Association of Antifolate Response Signature Status and Clinical Activity of Pemetrexed-Platinum Chemotherapy in Non–Small Cell Lung Cancer: The Piedmont Study

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ABSTRACT

Purpose: The Piedmont study is a prospectively designed retrospective evaluation of a new 48-gene antifolate response signature (AF-PRS) in patients with locally advanced/metastatic nonsquamous (NS) non–small cell lung cancer (NSCLC) treated with pemetrexed-containing platinum doublet chemotherapy (PMX-PDC). The study tested the hypothesis that AF-PRS identifies patients with NS-NSCLC who have a higher likelihood of responding positively to PMX-PDC. The goal was to gather clinical evidence supporting AF-PRS as a potential diagnostic test.

Experimental Design: Residual pretreatment FFPE tumor samples and clinical data were analyzed from 105 patients treated with first-line (1L) PMX-PDC. Ninety-five patients had sufficient RNA sequencing (RNA-seq) data quality and clinical annotation for inclusion in the analysis. Associations between AF-PRS status and associate genes and outcome measures including progression-free survival (PFS) and clinical response were evaluated.

Results: Overall, 53% of patients were AF-PRS(+), which was associated with extended PFS, but not overall survival, versus AF-PRS(−) (16.6 months vs. 6.6 months; $P = 0.025$). In patients who were stage I to III patients at the time of treatment, PFS was further extended in AF-PRS(+) versus AF-PRS(−) (36.2 months vs. 9.3 months; $P = 0.03$). Complete response (CR) to therapy was noted in 14 of 95 patients. AF-PRS(+) preferentially selected a majority (79%) of CRs, which were evenly split between patients stage I to III (six of seven) and stage IV (five of seven) at the time of treatment.

Conclusions: AF-PRS identified a significant population of patients with extended PFS and/or clinical response following PMX-PDC treatment. AF-PRS may be a useful diagnostic test for patients indicated for systemic chemotherapy, especially when determining the optimal PDC regimen for locally advanced disease.

Introduction

It is estimated that there were 235,760 new cases of lung cancer and 131,800 deaths in the United States in 2021 (www.cancer.gov). In both men and women, lung cancer is the second most common cancer but results in the greatest number of cancer-related deaths. The majority (84%; 198,038) of lung cancer diagnoses are non–small cell lung cancer (NSCLC; www.cancer.gov). Most patients (53.9%) are diagnosed with metastatic (stage IV) NS-NSCLC, while the remaining patients are diagnosed with stage I to III NS-NSCLC (1). For newly diagnosed, relapsed, or recurrent patients with stage IV NSCLC, treatments include surgery, radiation, and/or systemic therapies (e.g., cytotoxic chemotherapy, targeted therapy, immune therapy). For patients with earlier-stage NSCLC (e.g., stage II–III), surgery is the primary treatment with the addition of radiation and/or systemic therapies.

Platinum doublet chemotherapy (PDC; cisplatin or carboplatin combined with a second chemotherapeutic agent) has been a mainstay systemic treatment of NSCLC because of the original approval of vinorelbine + cisplatin in 1989 and subsequent approval of other PDC combinations including gemcitabine and taxanes. These PDC options were administered to the wider population of patients with NSCLC regardless of histology. These options demonstrated a comparable, albeit modest, yet clinically significant enhancement in survival rates when compared to nonsystemic conventional treatments such as surgery and radiation (2). The particular PDC used was typically based on the tolerability profile and not on histology or molecular characteristics.

Pemetrexed belongs to a class of chemotherapy agents that target the folate pathway by interfering with the production of purine and pyrimidine nucleotides—and hence DNA and RNA synthesis—by inhibiting shared enzymes, thymidylate synthase (TYMS) and dihydrofolate reductase (DHFR) as well as the purine biosynthetic pathway-specific enzymes phosphoribosylglycinamide formyltransferase (GART) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), thereby disrupting folate-dependent metabolism essential to proliferating cancer cells (3, 4). The initial approval of pemetrexed-containing PDC (PMX-PDC) in 2008 was the first PDC regimen to be approved where patients were selected by histology [patients with nonsquamous (NS)-NSCLC]. This approval was based on a noninferiority study of pemetrexed + cisplatin versus gemcitabine + cisplatin in patients with stage IIIB or IV NSCLC (5). Although survival was similar between both treatment groups, patients with nonsquamous histology (large cell or adenocarcinoma) had superior survival with pemetrexed + cisplatin, yet those with squamous histology had

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Translational Relevance

Platinum doublet chemotherapy (PDC) is an established therapeutic option for patients diagnosed with non–small cell lung cancer (NSCLC), including pemetrexed-containing PDC (PMX-PDC) for those with nonsquamous (NS) NSCLC. Which PDC regimen to employ is mainly chosen based upon tumor pathology or general tolerability profile of a particular regimen and not typically guided by molecular diagnostic tests. In the prospectively designed retrospective Piedmont study, a new RNA-based antifolate response signature (AF-PRS) was evaluated in NS-NSCLC patients treated with PMX-PDC. Extended survival and clinical response to therapy was associated with signature positivity in the overall study population, as well as those who were nonmetastatic at time of treatment. Genomic features of PMX activity in AF-PRS(+) tumors were evaluated in this study cohort, in addition to TCGA, providing additional support for potential use of AF-PRS as a diagnostic test to guide therapy selection in patients with NSCLC.

inferior survival. PMX-PDC garnered wide use in NS-NSCLC patients, but the approval of single-agent pembrolizumab in PD-L1–positive patients or in combination with PMX-PDC in patients with metastasis regardless of PD-L1 status has resulted in decreased PMX-PDC use as a standalone regimen in stage IV disease. However, it is still used frequently in patients at earlier stages of the disease who require systemic chemotherapy.

Prior attempts at developing new biomarkers that could be used to predict PMX-PDC response include IHC expression of target proteins such as thymidylate synthase or RNA expression analysis of its gene (TYMS), with a demonstration that protein and/or gene expression is inversely related with pemetrexed activity (6–9). Early work by Hayes and colleagues (10, 11) evaluated the use of RNA gene expression analysis to identify lung adenocarcinoma (LUAD) molecular subtypes [i.e., bronchioid (terminal respiratory unit), magnoid (aka proximal peripheral) and squamoid (proximal inflammatory)] that could be useful in predicting treatment response to various NSCLC treatment options, but this work was not tied directly with PMX-PDC response per se. With the blinded phase II study of TS molecular and protein expression relationship with PMX-PDC response (12) and subsequent molecular subtype analysis by Fennell and colleagues (13), the LUAD subtypes developed by Hayes and colleagues (10, 11) were utilized to evaluate pemetrexed response in patients with NS-NSCLC, showing that the bronchioid molecular subtype had more favorable response to PMX-PDC compared with the other subtypes. The Piedmont study builds upon these foundational RNA subtyping findings and examines a new reduced gene version of these gene signatures—a 48-gene antifolate response signature (AF-PRS) that could be implemented as a future diagnostic test.

As part of a larger retrospective study involving patients with NS-NSCLC who received standard-of-care systemic therapies, this specific analysis concentrated on patients who were administered PMX-PDC in both the early (stage I) and advanced (stage IV) stages of the disease. A primary objective was to evaluate a new RNA-based 48-gene antifolate response signature (AF-PRS) based upon established molecular subtypes and test the hypothesis that patients who are AF-PRS positive (+) will demonstrate preferential response to PMX-PDC compared with those who are AF-PRS negative (−). The clinical findings were put in context of key genes associated with pemetrexed activity and metabolism to better explain potential preferential responsiveness in AF-PRS(+) patients. The clinical importance of this study is the potential demonstration of initial utility of the AF-PRS, which may be further developed as a diagnostic test to aid in the selection of patients who are indicated for systemic chemotherapy that are most likely to respond to PMX-PDC.

Materials and Methods

IRB approval

The Piedmont study was a prospectively designed retrospective study. Patient samples and corresponding clinical data collected under an IRB-approved protocol (Levine Cancer Institute) that allowed for the waiver of informed consent for combined analysis of molecular data and relevant clinical and demographic data, provided that necessary protected health information (PHI) was removed, and dates were shifted prior to data transfer and subsequent analysis. Furthermore, the study was conducted in accordance with the Declaration of Helsinki.

Patient eligibility and tumor sample collection

The main inclusion criteria for patients in this analysis were as follows: (i) patients received 1 L PMX-PDC as their primary treatment for locally advanced or metastatic disease and did not receive any other concurrent systemic therapy, (ii) patients had available baseline demographic, treatment, and clinical response data, and (iii) patients had archived residual pretreatment formalin-fixed paraffin-embedded (FFPE) tumor tissue samples from either the primary tumor or metastatic site, which were considered sufficient for RNA extraction (see Materials and Methods). A total of 105 patients met these entry criteria. All were treated within the Levine Cancer Institute - Atrium Health Hospital system between 2012 and 2020.

Clinical annotation

Demographic and clinical variables were collected from medical records and entered into a dedicated auditable database (REDCap; www.project-redcap.org) designed around a predefined data dictionary. Data entry and subsequent QC were performed by separate individuals. Baseline clinical variables included information recorded at the time of initiation of PMX-PDC, which was administered as standard of care alone or in combination with other interventions such as surgery or radiation. Overall survival (OS) was defined as the interval from PMX-PDC initiation to patient death. The Social Security Death Index was consulted whenever possible if death date was not available. Progression-free survival (PFS) from PDC-PMX was defined as the interval between initiation of initial PMX-PDC treatment and disease progression, or the date of death in the absence of noted disease progression. In cases where a patient was still alive or the date of death was unknown, the date of last contact was used in place to estimate the censored OS/PFS. Clinical benefit was defined as complete response (CR), partial response (PR), or stable disease (SD).

RNA sequencing

H&E-stained FFPE sections underwent microscopic QC review by an anatomical pathologist to confirm histology diagnosis, evaluate percent tumor nuclei (≥5% required), percent necrosis, and cellularity prior to macrodissection and dual DNA/RNA extraction using the truXTRAC FFPE Total Nucleic Acid Kit (Covaris). RNA quantification was performed by Qubit measurement using ribogreen staining.

RNA was qualitatively assessed for integrity by Agilent TapeStation gel electrophoresis (optimal samples included 10 ng by ribogreen quantification and a TapeStation DV200 value ≥20%). Library preparation was performed using AmpliSeq for Illumina Transcriptome Human Gene Expression Panel Kit. A no-template control (NTC) and a positive control sample (NA12878 FFPE RNA) were included in each run. Libraries were individually captured, reviewed for appropriate size using a Bioanalyzer or TapeStation trace, and quantified (KAPA library quantification) prior to equal molar pooling. Sequencing was performed on an Illumina NovaSeq6000 sequencer using an S2 flow cell to generate ∼50 M, 2 × 50-bp paired-end reads. RNA-seq data were qualified and analyzed against other datasets within GeneCentric's archive. All samples in which the RNA-seq data met a minimum of a median pairwise (i.e., sample–sample) transcriptome-wide correlation of >0.8 and >25% of reads mapped to mRNA bases were included in downstream analyses.

RNA expression analyses

Expression values for the samples were derived from raw RNA-seq fastq files. Reads were aligned with STAR-aligner (GrCH38 ver. 22) to human assembly using the STAR/Salmon pipeline (14). Expression was quantified using the Salmon package (15) and the GrCH38 human transcriptome reference. Genes were filtered for a minimum expression count (at least 10 reads in at least five samples) and for a protein coding annotation by Ensemble (final set of genes = 16,901). Differential expression was assessed using the DESeq2 package (16) on this filtered set of genes. For all other analyses, expression values were upper quartile normalized and log2 transformed.

Analysis of TCGA LUAD dataset

As part of the development of the 48-gene AF-PRS and associated LUAD classifier, as well as application of the signatures to genes associated with antifolate activity, the $n = 515$ The Cancer Genome Atlas (TCGA) LUAD upper quantile normalized RSEM data were downloaded from Firehose and log2 transformed (17).

Gene signatures

48-Gene LUAD nearest centroid classifier

Prior to the analysis of the Piedmont study data, a new reduced gene-set LUAD classifier (and associated AF-PRS signature noted below) was developed that could be used in this study and ultimately validated as a clinical diagnostic test. The classifier was developed as described here as well as the related supplemental methods and uses the gold standard LUAD molecular subtypes [bronchioid (aka Terminal Respiratory Unit), magnoid (Proximal Peripheral), and squamoid (Proximal Inflammatory)] as defined by Wilkerson and colleagues (2012) for their 506-gene LUAD classifier (10, 11). Using the $n = 515$ TCGA LUAD dataset for training (17), the Classifying arrays to Nearest Centroid (CLaNC; ref. 18) algorithm was used with modification to select an equal number of negatively and positively correlated genes for each LUAD subtype. This was performed as an unsupervised analysis, and the genes in the signature were not curated from the literature. Fivefold cross-validation using TCGA LUAD suggested 16 per subtype (48 genes in total) was suitable for achieving optimal agreement with gold standard calls. To obtain the final gene list and the nearest centroid coefficients, the following steps were taken: all of the TCGA LUAD dataset was considered, except for 20% of the samples with the lowest gold standard subtype prediction strength. To describe the magnitude of differences among the subtypes in the 48 classifier genes in the Piedmont study, we calculated pairwise (bronchioid vs. squamoid, bronchioid vs. magnoid, squamoid vs. magnoid) t test P values and ratios of subtype gene means for each gene. We then recorded the most extreme P value and ratio per gene, where if the ratio was less than one, we took the inverse. Forty-one of the genes had ratios greater than 1.1 (median, 1.16; maximum, 9.09), and 38 had P values less than 0.01 (median = 0.00008, minimum = 4.45e−09). The expected performance of the 48-gene signature (Supplementary Table S1) was then confirmed across several fresh-frozen publicly available array and RNA-seq datasets (11, 19, 20) using gold standard subtype calls as defined by the previously published 506-gene signature (11). Further validation of the 48-gene signature was then performed in a newly collected RNA-seq dataset of archived FFPE adenocarcinoma samples to ensure comparable performance in FFPE samples (see Supplementary Materials and Methods for additional detail).

AF-PRS signature

The AF-PRS utilizes the new 48-gene LUAD nearest centroid classifier described above, with AF-PRS (+) samples comprising the bronchioid subtype and AF-PRS (−) comprising the remaining two subtypes (magnoid and squamoid).

Statistical analysis

Associations between clinical characteristics and subtype (AF-PRS) were evaluated using Fisher exact test and the Wilcoxon test for categorical and continuous variables. Gene expression–subtype associations were evaluated using box plots and the Kruskal–Wallis test. Cox proportional hazards models, log-rank tests, and Kaplan–Meier curves were used to examine associations with overall survival and PFS. All statistical analyses were conducted using R 3.6 software (http://cran.R-project.org).

Data availability

The raw RNA-seq data for this study were generated at OmniSeq and were used to generate the 48-gene LUAD nearest centroid classifier and related AF-PRS signature. The RNA-seq gene expression matrix for each patient can be found in Supplementary Table S2. Additionally, these matrices have been deposited in the Gene Expression Omnibus database under the accession ID GSE232569.

Results

Overall, 95 of the 105 (90.4%) FFPE samples that underwent RNA-seq met the minimum transcriptome-wide correlation and reads mapped to mRNA bases and were included in downstream analyses (Supplementary Fig. S1).

Baseline demographics and disease status abstracted from relevant patient records are presented in **Table 1** and include a comparison of those who were AF-PRS(+) and AF-PRS(−) based on the new 48-gene signature described in the Materials and Methods.

Consistent with other findings (12), a majority of the patients with NS-NSCLC had a primary diagnosis of adenocarcinoma (88%) with the remainder of diagnoses that included NSCLC NOS, poorly differentiated NSCLC, undifferentiated large cell carcinoma, and so on. Overall, patient demographics were well balanced by AF-PRS status. Fifty-three percent of patients were AF-PRS(+) (bronchioid molecular subtype), while the remaining 47% were AF-PRS(−) (magnoid/squamoid molecular subtype). This finding contrasts with 37% and 45% of bronchioid molecular subtype calls in the similar cohorts described by Wilkerson and colleagues (2012) or Fennel and colleagues (2014). Although there were no significant differences in demographics by AF-PRS status, patients who were AF-PRS(+) generally had a lower stage

Table 1. Baseline demographics and disease status of the study population by AF-PRS status.

Baseline characteristics	All ($n = 95$)	AF-PRS(+) [$n = 50$ (53%)]	AF-PRS(-) [$n = 45$ (47%)]	P[b]
Gender, *n* (%[a])				
Female	47 (49%)	28 (56%)	19 (42%)	0.22
Male	48 (51%)	22 (44%)	26 (58%)	
Race, *n* (%)				
White	82 (86%)	43 (86%)	39 (87%)	0.65
African American	12 (13%)	7 (14%)	5 (11%)	
Other	1 (1%)	0 (0%)	1 (2%)	
Age (y)				
Median	68	70	66	0.45
Age category [min, max]				
[43, 66]	42 (44%)	20 (40%)	22 (49%)	0.41
[66, 90]	53 (56%)	30 (60%)	23 (51%)	
History of smoking, *n* (%)				
Yes	85 (89%)	44 (88%)	41 (91%)	0.74
No	10 (11%)	6 (12%)	4 (9%)	
NSCLC dx, *n* (%)				
Adenocarcinoma	84 (88%)	45 (90%)	39 (87%)	0.75
Other	11 (12%)	5 (10%)	6 (13%)	
T at dx, *n* (%)				
T1	17 (35%)	9 (31%)	8 (42%)	0.83
T2	14 (29%)	9 (31%)	5 (26%)	
T3	12 (25%)	7 (24%)	5 (26%)	
T4	5 (10%)	4 (14%)	1 (5%)	
NA	47	21	26	
N at dx, *n* (%)				
N0	13 (28%)	9 (31%)	4 (24%)	0.06
N1	15 (33%)	12 (41%)	3 (18%)	
N2	12 (26%)	7 (24%)	5 (29%)	
N3	6 (13%)	1 (3%)	5 (29%)	
NA	49	21	28	
M at dx, *n* (%)				
M0	28 (61%)	18 (72%)	10 (48%)	0.13
M1	18 (39%)	7 (28%)	11 (52%)	
NA	49	25	24	
Stage at dx, *n* (%)				
I	2 (2%)	0 (0%)	2 (10%)	0.022
II	19 (38%)	15 (50%)	4 (19%)	
III	12 (24%)	8 (27%)	4 (19%)	
IV	17 (34%)	7 (23%)	11 (52%)	
NA	45 (47%)	20	24	
Stage at treatment, *n* (%)				
I–III	26 (27%)	19 (38%)	7 (16%)	0.021
IV	69 (73%)	31 (62%)	38 (84%)	
Molecular subtype, *n* (%)				
Bronchioid	50 (53%)	50 (100%)	0 (0%)	
Magnoid	27 (28%)	0 (0%)	27 (60%)	
Squamoid	18 (19%)	0 (0%)	18 (40%)	
PDL1 status, *n* (%)				
+	39 (58%)	21 (62%)	18 (55%)	0.62
−	28 (42%)	13 (38%)	15 (45%)	
NA	28	16	12	
PD-L1 staining, *n* (%)				
<1%	28 (42%)	13 (38%)	15 (45%)	1.0
1%–50%	26 (39%)	13 (38%)	13 (39%)	
>50%	13 (19%)	8 (24%)	5 (15%)	
NA	28	16	12	

[a]Calculated as the percentage of the overall group with data available.
[b]*P* value comparing AF-PRS(+) and AF-PRS(−) patients using Fisher exact or Wilcoxon test (NA, not available).

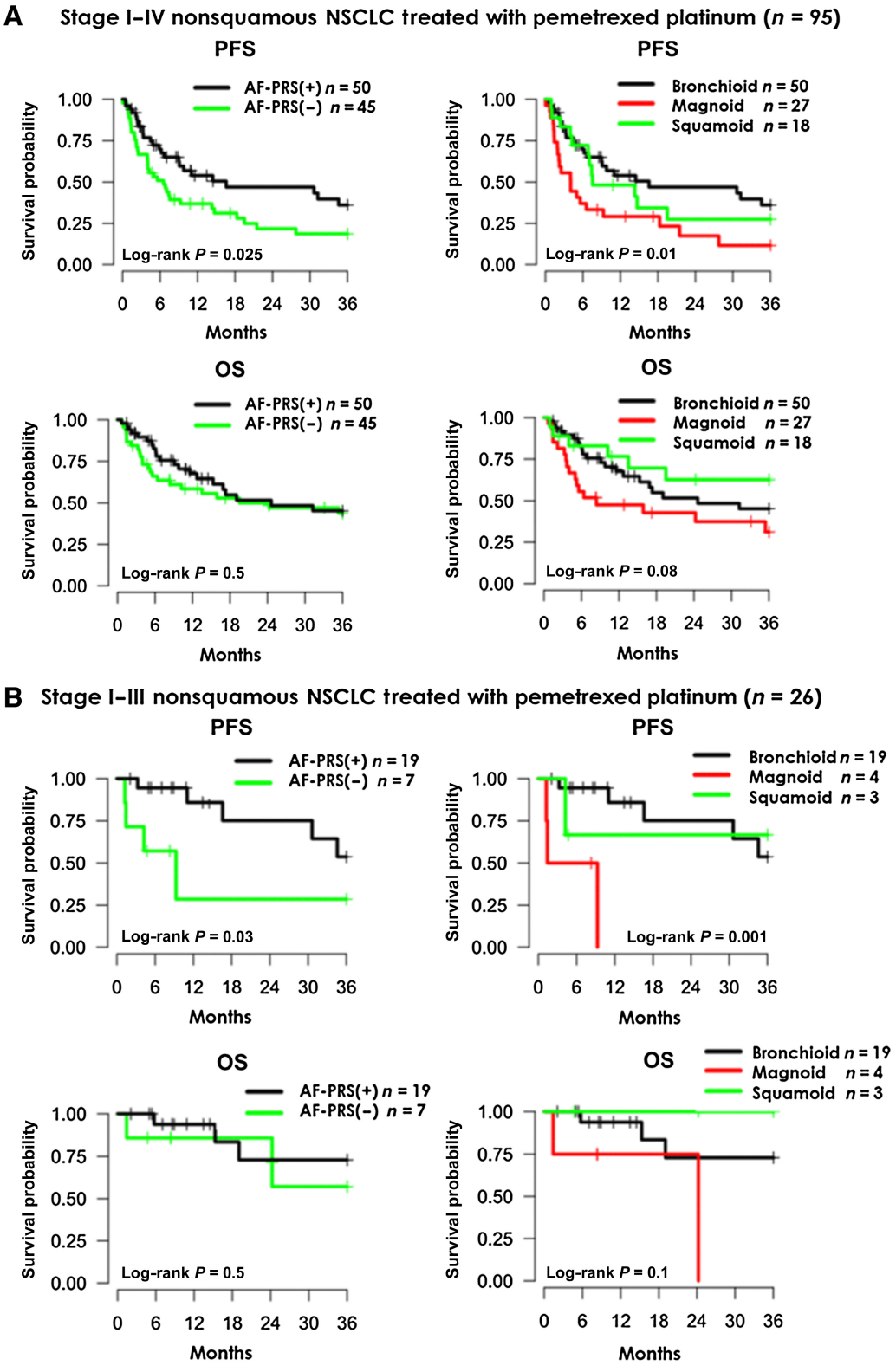

Figure 1.
Progression-free and overall survival probability by AF-PRS status or LUAD subtype in patients stage I to IV at time of treatment (**A**) or stage I to III at time of treatment (**B**).

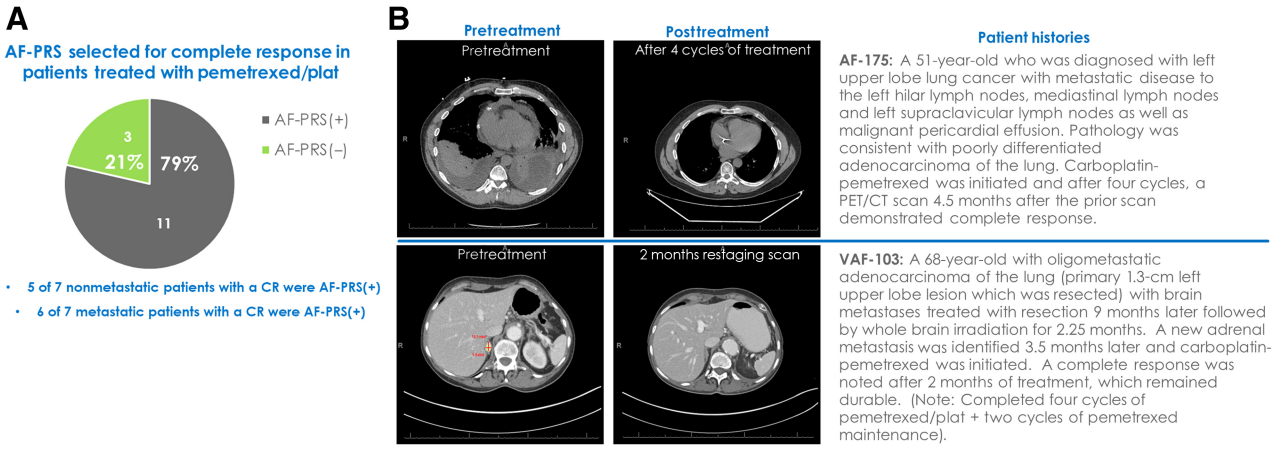

Figure 2.
Evaluation of CRs in patients stage I to IV at the time of treatment (**A**) with representative scans from stage IV patients (**B**).

of disease. This included a noticeable trend toward reduced node involvement at the time of diagnosis, as well as significant differences in overall stage at diagnosis and stage at treatment. Thus, in the survival and clinical response analyses described in **Figs. 1** and **2**, the subset of patients who were stage I to III at the time of treatment were evaluated independently of those who were stage IV. Because this study includes patients diagnosed with NS-NSCLC prior to FDA approval of anti–PD-(L)1 therapy, only 71% of the patients treated with PMX-PDC had PD-L1 status recorded; of these patients, just over half (58%) were PD-L1(+) (≥1% TPS), which is consistent with other investigations (21). Within the Piedmont dataset, detected mutations for KRAS, TP53, KEAP1, and EGFR were sparse partly because of mutation analysis not being performed in these patients as part of their standard of care. For the mutations that were detected, there did not appear to be a significant difference in oncogenotypes detected between AF-PRS subtypes (data not shown).

The median duration of follow-up for this retrospective analysis was 43.7 months (37.9–63.8) for the overall cohort and 40.9 months (14.5–55.9) and 50.7 (41.1–NR) for AF-PRS(+) and AF-PRS(−), respectively. This exceeded the median duration of follow-up for phase III studies that included the evaluation of PMX-PDC (10.5–12.5 months; refs. 21–23). Because median duration of follow-up for the overall cohort was less than 4 years, censoring was performed at 3 years as reflected in the survival curves.

Clinical outcomes following treatment with PMX-PDC for the overall study population ($n = 95$) as well as those who were AF-PRS(+) and AF-PRS(−) are summarized **Table 2**.

A significant difference in the proportion of patients in each clinical response category (e.g., CR, PR, SD, PD) was observed between AF-PRS(+) vs. AF-PRS(−) patients ($P = 0.009$), with a greater proportion of AF-PRS(+) patients having a CR to PMX-PDC (described in further detail in **Fig. 2**). Also, a greater median PFS (~2.5× longer) was observed in AF-PRS(+) versus AF-PRS(−) patients, which was consistent with the significant PFS difference noted in **Fig. 1A**. The rates of PFS in the AF-PRS(+) patients at 6 and 12 months were numerically greater than the rates observed in the AF-PRS(−) patients at these respective timepoints. Survival analyses for both OS and PFS from time of treatment start are presented in **Fig. 1** and **Table 2**. Although the rate of OS at 6 months was numerically greater in those who were AF-PRS(+), the median OS was similar between AF-PRS(+) and (−) patients; however, this observation was not unexpected given the retrospective nature of the study, and many patients were treated with additional systemic therapies upon progression **Fig. 1A**). The Kaplan–Meier PFS curves

Table 2. Clinical treatment outcomes by AF-PRS status.

Outcomes	All ($n = 95$)	AF-PRS(+) ($n = 50$)	AF-PRS(−) ($n = 45$)
Best response, *n* (%)			
CR	14 (15%)	11 (22%)	3 (7%)
PR	33 (37%)	12 (24%)	21 (51%)
SD	25 (28%)	18 (37%)	7 (17%)
PD	18 (20%)	8 (16%)	10 (24%)
NA	5	1	4
ORR, *n* (%)	47 (52)	23 (47)	24 (58)
Clinical benefit, *n* (%)			
Yes	72 (80%)	41 (84%)	31 (77%)
Median PFS, mo (95% CI)	9.07 (6.54–19.5)	16.57 (8.98–NR)	6.54 (4.01–14.7)
Rate of PFS at 6 mo (95% CI)	60.7% (51.4–71.6)	69.9% (57.8–84.5)	50.9% (38.1–67.9)
Rate of PFS at 12 mo (95% CI)	45.7% (36.2–57.6)	53.9% (40.7–71.6)	36.9% (25.0–54.4)
Median OS, mo (95% CI)	24.2 (15.3–NR)	24.59 (15.3–NR)	24.23 (8.4–NR)
Rate of OS at 6 mo, *n* (%)	74.5% (66.0–84.1)	82.6% (72.3–94.4)	66.0% (53.3–81.6)
Rate of OS at 12 mo, *n* (%)	63.3% (53.8–74.4)	67.5% (54.7–83.3)	58.5% (45.5–75.3)

for the overall cohort were significantly different based upon AF-PRS status or when split by the associated LUAD subtype classifier. Because there was a difference by AF-PRS status in the relative proportion of patients who were stage I to III versus stage IV at time of treatment, stage I to III patients were evaluated independently (**Fig. 1B**). Despite the reduced number of patients, the subanalysis of stage I to III patients resulted in a similar, if not greater, separation of the PFS survival curves. Notably, whereas **Fig. 1B** includes those who were stage I to III at treatment, only two patients in the entire cohort were stage I at diagnosis.

When evaluating the site of progression for the patients across all stages with an event during the 36 months interval following initiation of pemetrexed-platinum treatment, it appears there may be a trend toward both liver and brain progression being greater in AF-PRS(−) patients compared with AF-PRS(+) patients (four vs. two and four vs. one occurrences for liver and brain, respectively). However, the AF-PRS(−) patients also had a greater overall rate of progression.

Although overall response rate (ORR) and the clinical response rate (CR+PR) were similar between AF-PRS(+) and (−) patients, further evaluation of the CR group revealed that AF-PRS positivity appears to select for patients with a CR (**Table 2**; **Fig. 2A**). For example, although the overall CR rate was 15%, 22% of the AF-PRS(+) patients, and 7% of the AF-PRS(−) patients had a CR. For the 14 of 95 (15%) patients with a CR to pemetrexed/platinum, the majority [11 of 14 (79%)] were AF-PRS(+), including five of seven and six of seven who were stage I to III and stage IV, respectively, at the time of treatment. Representative scans, along with detailed patient histories, are provided for two of the AF-PRS(+) patients who were stage IV at the time of treatment (**Fig. 2B**).

Consistent with and extending the findings from previous reports (9, 13, 24–27), differential gene expression of pemetrexed target genes as well as genes for transporters involved in its cellular influx/efflux was evaluated to gain insight into the molecular mechanisms that may contribute to the pemetrexed differential responses observed based upon AF-PRS status. Pemetrexed/antifolate target genes of interest included *ATIC*, *DHFR, GART, MTHFD1L, TYMS*, and *GART* and their relative expression levels by AF-PRS status/LUAD subtype are presented in **Fig. 3A**; Supplementary Fig. S2, respectively as well as genes associated with pemetrexed/antifolate metabolism (**Fig. 3B**; *FOLR1, FOLR2, ABCC2, GGH*, and *SLC46A1*). Expression of *TYMS, ATIC*, and *GART* was significantly lower in AF-PRS(+) relative to AF-PRS(−) samples in both the Piedmont study and TCGA LUAD cohorts. *MTHFD1 L* and *DHFR* expression was similarly decreased in the larger TCGA LUAD cohort. Similar differences were noted when split by LUAD subtype.

To further elucidate potential biological underpinnings that may contribute to pemetrexed response in patients with AF-PRS(+) tumors, genes associated with cellular trafficking and detoxification of pemetrexed were also interrogated (**Fig. 3B**). Significantly higher expression of folate receptor genes (*FOLR1* and *FOLR2*) in AF-PRS(+) tumors were observed in both the Piedmont study and TCGA LUAD cohorts. *ABCC2*, which is responsible for folate efflux, was significantly lower in AF-PRS(+) samples from the larger TCGA LUAD cohort. Similarly, lower expression levels of gamma-glutamyl hydrolase (*GGH*) were observed in AF-PRS(+) samples. Although several of the genes noted above (*GGH*, *TYMS*, *FOLR2*, and *FOLR1*) were included in the original 506 gene subtype classifier developed by Wilkerson and colleagues (11) with relative subtype associations, this study mapped their activities to the metabolism of pemetrexed in the context of preferential PMX-PDC response in AF-PRS(+)/bronchioid tumors. When evaluating the relationship of the expression of individual genes (*ATIC, GART, DHFR, MTHFD1 L*, or *TYMS)* with survival (OS or PFS), there was no significant difference in OS, and the only significant difference observed for PFS was for *ATIC* and *MTHFD1 L* (Supplementary Table S3).

Discussion

The Piedmont study is the first to evaluate the molecular characteristics of PMX-PDC response using a multigene RNA-based response signature, building upon the foundational NSCLC molecular subtype analysis of Hayes and colleagues (10) and Wilkerson and colleagues (11), as well as the exploratory PMX-PDC study by Fennell and colleagues (12, 13). Here we employed a new 48-gene AF-PRS, which identified patients who demonstrated extended survival and clinical response to PMX-PDC, whether applied to the entire cohort of patients (stage I–IV at the time of treatment) or those who had earlier stage or locally advanced disease (stage I–III at the time of treatment). Further, we provided a molecular rationale for this preferential PMX-PDC response by showing that genes and related pathways associated with antifolate activity and metabolism were differentially expressed.

This study includes the evaluation of real-world PMX-PDC use and provides unique insights into its activity across a broader NS-NSCLC population. Although the initial approval of PMX-PDC in NS-NSCLC was for patients with advanced disease (stage IIIB–IV; ref. 5) and subsequently in combination with pembrolizumab for patients with metastasis (stage IV; ref. 21), current PMX-PDC use independent of I–O combination is often in earlier-stage patients (stage I–III), including in the adjuvant setting (e.g., with surgery and/or radiation). Although not statistically compared across studies, median survival was numerically longer in this study compared with prospective studies of PMX-PDC clinical activity, including the pivotal studies such as PMX-PDC used alone [pemetrexed-cisplatin vs. gemcitabine-cisplatin (5) or in combination with anti–PD-(L)1 (PMX-PDC vs. PMX-PDC+ pembrolizumab (21)], as well as the blinded single-arm study of pemetrexed-cisplatin investigating biomarkers of response (13). Median PFS and OS in the overall Piedmont patient population were 9.07 and 24.2 months, compared with the aforementioned studies, 5.5 to 4.8 months and 11.3 to 9.6 months, respectively. These differences are not unexpected because real-world evidence (RWE) studies reflect real-world therapeutic use, including patients with earlier-stage cancer as is the case in this study, which likely contributed to the survival differences across studies.

Prior to the approval of PMX-PDC in the first-line setting for patients with NS-NSCLC (5), treatment was not typically guided by a specific NSCLC histology (e.g., patients with NS-NSCLC), but instead often by the PDC regimen tolerability (2). When the pivotal study by Scagliotti and colleagues was nearing completion, interest built around the use of gene expression profiling to identify lung cancer molecular subtypes as a potential aid in determining prognosis and/or treatment response across multiple NSCLC systemic therapies. This included initial work by Hayes and colleagues (10), who employed consensus clustering to LUAD subtypes of bronchioid, magnoid, and squamoid, and their relative prevalence and stage-specific survival, including patients with the bronchioid molecular subtype having a better prognosis than those with the magnoid/squamoid subtype. The study was expanded by creating a gene LUAD subtype classifier involving 506 genes. This classifier not only validated the prognostic findings related to the bronchioid subtype in LUAD, but also demonstrated for

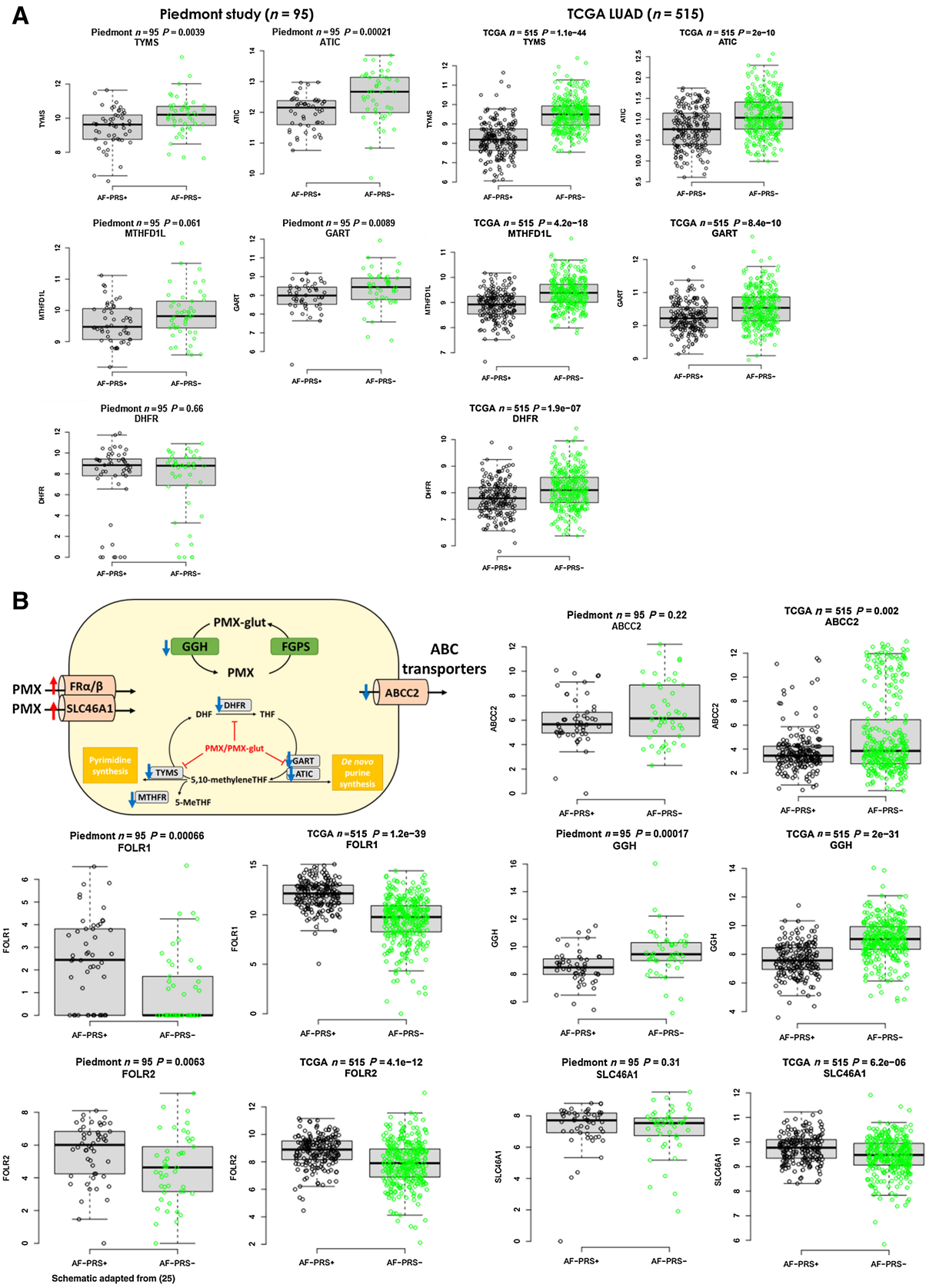

Figure 3.
Expression of genes associated with antifolate (Pemetrexed) activity (**A**) and cellular influx/efflux (**B**).

the first time that the response to PDC varied depending on the molecular subtype of NSCLC. For example, patients with the magnoid subtype treated with adjuvant vinorelbine + cisplatin showed a better response compared to those receiving best supportive care (11). Fennel and colleagues (2014) were the first to investigate PMX-PDC clinical response in context of molecular subtype using RNA expression analysis. In that exploratory study, patients with NS-NSCLC with bronchioid (cluster 1) subtype had a two to three times increase in survival following PMX-PDC treatment compared with those with a magnoid (Cluster 2) or squamoid (Cluster 3) subtype. Our current study confirms these results in a real-world setting, with the demonstration of AF-PRS(+) (bronchioid subtype) patients having a similar two to three times longer survival (PFS) following PMX-PDC, compared with AF-PRS(−) (magnoid/squamoid subtype) patients. Because there was a significant difference in disease stage at diagnosis and treatment by AF-PRS status in this study with more stage IV patients being AF-PRS(−), we also evaluated survival in patients who were stage I to III at the time of treatment; and there was an equal, if not greater PFS advantage for AF-PRS(+) patients compared with those who were AF-PRS(−), despite the smaller sample size. Although both PFS and OS were used for evaluation of activity PMX-PDC in the original prospective studies, this study utilized PFS as the primary survival endpoint because OS is often confounded by subsequent therapies such as anti–PD-(L)1 or targeted therapies that were not available at the time of PMX-PDC approval.

Since the approval of pembrolizumab in combination with PMX-PDC for the treatment of patients with metastatic NS-NSCLC in 2018 (21), the use of PMX-PDC alone in patients with advanced disease has decreased, and the choice to use PMX-PDC in the absence of anti–PD-(L)1 therapy is often dictated by chronic immune suppression, active autoimmune diagnoses, or other medically driven limitations to immunotherapy utilization. However, PMX-PDC use along with other PDC regimens continues to be prevalent in patients with earlier-stage disease where there is clinically meaningful improvement in survival/response when combined with nonsystemic treatments such as radiation and surgery (28, 29). A question that remains is how best to select which PDC regimen to use in the adjuvant setting (30–32). In addition to the extended PFS in stage I to III or the broader stage I to IV AF-PRS(+) patients, AF-PRS positivity was associated with a majority of the patients (79%) who demonstrated a CR to therapy. Importantly, this analysis included patients with metastatic disease (stage IV) or those with nonmetastatic disease (stage I–III) at the time of treatment [i.e., six of seven and five of seven patients with stage IV and stage I–III disease, respectively, with AF-PRS(+)CR]. The clinical response findings, in addition to extended PFS, support use of AF-PRS status to help select stage I to III patients indicated for systemic chemotherapy who are most likely to respond to PMX-PDC.

Along those lines, a great deal of work has gone into identifying patients who are likely to respond to PMX-PDC, from the initial retrospective (6) and prospective (12) clinical observation that low *TYMS* protein expression by IHC predicts response. It was the subsequent analysis by Fennel and colleagues (13) that also provided for *TYMS* mRNA expression being inversely related to clinical activity. Others have also demonstrated that low expression of TYMS and other related pemetrexed targets are associated with sensitivity (9, 24, 25). The study by Fennel and colleagues was significant, as it emphasized the importance of developing a molecularly based biomarker to effectively identify patients who are best suited for receiving pemetrexed treatment, despite being exploratory in nature and having limitations in sample size.

Although the bronchioid molecular subtype [AF-PRS(+)] is associated with improved prognosis in patients with LUAD (10, 11), this does not appear to be a sign of indolent disease. Molecular features related to antifolate activity and metabolism are associated with the AF-PRS(+) status (bronchioid subtype) and may contribute to preferential responsiveness to pemetrexed compared with AF-PRS(−) (magnoid/squamoid molecular subtypes).

Similar to the findings reported in the molecular subtype analysis by Fennel and colleagues, which showed that patients with the bronchoid subtype (cluster 1) had the lowest TYMS expression and longest survival, and in line with the subtypes identified by Wilkerson and colleagues (11), we observed a comparable trend in the Piedmont cohort. Specifically, AF-PRS(+) patients in the Piedmont cohort showed significantly lower *TYMS* expression levels and experienced extended survival. Our findings extend these observations into other genes that are related to antifolate activity, including *ATIC, MTHF D1L*, and *GART*, where they also have lower expression AF-PRS(+) tumors. Furthermore, these findings were nearly identical to those from a similar analysis of the TCGA LUAD cohort. Extending the rationale for AF-PRS(+) sensitivity to PMX-PDC, genes associated with PMX cellular uptake, disposition, and metabolism (26, 27, 33–37) were also differentially regulated. Together, these data may suggest a molecular mechanism in which AF-PRS(+) tumors represent ideal targets for pemetrexed treatment due to their low expression of genes directly involved in folate metabolism for *de novo* purine synthesis (*TYMS, DHFR, ATIC, GART*) and perhaps exhibit increased uptake of pemetrexed (supported by *FOLR1* and *FOLR2* expression) and a decreased ability to attenuate its activity (supported by GGH) and potential decrease in its efflux (supported by *ABCC2*).

There are potential limitations of this study as a retrospective cohort reflecting real-world PMX-PDC use within a single institution. Staging was not available for all the patients at diagnosis; however, metastatic disease status (e.g., stage I–III vs. stage IV) was known at the time of treatment for all patients included in this analysis. Therefore, stage at time of treatment was used as a primary variable in the analysis. Another potential limitation of the study is an apparent lack of concordance between median PFS and OS regarding their association with AF-PRS status. Median OS was not extended in AF-PRS(+) patients, as was the case with median PFS. However, with a focus on short-term survival analysis, the 6- and 12-month PFS and OS rates were both numerically greater in patients who were AF-PRS(+). Significant progress has been made over time regarding NSCLC care, and there have been increases in post-progression survival (PPS; refs. 38, 39). With increasing PPS, there is weaker correlation between PFS and OS, which has been demonstrated in a clinical trial setting (40). In this study, the PPS was relatively long, which may be partially responsible for the discordant findings between AF-PRS(+) patients having extended PFS but having an OS that is not different than AF-PRS(−) patients. As previously reported in patients with NSCLC and small cell lung cancer, PPS is strongly associated with OS after first- and second-line chemotherapy, which suggests subsequent treatment after disease progression following early-line treatments influences OS in evaluating efficacy of first-line chemotherapy (41). Therefore, discordance between PFS and OS from the start of first-line chemotherapy in AF-PRS subtypes does not necessarily invalidate the clinical utility of the AF-PRS gene signature but is an area for further evaluation in subsequent studies. In conclusion, the Piedmont study identified a population of patients with NS-NSCLC who were AF-PRS(+) and had significantly extended PFS and increased clinical response following

treatment with PMX-PDC. These findings were not only observed in the overall cohort of patients, but also in patients with earlier-stage disease when PMX-PDC is administered in conjunction with nonsystemic therapy. The clinical findings were supported by molecular differences in AF-PRS(+) tumors, namely preferential pemetrexed activity and metabolism, which likely contributes to clinical benefit. Although the current analysis provides initial clinical utility for the prognostic aspects of AF-PRS as the Piedmont study was retrospective in nature, its further development as a diagnostic test to aid in identifying patients as to whom are most likely to respond to PMX-PDC is warranted. This includes the approximately 70,000 patients diagnosed with stage II to IV NS-NSCLC annually in the United States, to many for which chemotherapy is indicated. As part of additional clinical validation of AF-PRS, prospective evaluation of patients treated with PMX-PDC and other PDC combinations will help support its use as a predictive test for selection of the optimal chemotherapy regimen in NSCLC. As demonstrated with the initial findings of Wilkerson and colleagues (11), molecular subtypes included in patients who were AF-PRS(−) may show preferential response to alternate PDC regimens. Thus, a future AF-PRS test may be useful in aiding in the selection of patients most likely to respond to PMX-PDC as well as other PDC regimens depending on AF-PRS status, resulting in potential increased clinical and health economic benefit.

Authors' Disclosures

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Authors' Contributions

J.R. Eisner: Conceptualization, resources, data curation, supervision, investigation, methodology, writing–original draft, project administration, writing–review and editing. **G.M. Mayhew:** Data curation, software, formal analysis, validation, visualization, methodology, writing–review and editing. **J.M. Davison:** Data curation, formal analysis, visualization, writing–review and editing. **K.D. Beebe:** Supervision, writing–original draft, project administration, writing–review and editing. **Y. Shibata:** Data curation, software, formal analysis, validation, methodology, writing–review and editing. **Y. Guo:** Data curation, software, formal analysis, validation, visualization, writing–review and editing. **C. Farhangfar:** Resources, supervision, project administration, writing–review and editing. **F. Farhangfar:** Resources, supervision, project administration, writing–review and editing. **J.M. Uronis:** Resources, project administration, writing–review and editing. **J.M. Conroy:** Resources, supervision, methodology, project administration, writing–review and editing. **M.V. Milburn:** Conceptualization, resources, funding acquisition, writing–review and editing. **D.N. Hayes:** Conceptualization, formal analysis, supervision, methodology, writing–review and editing. **K.F. Mileham:** Conceptualization, resources, data curation, supervision, investigation, methodology, writing–original draft, project administration, writing–review and editing.

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