring that treatment remains aligned with the patient's evolving response.

Our study has limitations. For example, the retrospective design and its reliance on non-randomized cohorts could introduce selection bias, limiting our capacity to precisely gauge the predictive power of TNBC-DX scores for specific therapeutic interventions. Additionally, in the ADAPT-TN trial, detailed information on adjuvant chemotherapy was not captured, which limits our ability to adjust for its impact on patient outcomes. Moreover, the routine administration of anthracycline-based therapy in the adjuvant setting for most patients with residual disease, the median follow-up period of about 5-6 years, and the absence of long-term outcome data beyond this timeframe necessitates further inquiry. However, it is important to note that TNBC recurrences are most common within the first 3-5 years post-treatment[40]. Additionally, TNBC-DX low-risk classification in patients without a pCR may currently lack clinical utility in guiding adjuvant systemic therapy, although it could aid in future patient selection for adjuvant trials. We also acknowledge that the ability of the TNBC-DX pCR score to predict pCR is lower compared to the HER2DX pCR score in HER2-positive breast cancer. This difference may be partially explained by the higher heterogeneity of HER2-positive disease[41], which involves more distinct molecular subtypes and therapeutic targets compared to the relatively homogeneous nature of TNBC. Finally, we have not explored the value of TNBC-DX in the 5-15% of patients with TNBC who have germline BRCA1/2 mutations and are candidates for receiving 1-year of adjuvant olaparib[42].

To conclude, the TNBC-DX genomic test offers a valuable tool for predicting pCR and survival outcomes in early-stage TNBC. This advancement supports the shift toward more personalized and potentially less intensive treatment options, helping to better align therapeutic strategies with the unique profiles and needs of patients with TNBC.

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Disclosures
Potential conflicts of interest are the following: L.P is an employee of Reveal Genomics and has patents filed: PCT/EP2021/070788, EP23382703 and EP23383369. G.V. has received a speaker's fee from MSD, Pfizer, GSK and Pierre Fabrer, has held an advisory role with AstraZeneca and received consultant fees from Reveal Genomics. M.M-A. is an employee of Reveal Genomics. P.G. reports part-time employment from Reveal Genomics. C.M.P. reports stockholder and consulting fees from Reveal Genomics. A.P. reports advisory and consulting fees from AstraZeneca, Roche, Pfizer, Novartis, Daiichi Sankyo, and Peptomyc, lecture fees from AstraZeneca, Roche, Novartis, and Daiichi Sankyo, institutional financial interests from AstraZeneca, Novartis, Roche, and Daiichi Sankyo; stockholder and employee of Reveal Genomics; patents filed PCT/EP2016/080056, PCT/EP2022/086493, PCT/EP2023/060810, EP23382703 and EP23383369. F.B-M. reports part-time employment from Reveal Genomics,

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Figure Legends

Figure 1. **Association of TNBC-DX pCR score with pCR endpoint in the combined external validation cohort of 418 patients treated without pembrolizumab.** (**A**) Univariable and multivariable logistic models to predict pCR. A separate multivariable model was estimated using TNBC-DX risk groups instead of TNBC-DX pCR score. To avoid multicollinearity, TNBC-DX pCR groups and TNBC-DX risk score cannot be included in the same model (**B**) Bar plots showing the pCR rates across the HER2DX pCR groups based on the study and chemotherapy regimen. The interaction test between the TNBC-DX pCR score and treatment (Taxane + Carbo vs. Taxane + Gem) resulted in a p-value of 0.07. OR: odds ratio; 95% CI: 95% confidence interval; pCR: pathological complete response; Tax: Taxane; Carbo: Carboplatin; Gem: Gemcitabine.

Figure 2. **Association of TNBC-DX risk score with survival endpoint in the combined external validation cohort of 418 patients without pembrolizumab.** (**A**) Univariable and multivariable Cox models to predict DDFS. (**B**) Kaplan-Meier curves by TNBC-DX risk group (low-risk vs high-risk) in the DDFS endpoint. (**C**) Kaplan-Meier curves by TNBC-DX risk group (low-risk vs high-risk) in the OS endpoint. HR: hazard ratio; 95% CI: 95% confidence interval. Tax: Taxane; Carbo: Carboplatin; Gem: Gemcitabine.

Figure 3. **TNBC-DX risk score association with clinical-pathological variables, pCR status and treatment information in the combined external validation cohort of 418 patients without pembrolizumab.** TNBC-DX risk score ranking and association with clinical-pathological variables, TNBC-DX pCR groups, pCR endpoint and type of treatment. Each column represents the information for a patient. T: Clinical tumor stage; N: Clinical nodal stage; Tax: Taxane; Carbo: Carboplatin; Gem: Gemcitabine.

Figure 4. **Distant disease-free survival (DDFS) an overall survival (OS) by pCR status and TNBC-DX risk group in patients treated without pembrolizumab.** (**A**) DDFS by pCR status across study (ADAPT-TN and MMJ-CAR-2014-01), (**B**) OS by pCR status across study (ADAPT-TN and MMJ-CAR-2014-01), (**C**) DDFS by pCR status and by TNBC-DX score, (**D**) OS by pCR status and by TNBC-DX score. Distant disease-free survival (DDFS); Overall survival (OS); HR: hazard ratio; 95% CI: 95% confidence interval; pCR: pathological complete response.

Figure 5. **Independent validation of the TNBC-DX pCR and risk scores in patients treated with neoadjuvant docetaxel, carboplatin and pembrolizumab in the NeoPACT phase II clinical trial.** (**A**) pCR rates according to the TNBC-DX pCR score groups (i.e., low, medium and high), (**B**) EFS by TNBC-DX risk score groups, (**C**) OS by TNBC-DX risk score groups, (**D**) EFS in patients with pCR or residual disease at surgery by TNBC-DX risk score groups, (**E**) OS in patients with pCR or residual disease at surgery by TNBC-DX risk score groups.

Tables

Table 1. Clinical-pathological characteristics of patients in the West German Group ADAPT-TN, the MMJ-CAR-2014-01, and the NeoPACT studies.

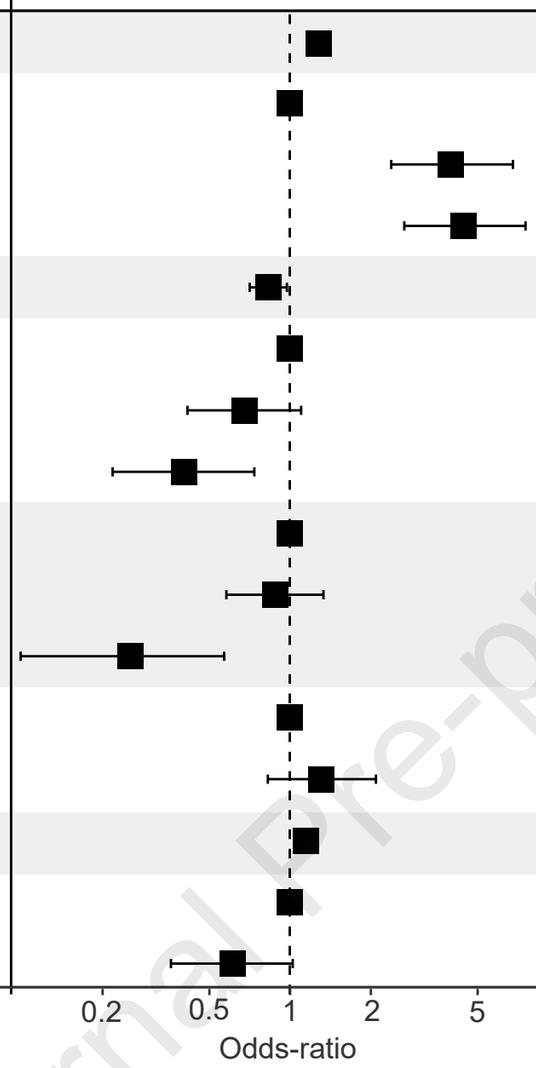
		Combined (n=527)		ADAPT-TN (n=126)		MMJ-CAR-2014-01 (n=292)		NeoPACT (n=109[1])	
Mean age (range)		52 (26-80)		52 (26-76)		53 (26-80)		50 (27-70)	
Tumor stage	cT1	110	20.9%	51	40.5%	39	13.4%	20	18.3%
	cT2	308	58.4%	66	52.4%	173	59.2%	69	63.3%
	cT3-4	109	20.7%	9	7.1%	80	27.4%	20	18.3%
Nodal stage	cN0	306	58.1%	91	72.2%	147	50.3%	68	62.4%
	cN1	178	33.8%	31	24.6%	112	38.4%	35	32.1%
	cN2-3	43	8.1%	4	3.2%	33	11.3%	6	5.5%
Overall stage	Stage 1	75	14.2%	40	31.7%	22	7.5%	13	11.9%
	Stage 2	367	69.7%	79	62.7%	205	70.2%	83	76.1%
	Stage 3	85	16.1%	7	5.6%	65	22.3%	13	11.9%
TILs mean (range)		26.5 (0 - 95)		31.3 (0 - 90)		21.8 (0 - 90)		33.3 (1 - 95)	
TILs group	[0% - 10%]	227	43.2%	34	27.0%	145	49.7%	48	44.4%
	[>10% -50%]	193	36.7%	67	53.2%	103	35.3%	23	21.3%
	>50%	106	20.1%	25	19.8%	44	15.1%	37	34.3%
Histological grade	1 - 2	111	21.1%	9	7.1%	88	30.1%	14	12.8%
	3	416	79.1%	117	92.9%	204	69.9%	95	87.2%
Neoadjuvant treatment	Taxane-carboplatin	343	65.1%	51	40.5%	292	100%	0	0%
	Nab-paclitaxel-gemcitabine	75	14.2%	75	59.5%	0	0%	0	0%
	Pembrolizumab, carboplatin, docetaxel,	109	20.7%	0	0%	0	0%	109	100%
Pathological response	pCR	231	43.8%	43	34.1%	140	49.5%	46	42.2%
	Residual disease	296	56.2%	83	65.9%	143	50.5%	63	57.8%
TNBCDX pCR groups	Low	174	33.0%	35	27.8%	103	35.3%	36	33.0%
	Medium	174	33.0%	32	25.4%	106	36.3%	36	33.0%
	High	179	34.0%	59	46.8%	83	28.4%	37	33.9%
TNBCDX risk groups	Low	292	55.4%	86	68.3%	131	44.9%	75	68.8%
	High	235	44.6%	40	31.7%	161	55.1%	34	31.2%

[1]109/115 (94.8%) of pCR evaluable population in Sharma, Stecklein et al., *JAMA Oncology*, 2024. sTILs unavailable for n=1 patient.

Figure 1

A

Variable		N	pCR rate	Univariable analysis OR (95%CI)	p-value	Multivariable analysis OR (95%CI)	p-value
TNBC-DX pCR score (10-units increment)		418	-	1.28 (1.18, 1.38)	<0.001	1.34 (1.20, 1.52)	**<0.001**
TNBC-DX pCR score groups	Low	138	22.5%	Reference		Reference*	
	Med	138	53.6%	3.99 (2.39, 6.79)	<0.001	3.74 (2.15, 6.64)*	**<0.001**
	High	142	56.3%	4.45 (2.67, 7.57)	<0.001	3.48 (1.72, 7.15)*	**<0.001**
Age (10-units increment)		418	-	0.83 (0.71, 0.98)	0.024	0.89 (0.75, 1.07)	0.221
Clinical tumor stage	T1	90	54.4%	Reference		Reference	
	T2	239	44.8%	0.68 (0.42, 1.10)	0.118	1.85 (0.93, 3.72)	0.083
	T3−T4	89	32.6%	0.40 (0.22, 0.74)	0.003	0.97 (0.44, 2.11)	0.934
Clinical nodal stage	N0	238	47.9%	Reference		Reference	
	N1	143	44.8%	0.88 (0.58, 1.34)	0.552	1.37 (0.84, 2.26)	0.206
	N2+	37	18.9%	0.25 (0.10, 0.57)	0.002	0.37 (0.13, 0.91)	**0.039**
Histological grade	1−2	97	39.2%	Reference		Reference	
	3	321	45.8%	1.31 (0.83, 2.10)	0.251	1.11 (0.65, 1.89)	0.696
TILs (%) (10-units increment)		418	-	1.14 (1.06, 1.24)	<0.001	1.03 (0.94, 1.14)	0.481
Chemotherapy regimen	Tax+Carbo	343	46.4%	Reference		Reference	
	Tax+Gem	75	34.7%	0.61 (0.36, 1.03)	0.066	0.40 (0.22, 0.72)	**0.003**



B

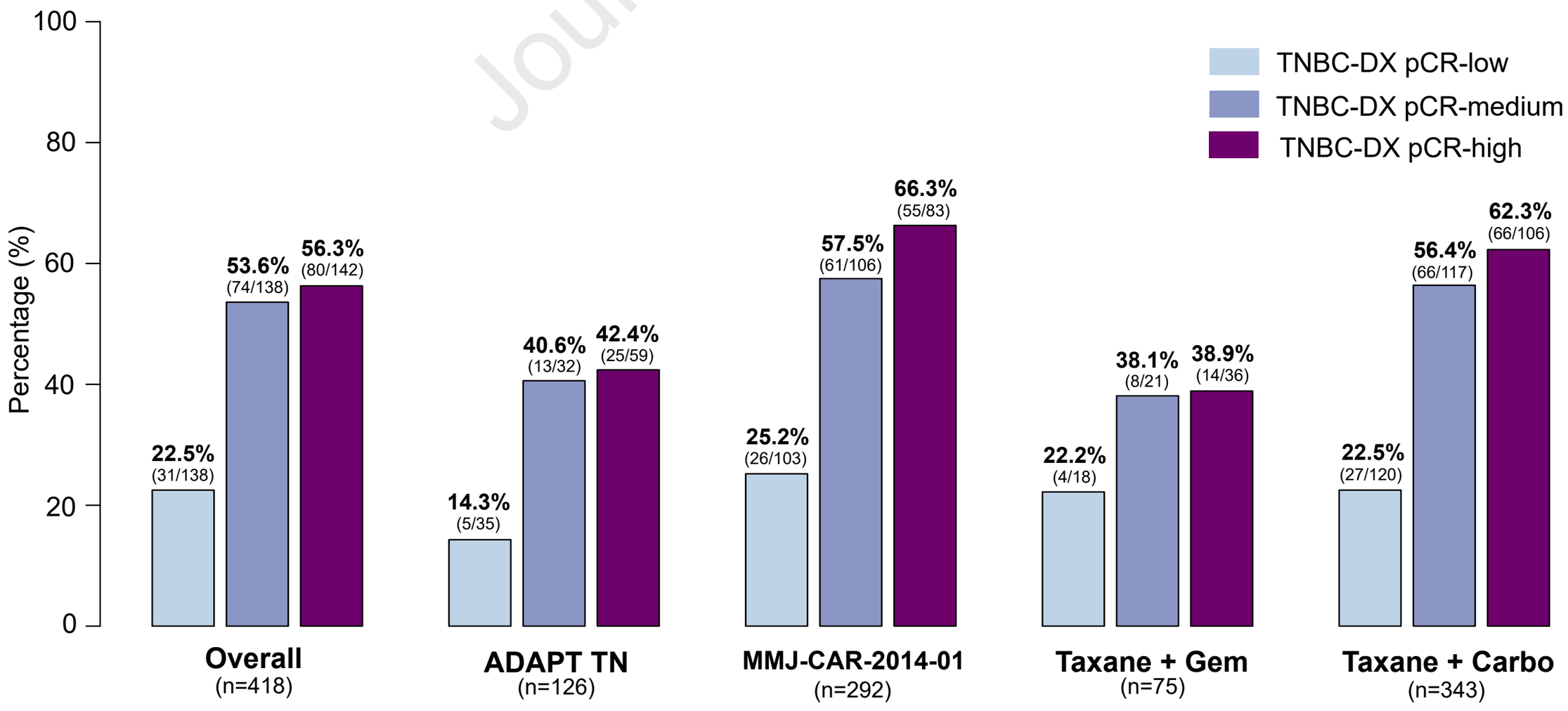
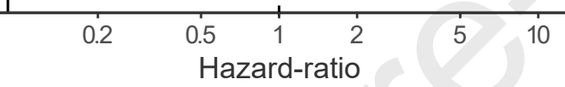


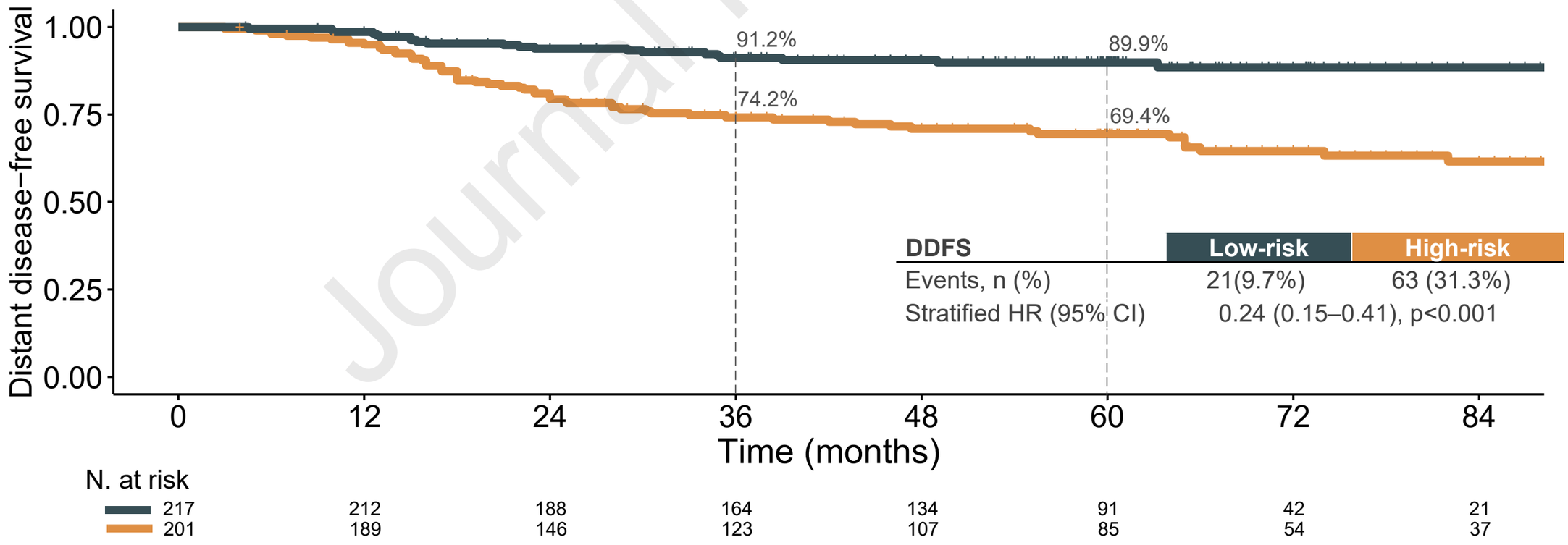
Figure 2

A

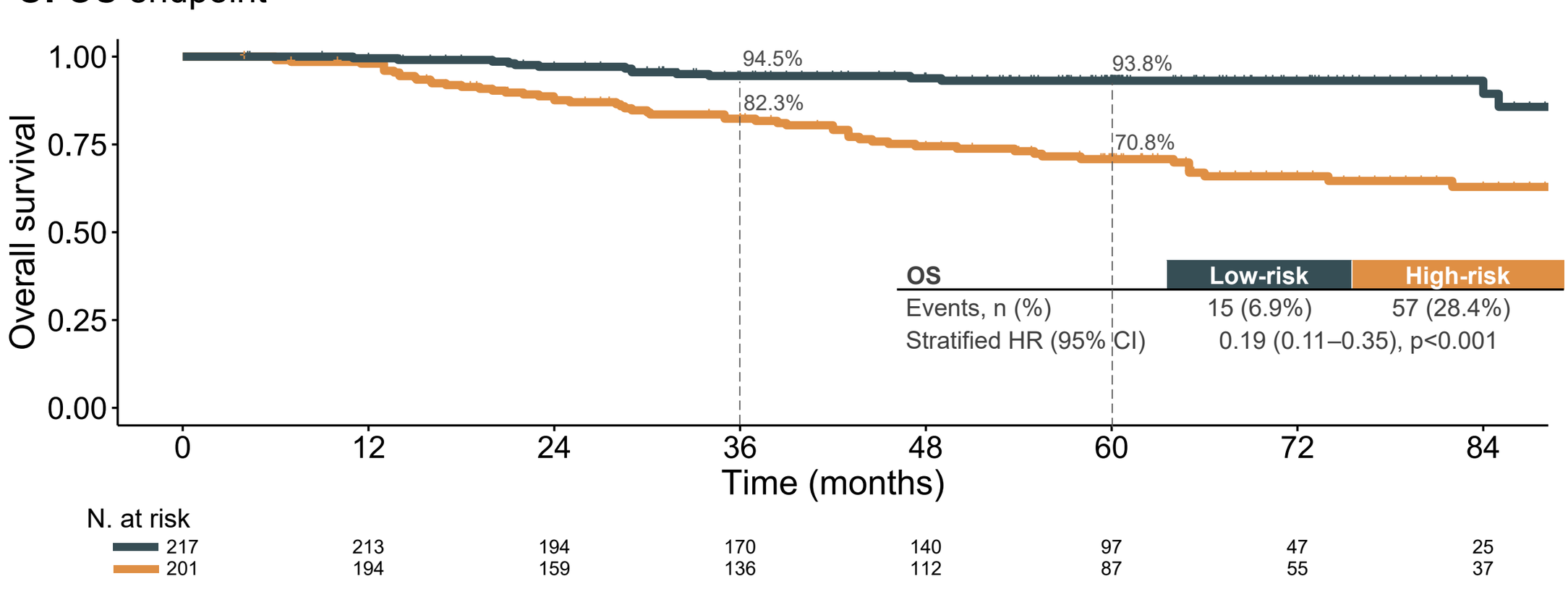
Variable		N	Hazard ratio	Univariable analysis HR (95%CI)	p-value	Multivariable analysis HR (95%CI)	p-value
TNBC-DX risk score (10-units increment)		418		1.37 (1.25, 1.51)	**<0.001**	1.33 (1.09, 1.61)	**0.004**
TNBC-DX risk groups	High	201		Reference		Reference*	
	Low	217		0.24 (0.15, 0.41)	**<0.001**	0.52 (0.22, 1.23)*	0.136
Age (10-units increment)		418		1.02 (0.85, 1.21)	0.86	1.06 (0.89, 1.27)	0.521
Clinical tumor stage	T1	90		Reference		Reference	
	T2	239		1.03 (0.55, 1.92)	0.93	0.68 (0.34, 1.37)	0.279
	T3−T4	89		3.55 (1.82, 6.91)	**<0.001**	1.48 (0.68, 3.23)	0.321
Clinical nodal stage	N0	238		Reference		Reference	
	N1	143		3.43 (2.06, 5.70)	**<0.001**	0.92 (0.37, 2.29)	0.863
	N2+	37		6.06 (3.14, 11.68)	**<0.001**	1.21 (0.41, 3.58)	0.734
Histological grade	1−2	97		Reference		Reference	
	3	321		0.62 (0.38, 1.03)	0.06	0.62 (0.37, 1.04)	0.069
TILs (%) (10-units increment)		418		0.93 (0.85, 1.03)	0.16	1.03 (0.92, 1.14)	0.614
Chemotherapy regimen	Tax+Carbo	343		Reference		Reference	
	Tax+Gem	75		0.41 (0.19, 0.88)	**0.02**	0.47 (0.22, 1.02)	0.056



B. DDFS endpoint



C. OS endpoint



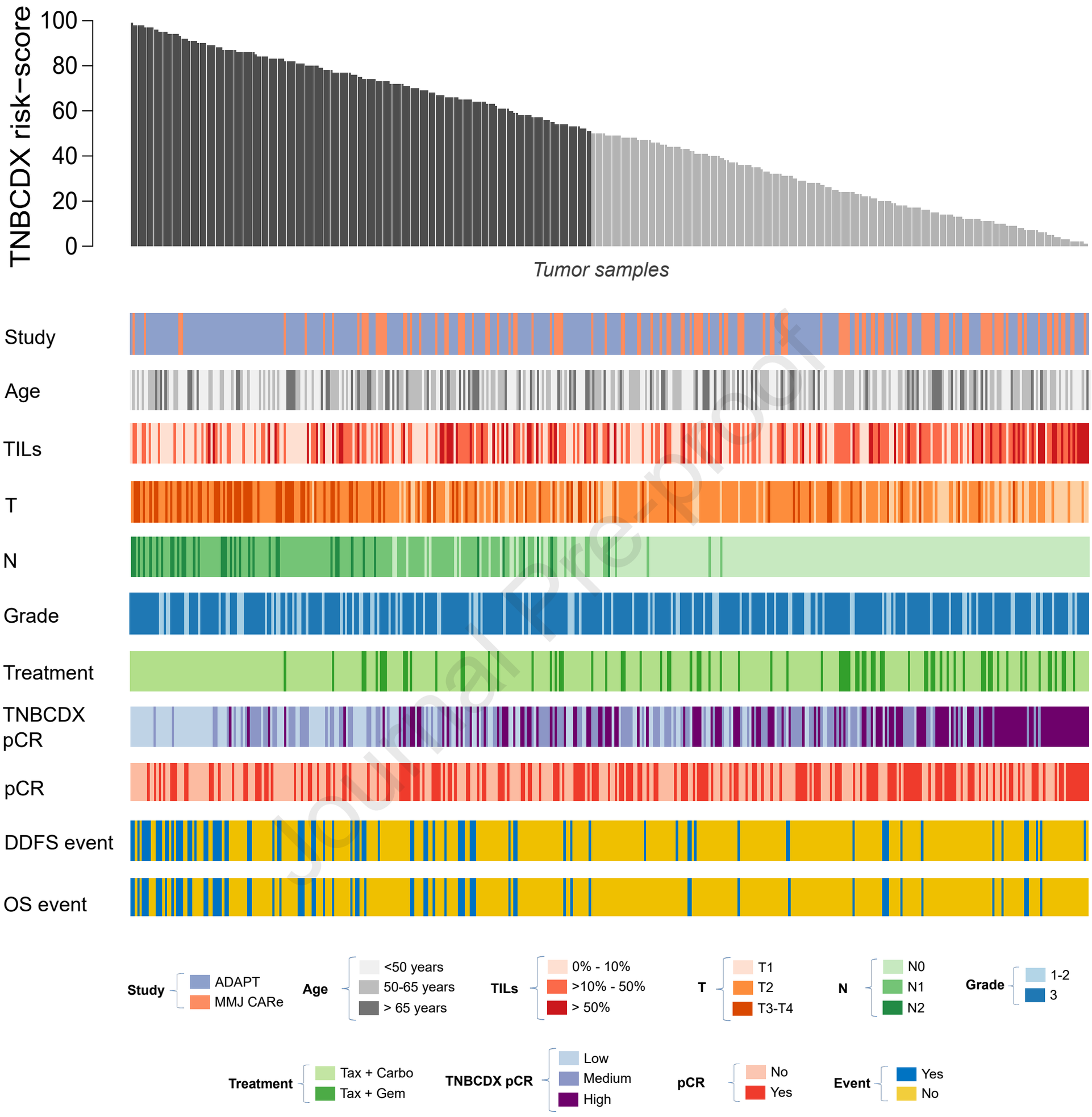
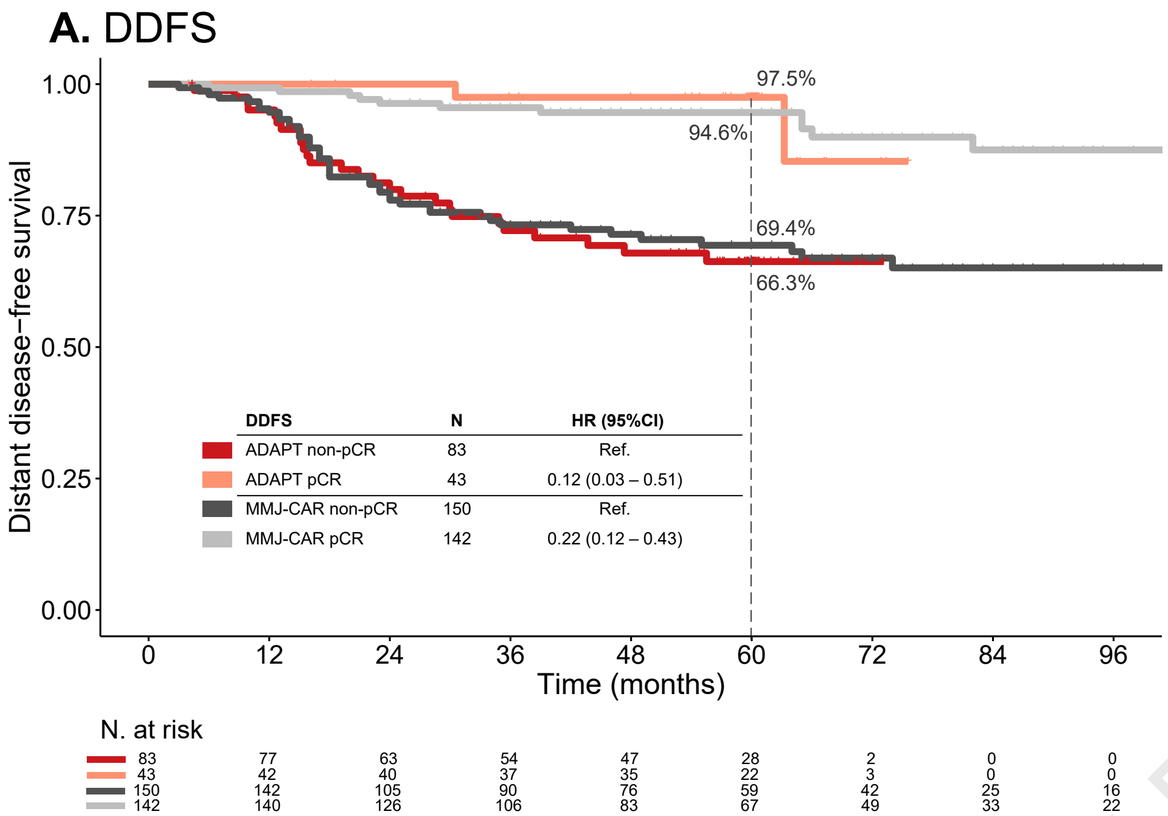
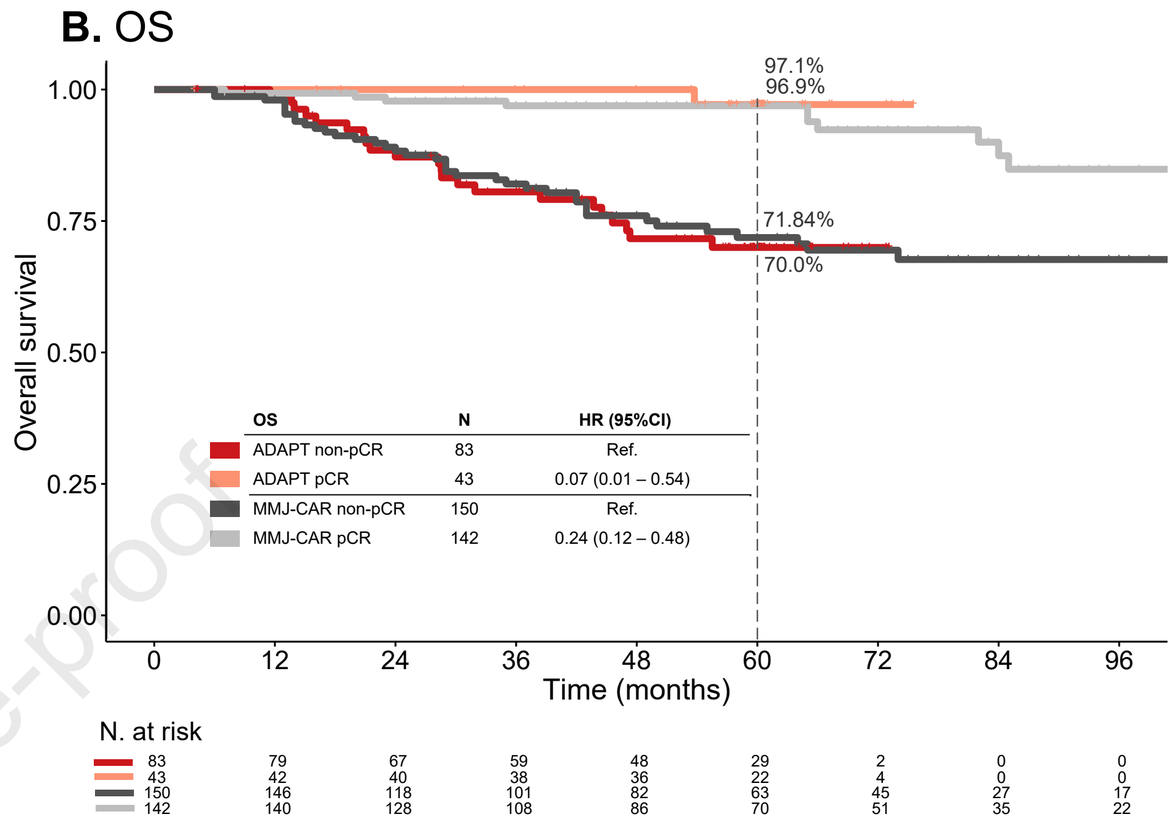
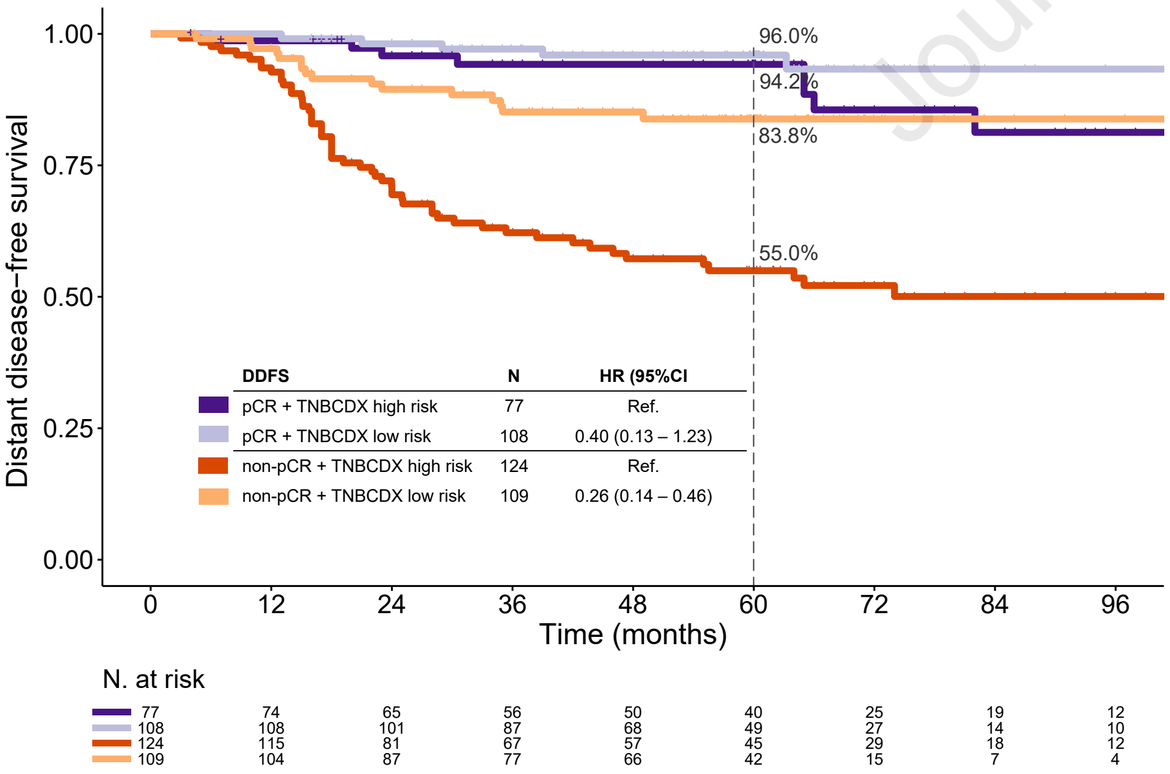


Figure 4







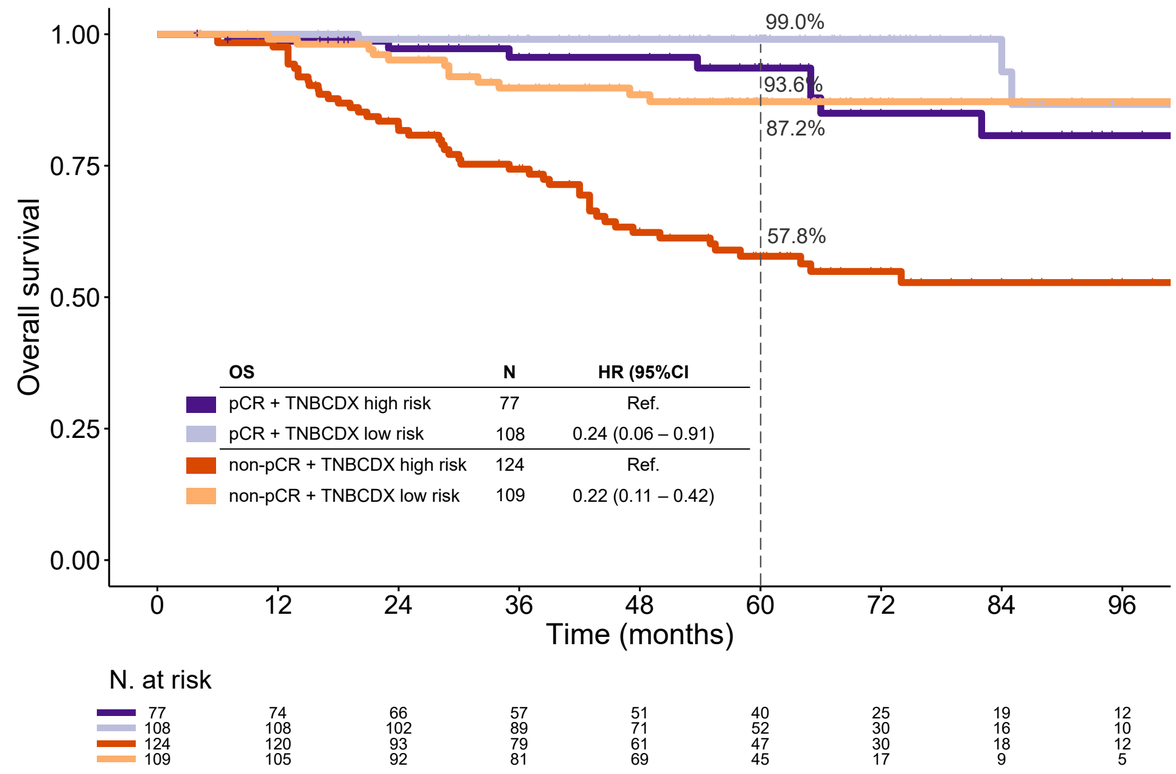


Figure 3