Issues in developing multivariable molecular signatures for guiding clinical care decisions

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Abstract

Omics technologies that generate a large amount of molecular data characterizing biospecimens have the potential to provide information about patients' disease characteristics beyond standard clinical features. By combining information from a large number of features into a multivariable model, called a biomarker signature, there is the opportunity to identify distinct subgroups of patients for whom treatment decisions can be personalized. The key challenge is to derive a signature with good performance and appropriately characterize its performance. We summarize the key statistical issues and methods for developing and validating biomarker signatures, using examples from the literature for illustration.

keywords: biomarkers; biomarker signatures; personalized medicine

Introduction

Omics technologies that generate a large amount of molecular data characterizing patient biospecimens have the potential to provide information about the patients' disease characteristics above and beyond standard clinical and pathological features. By combining the information from a large number of molecular features into a multivariable model, hereafter referred to as a *biomarker signature*, there is the opportunity to identify distinct subgroups of patients for whom clinical management decisions can be personalized. In early-stage disease, for example, a highly prognostic signature might identify a subpopulation of good risk patients that has such a high probability of long survival or disease-free survival that they do not require additional treatment beyond some standard base therapy. Therefore these good risk patients can be spared the risks and side-effects associated with additional therapy. In the context of a specific therapy that targets a particular molecular pathway, a signature may identify a subpopulation of patients that does or does not benefit from that therapy, thereby guiding the decision of whether or not to administer the therapy.

The key challenge we address in this paper is how to combine feature mneasurements generated by a high dimensional multiplex molecular assay to derive a signature that is fit for a specified clinical purpose and additionally provide valid estimates of its performance characteristics. A signature that has clinical value must have statistical performance (as quantified by one or more metrics) that is fit for the clinical context, and it must provide information that can be acted upon clinically to provide some benefit to a patient. For example, a biomarker signature that is well calibrated may accurately predict a clinical outcome, but that does not neccessarily imply that the prediction provides clinically useful information; the signature may divide patients into subgroups with different prognosis, but if there are no therapies available to improve the outcome of either subgroup, then the signature may have little clinical value. While the focus here is statistical estimation of a numerical performance metric, it should be kept in mind that clinical usefulness may depend on a variety of additional factors.

Terminology and Notation

A **biomarker signature** is a transformation of multiple individual feature measurements, typically molecular characteristics measured on a multiplex assay, to a one-dimensional space. Specifically, let X denote the set of p feature measurements under consideration. The signature is an unknown function $f(X) : \mathbb{R}^p \mapsto \mathbb{R}^1$. The signature result may be continuous, take multiple discrete values, or be dichotomous. In principle, the signature could also depend on other clinical or pathological variables in addition to the molecular measurements, but to simplify the discussion we will focus on signatures that depend on molecular feature data only. Let S denote the development dataset which includes, for each of n represented individuals, a feature vector X, an outcome Y, and a treatment Z. S is a sample of size n from distribution $\mathcal{P}$ with domain $\mathcal{S}$. Let $\mathcal{F}$ be a mapping from $\mathcal{S}$ to the space of continuous functions, $\mathcal{D}$, with domain $\mathbb{R}^p$ and range $\mathbb{R}$. Thus $\mathcal{F} : \mathcal{S} \mapsto \mathcal{D}$ denotes the process or algorithm through which a particular f is estimated. We do not place any other restrictions on $\mathcal{F}$, it could be a clustering approach, a regression approach, a combination of both, or something else entirely. We will use $\mathcal{F}$ to denote the manner in which f is estimated and will write $f \in \mathcal{F}$ to denote that f is estimated with the class of methods $\mathcal{F}$.

Let $\phi : \mathcal{D} \times \mathcal{S} \mapsto \mathbb{R}$ denote the statistic that quantifies the performance of the function f. This is a function of both f and S. We are interested in estimating $E_{\mathcal{P}}[\phi_{f^*}(S)]$, which is the expected performance under the data generation mechanism (distribution $\mathcal{P}$), for a particular $f^* \in \mathcal{F}$ when f^* is applied to X for the purpose of predicting Y. This expectation is with respect to $\mathcal{P}$, the distribution of the random variable S. This allows us to understand how the signature will perform on future observations generated from $\mathcal{P}$. The difficulty in estimating this quantity is that generally only a single sample from $\mathcal{P}$ is available to both develop f^* and to estimate its expected performance $E_{\mathcal{P}}[\phi_{f^*}(S)]$.

We may also be interested in estimating $E_{\mathcal{P}}[\phi_f(S)]$ **for all** $f \in \mathcal{F}$, which is the generalization performance for f generated using mechanism $\mathcal{F}$. This doesn't guide outside researchers as to which specific f to use, yet it is useful for development because it tells us how much signal is in the data and how well it can be captured with $f \in \mathcal{F}$. As shorthand we will write this as $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$.

Overview of biomarker signature development

The inherent statistical challenge is how to both develop a signature $f \in \mathcal{F}$ and obtain a valid assessment of its performance. Additionally, one should provide a specification of f for others to use. Typically, a specific f is estimated using $\mathcal{F}$ based on some training data. The class of methods $\mathcal{F}$ can comprise a variety of different computational approaches. In recent years there has been an explosion in the literature of computational approaches to classification and prediction, and we do not intend to summarize them all here. Some excellent reviews are provided by Hastie et al. [2009] and Moons et al. [2012]. The main considerations in signature estimation are identifying the features to include, deciding what transformations to apply to the feature measurements, determining how to combine the feature measurements, and determining what, if any, thresholds or cutoffs should be applied to the resulting signature value.

Our focus here will be mainly on the development of signatures using supervised methods, meaning methods that use information about an outcome variable that it is desired to predict. Regression methods are a commonly used class of supervised methods. Oncotype DX [Paik et al., 2004] is an example of a prognostic signature used for clinical decision making in early stage breast cancer that was developed using supervised methods. In the case of Oncotype DX, the outcome Y was time to distant recurrence of breast cancer and the feature data were gene expression values measured in breast tumor specimens. Regression methods can also be used to develop treatment-selection (also called predictive) signatures that exhibit a strong interaction with a particular treatment.

Supervised methods are contrasted with unsupervised methods which use only the feature data to derive a signature. An example of a signature developed by unsupervised methods is the signature that identifies biological subclasses of diffuse large B-cell lymphoma, which were originally developed using clustering methods and subsequently were found to be associated with prognosis [Alizadeh et al., 2000]. It is possible, and quite common in high-dimensional data settings, to combine multiple approaches when estimating a signature. For instance, a data-reduction step by variable selection or clustering may be performed before doing regression analysis on the resulting components. A review by Subramanian and Simon identified a large collection of gene expression–based prognostic signatures in lung cancer which had been developed using a wide variety of methods [Subramanian and Simon, 2010].

The focus of this paper is how to obtain a valid estimate of signature performance as quantified by a metric ϕ, that is, a good estimate of $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$. The principles discussed apply no matter what signature development method is used. Performance depends on the true signal in the data and the specific algorithm $\mathcal{F}$ used for development. The performance will reflect that expected of signatures developed applying the prescribed methods to data drawn from the distribution $\mathcal{P}$. For signature deployment, one would also want a particular specification of f for others to use on independent data (e.g., in clinical practice). It is not trivial to conduct this evaluation in a valid manner when there is a limited data set available to both define a signature and assess its performance. We illustrate the potential for bias to creep into the performance estimation and review some strategies to avoid the pitfalls that lead to those biases. We evaluate various strategies through a simulation study and compare the performance of some of those strategies using a real data exmaple involving the development of a signature for use in making treatment decisions for patients with lung cancer. All simulations and analyses were conducted using `R` version 3.2.4 [R Core Team, 2016].

Issues

$E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$ can be estimated with the in-sample empirical estimate: $\hat{E}[\phi_f(S)] = \frac{1}{n}\sum_{i=1}^{n}\phi_f(s_i)$ for a particular f. However, if S is used to estimate f then the estimate will be biased due to overfitting, that is, $|E_{\mathcal{P}}[\phi_f(S)] - \hat{E}[\phi_f(S)]|$ will be large. Overfitting occurs when a model is fit to noise in the data. This often occurs when fitting a model that is overly complex relative to the amount of signal in the available data. Overfitting bias results from the fact that $\phi_f(S)$ depends on f which depends on S, and thus the statistic $\phi_f(S)$ is being adaptively defined based on the observed data S. Any estimate of signature performance is potentially subject to bias if overfitting occurs, but such biases frequently go unrecognized in the medical literature.

Typically a performance metric ϕ used during signature development is designed to quantify either calibration or discrimination, or some combination of the two. Calibration assesses how well the signature-based predictions compare to the observed outcomes. Discrimination assesses how well the signature distinguishes between groups of patients who do or do not experience an event or have a particular phenotype. To assess discrimination, a different metric ϕ may be used, such as

the area under the ROC curve. Measures of association such as the odds ratio, hazard ratio, or difference in survival probabilities are also commonly used performance metrics, although their value for biomarker signatures has been debated [Pepe et al., 2004]. In Zhu et al. [2010], ϕ was the hazard ratio comparing disease-specific survival between the groups identified as high risk and low risk. The risk groups were determined by the signature f, which was estimated using the JBL.10 observation arm data. The signature was then applied to the same data that were used to build it to define risk groups, producing the hazard ratio estimate of 15 for the prognostic effect comparing signature-defined risk groups in the observation arm. Subsequent evaluations of the signature's performance on independent data resulted in hazard ratio estimates in the 2 - 3 range, substantially smaller than the original estimate of 15. This disparity is due, at least in part, to the bias in the original hazard ratio estimate of 15 resulting from reuse of the data used to build the signature. Next we discuss some methods to avoid bias in evaluation of signature performance.

Avoiding Overfitting

A traditional method to avoid overfitting is the split sample holdout approach. First, randomly partition S into the training sample S_t and the holdout sample S_h with sample sizes n_t and n_h, respectively. Then, S_h is hidden from the analyst while $\mathcal{F}$ is applied to S_t to estimate the signature function f_t. For fixed f_t, $\hat{E}[\phi_{f_t}(S_h)]$ is an unbiased estimator of $E_{\mathcal{P}}[\phi_{f_t}(S)]$. Dobbin and Simon [2011] investigate how to optimally split a dataset into training and holdout partitions. The specific form of f_t that is fixed using S_t can be reported as the function for others to use, therefore the aforementioned estimator applies for that specific f_t. Alternatively, f^*, the signature derived using the entire set S can be reported, accompanied by the estimate of the performance of f_t. The drawback of this approach is that the performance of f_t is likely to be inferior to the performance of a function f^* derived using the same approach applied to the entire data set. Dobbin and Simon [2007] investigate how to choose the sample size n so that there is high probability that the signature developed will have expected performance within some specified margin of the the optimal signature that could be developed if the development data set had infinite sample size.

Another approach to avoid overfitting is cross-validation, which is a resampling based approach. For a fixed integer k, which can be between 1 and n, we randomly partition the full data set S into

subgroups of size k (assume for simplicity here that n is evenly divisible by k so that $K = n/k$ subgroups are formed). For each k, f_{-k} is estimated and fixed by appling $\mathcal{F}$ on S_{-k} which is the subset of S that is disjoint from S_k. Then, we get an estimate $\hat{E}[\phi_{f_{-k}}(S_k)]$ which is an estimate of $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$. Then one iterates through the K subgroups to yield K estimates and then these are averaged to obtain the performance estimate. This process is called "leave k out" or "K fold" cross-validation. Note that for each subgroup in the partition, we obtain a new estimate of f, therefore we are only estimating $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$ and not the performance for a single specified signature. Typically, if a specific form for f is desired, it would be estimated using the entire dataset S to yield f^* as above. Importantly one should report the cross-validated estimate just described and not the naïve estimate that would be obtained by substituting the full data S into the performance metric calculation for the signature f^*. The problematic aspects of such naive "resubstitution estimates" of performance are discussed in more detail below.

A variation on the cross-validation approach is bootstrapping. In that case, a sample S_b of size n is sampled *with replacement* from S. Then f_b is estimated and fixed by applying $\mathcal{F}$ to S_b. The performance metric ϕ is calculated on the subset of S that is disjoint from S_b, which we denote by S_{-b}, to yield an estimate of $E[\phi_{f_b}(S_{-b})]$. This process is repeated K times to yield K estimates. These K performance metric estimates are averaged to obtain the mean over the bootstrap replicates. Efron and Tibshirani [1997] suggest a variation, the 0.632 estimate:

$$\hat{E}^*[\phi_{\mathcal{F}}(S)] = .368\hat{E}[\phi_f(S)] + 0.632\hat{E}[\phi_{f_b}(S_{-b})],$$

where $\hat{E}[\phi_f(S)]$ is the naïve resubstitution estimate of ϕ_f using the entire dataset.

Another variation on all of these methods is the concept of pre-validation [Tibshirani and Efron, 2002]. With pre-validation, instead of computing the statistic ϕ for each of the held-out subsets (S_{-b} for the bootstrap or S_k for cross-validation), the fitted signature $\hat{f}(X_i)$ is estimated for $X_i \in S_{-b}$ where $\hat{f}$ is estimated using S_b. This process is repeated to obtain a set of pre-validated signature estimates $\{\hat{f}_i, i = 1, \ldots, n\}$. Then some measure of the association between these signature estimates and the withheld true outcomes, $Y_i, i = 1, \ldots, n$, is estimated to serve as a measure of the signature performance. The association measure ϕ could be estimated as a coefficient in a linear, Cox

Table 1: Description of various commonly used but biased approaches to signature performance evaluation. This are used for illustration in the simulations.

Name	Description
Partial Holdout	Select features on full dataset S. Split data into S_t and S_h. Build model on S_t using only features pre-selected from full dataset S. Then test that model on S_h.
Partial CV	Select features on full dataset S. Fit regression model inside a cross-validation loop, where at each iteration S_{-k} restricted to pre-selected features is used to build and S_k is used to test.
Naïve Resubstitution	Select features on full dataset S and build model on S using features pre-selected from S. Then test that model on S.
Partial Resubstitution	Split data into S_t and S_h. Select features on S_t. Build model on S_t using only features pre-selected from S_t. Then test that model on the full dataset S.

proportional hazards, or logistic regression model, with or without adjustment for other covariates, as appropriate for the situation. The idea behind this pre-validation approach is that if the signature has good performance, then it should produce values that are strongly associated with the true outcomes. In contrast to cross-validation and the bootstrap, this process avoids the problem of having too few cases to estimate the statistic ϕ on each of the smaller held-out datasets.

These resampling-based procedures described above provide unbiased estimates of predictive accuracy for the procedure, that is $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$, as opposed to the accuracy for a particular signature that others can use. Often, we will provide the signature developed on the entire dataset as the one for future use, as using the full data will estimate signature coefficients with the most precision. Still, it is important to avoid the temptation to report the estimate of performance on this full data signature, as it can be severly biased.

Simulation Study

To illustrate some of the different properties of these estimates and how they help to avoid overfitting, we conduct a limited simulation study. Data were generated with $N = 200$ or 1000 observations, each with a binary outcome Y with prevalence 0.3, and $p = 10, 100, 500$, or 5000 mutually independent

features sampled from the standard normal distribution. This is the null case where no features are associated with Y. The signature development procedure entails a feature selection step, in which each feature is regressed against Y in a univariate logistic regression model. The 25 (10 in the case where $p = 10$) features with the smallest p-values are selected for inclusion in a multivariable logistic regression model which defines the final signature.

We compare each of the methods described above: split-sample holdout, cross-validation, bootstrap, and pre-validation, along with several commonly used but biased approaches. The biased approaches are described in Table 1. Two of the biased approaches use the full sample to select the features, followed by fitting the multivariable model on holdout subsets. This is referred to as "parital holdout" or "partial CV" when using split-sample holdout or cross-validation as the validation step, respectively. We also implemented the naïve resubstitution approach, wherein the model is trained and evaluated on the same dataset, and the partial resubstitution approach wherein the model is developed on a training subset but then evaluated on the combined training and holdout data sets. Our main interest is in comparing the bias and variance of the resulting estimates of $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$. In our simulation, we look at two different performance metrics, the area under the ROC curve (AUC) and the odds ratio for the binary outcome comparing the signature groups.

Table 2: Comparison of different approaches to estimating the Area Under the ROC Curve (AUC) and the log odds ratio (OR) in the setting where a dataset is used to both develop the signature and evaluate its performance. The true value of the AUC is 0.5 and the true value of the Log OR is 0.0. Estimates are based on 1000 replicates of the numerical experiment. In each replicate, there are 1000 observations and 500 features. CV = Cross validation. Additional results for different N and p are in the appendix.

Approach	mean AUC	std.dev AUC	Bias AUC	mean OR	std.dev OR	Bias OR
Resubstitution	0.72	0.01	0.22	1.33	0.12	1.33
Partial CV	0.68	0.01	0.18	1.02	0.12	1.02

Approach	mean AUC	std.dev AUC	Bias AUC	mean OR	std.dev OR	Bias OR
Partial Holdout	0.67	0.02	0.17	0.97	0.19	0.97
Partial Resubstitution	0.65	0.02	0.15	0.88	0.12	0.88
Pre-validation	0.50	0.05	0.00	-0.01	0.32	-0.01
Leave 10 out CV	0.50	0.04	0.00	0.00	0.25	0.00
Leave 100 out CV	0.50	0.03	0.00	0.00	0.20	0.00
30% Holdout	0.50	0.03	0.00	0.00	0.24	0.00
50% Holdout	0.50	0.03	0.00	0.00	0.20	0.00
Bootstrap	0.50	0.01	0.00	0.00	0.08	0.00

Not surprisingly, the resubstitution estimates are optimistically biased: for $N = 1000$ and $p = 500$ the naive resubstitution estimate of the AUC is 44% larger than it should be and the log OR estimate is over 2 times higher than it should be, on average. Partial resubstitution, partial holdout, and partial cross-validation estimates do not ameliorate the bias very much. Investigators are tempted to use partial holdout estimates as it is more convenient to not have to carry along a large number of feature measurements into the modeling part of the signature development process, and they may think that the resulting performance estimates are still close to valid, as only half of the data are used to form the estimates. However, here we see that these versions are still severely biased and should not be reported as valid assessments of the performance of biomarker signatures.

It is evident in Figures 1 and 2 that the amount of bias tends to increase as the number of features increases. Even with only 10 features to select from, the resubstitution estimates are still biased. A larger sample size does not help with the bias, though it does improve precision of the performance estimates. With $N = 200$ and $p = 5000$, which is the situation closest to our real data example, we see that the resubstitution estimate indicates nearly perfect prediction performance, when in fact there is no signal in the data.

The split-sample holdout, cross-validation, bootstrap, and pre-validation methods all produce esentially unbiased estimates in the simulated example, with their mean AUCs being nearly 0.5 and the mean ORs being nearly 1. We can compare the spread of the distributions to get a sense of the

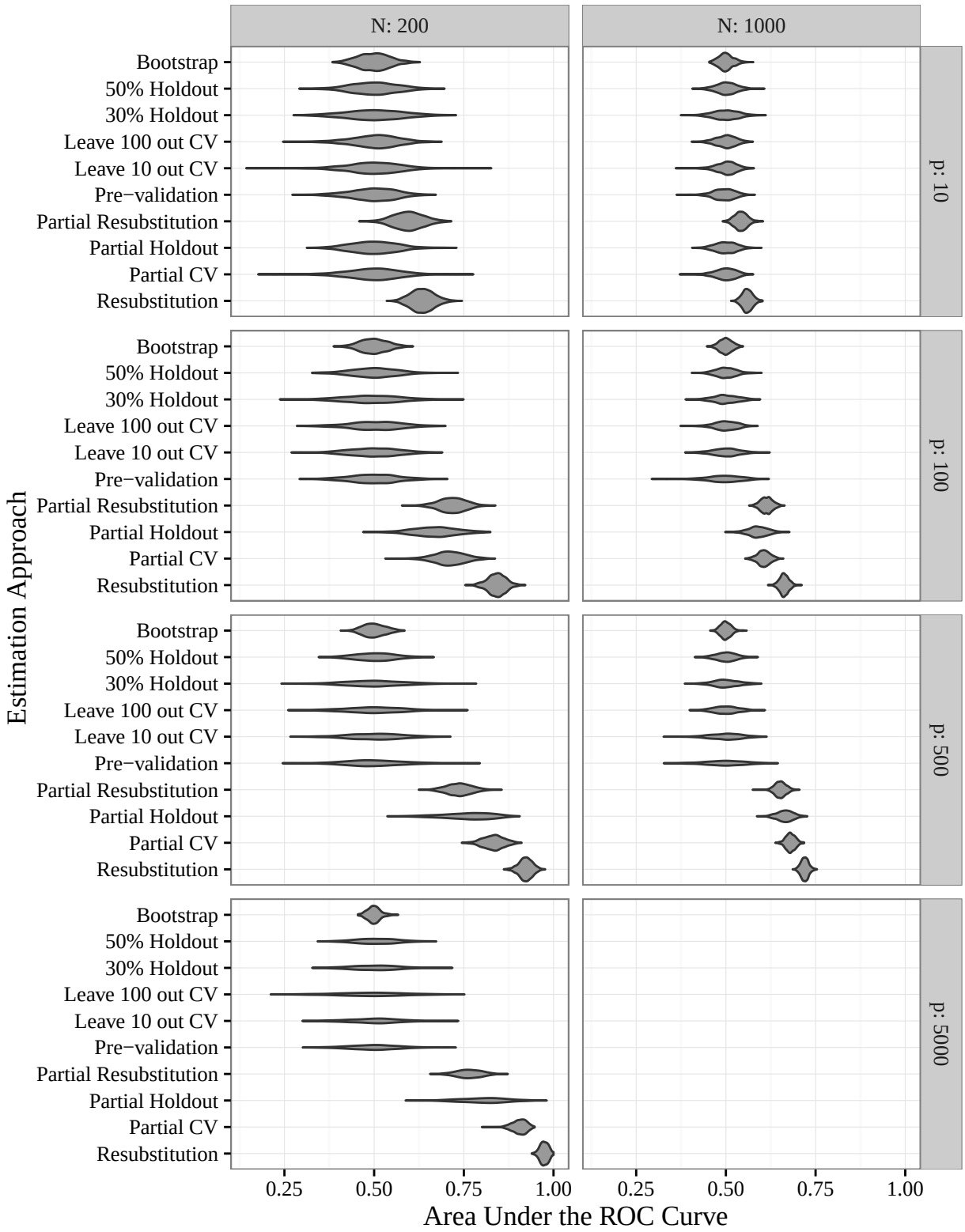

Figure 1: Comparison of different approaches to estimating the Area Under the ROC Curve (AUC) in the setting where a dataset is used to both develop the signature and evaluate its performance. The violin plots show mirrored density estimates for the AUC for 1000 replicates of the numerical experiment. In each replicate, there are N observations and p features. The true value of the AUC is 0.5. CV = Cross validation.

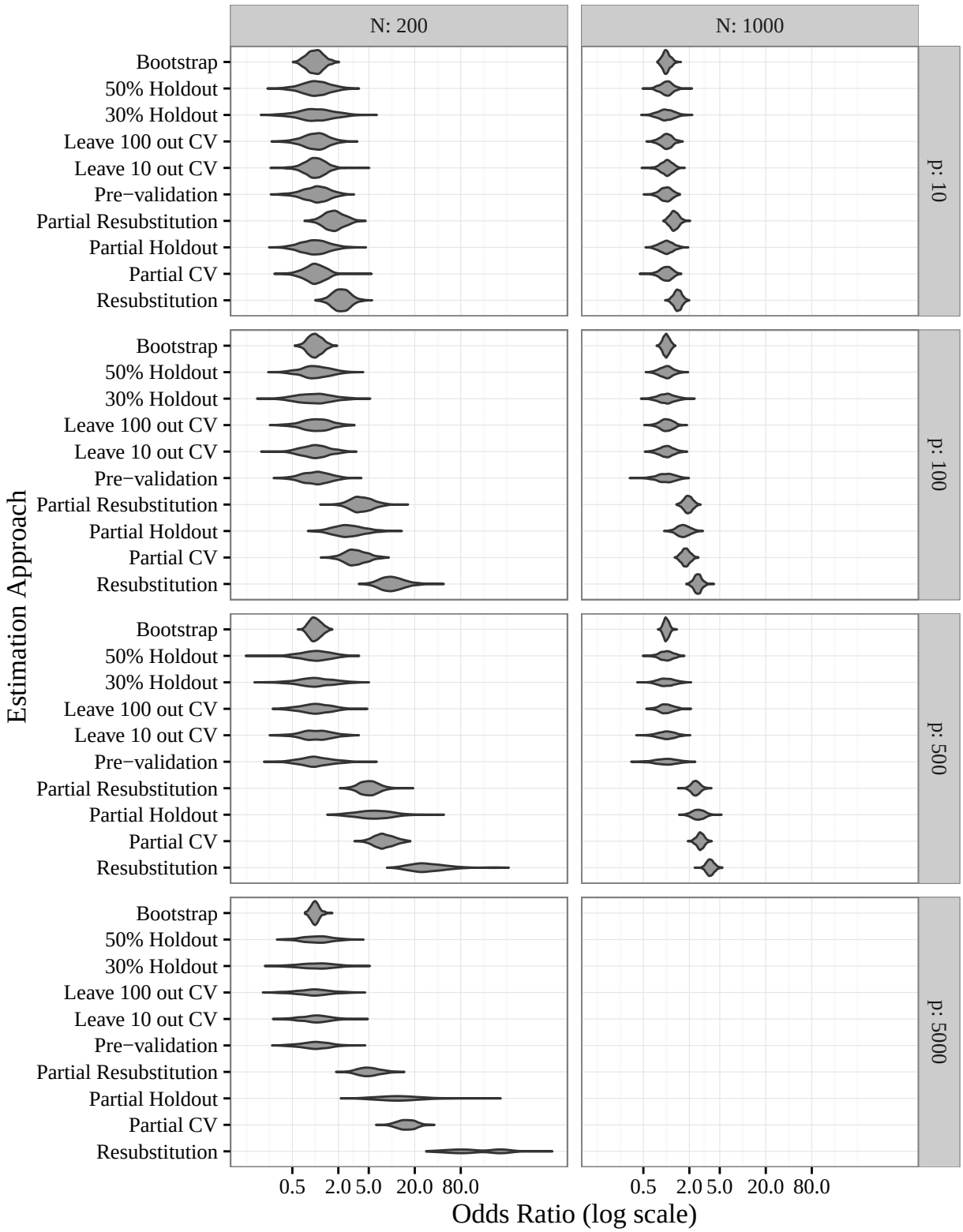

Figure 2: Comparison of different approaches to estimating the odds ratio (OR) in the setting where a dataset is used to both develop the signature and evaluate its performance. The violin plots show mirrored density estimates for the log OR for 1000 replicates of the numerical experiment. In each replicate, there are N observations and p features. The true value of the OR is 1.0. CV = Cross validation.

differences in precision of the estimates. The bootstrap approach appears to be the most precise, followed by the cross-validation, holdout, and finally the pre-validation. The bootstrap, as intended, is a more efficient, smoothed version of the cross validation estimate (Figure 1, 2). It provides the best balance between allocating data to train the signature and having independent data remaining to precisely estimate the statistic ϕ.

Data Analysis

Data analysis example

We now illustrate the concepts and methods just discussed by reanalyzing data that had been used to build a previously published lung cancer prognostic signature Zhu et al. [2010]. Briefly, the data of interest are from the JBR.10 trial, which was a randomized controlled trial of the adjuvant chemotherapy regimen vinorelbine/cisplatin (ACT) versus observation alone (OBS) in 482 participants with non small cell lung cancer (NSCLC) who had undergone surgery. Of those 482 participants, 169 had frozen tumor tissue collected, and of those tumor samples, 133 (71 in ACT and 62 in OBS) had gene-expression profiling performed using U133A oligonucleotide microarrays (Affymetrix, Santa Clara, CA).

The goal of the Zhu et al. [2010] paper was to identify a multi-gene signature that strongly predicts prognosis, and the hypothesis was that the poor prognosis subgroup would benefit more from ACT than the good prognosis subgroup. The signature was developed on a training data set ("trained") to predict disease specific survival. The annotated gene expression data and clinical information are available from the Gene Expression Omnibus database(identifier: GSE14814, Edgar et al. [2002]).

Zhu et al. [2010] present results that mainly focus on the association of their signature with outcome as quantified by a hazard ratio, albeit a less than ideal measure of discrimination ability. They demonstrate that the two risk subgroups predicted by their signature (high risk and low risk) have separation in their survival curves and that the hazard ratio for their signature is large and significant even when adjusting for other risk factors. They do not directly address calibration, that is, whether their signature accurately predicts survival times.

We used a similar approach to preprocessing as did Zhu et al. [2010], although we could not reproduce

their workflow exactly due to outdated software. Batch effects were removed using the `ComBat` function in the `sva` `R` package [Leek et al., 2016] and then the gene expression values were centered by their means and scaled by their standard deviations. Our signature development approach is similar but not identical to that in Zhu et al. [2010]. For purposes of illustrating the concepts we used a simplified approach to signature development that retains the main characteristics of the original method. The exact approach to signature development does not have a major impact on the main conclusions of our evaluation of the various approaches to signature performance assessment.

After processing the data as described above, we performed a gene selection step wherein we fit univariate Cox regression models with disease specific survival as the outcome and each gene as the single predictor. Genes with univariate p-values less than 0.005 were preliminarily selected for further analysis. Then, each gene from the preliminary list was weighted by its univariate Cox regression coefficient, and the resulting weighted gene expression values were summed to form risk scores. Genes were selected for inclusion in the risk score in a forward selection manner. Starting with the most significant weighted gene, the gene that when added to the risk score improved the concordance between survival times and the risk score most was selected next. If no gene improved the concordance, the process was stopped. All genes on the final selected list were included in a multivariable Cox regression model to fit the final risk score with new coefficients. We selected the cutoff that split the patients into two risk groups corresponding to the smallest log-rank statistic p-value when applied to the continuous risk score.

Now recall the general framework for signature development that was described in the introduction. There is both a feature selection step, and a multivariable estimation step. This results in a continuous signature which is the linear predictor of a Cox regression model. The signature is dichotomized by selecting the cutoff that yields the most significant log-rank statistic for comparing the resulting risk groups. Discrimination of the signature is assessed using the concordance statistic as implemented in the `survival` package in `R` [Therneau, 2015]. To paraphrase the help file: the concordance statistic is defined as the probability of agreement for any two randomly chosen observations, which in this case means that the observation with the shorter survival time also has the larger signature value. This is similar to an interpretation of the AUC for binary data.

First we fit the signature using the entire observation arm (n = 62). The signature was then evaluated

on the same dataset. The survival plot on the left side of Figure 3 shows extreme separation between the two risk groups (HR = 20, p < 0.001), consistent with the reported JBL.10 signature, and the estimated concordance is 0.87. After correctly accounting for the selection process, our estimates of association and discrimination are much less impressive.

The right plot in Figure 3 shows the survival curves for the two risk groups using the pre-validated estimates of the risk score. We partitioned the 62 observations into 8 groups of 6 and 2 groups of 7. Then for each group b, we fit the model using S_{-b} and obtained prevalidated estimates for S_b. The survival curves plot the survival times for comparing risk groups using the prevalidated estimates. The separation is much less impressive. The concordance between the prevalidated signature and the survival times is 0.61, indicating much worse discrimination.

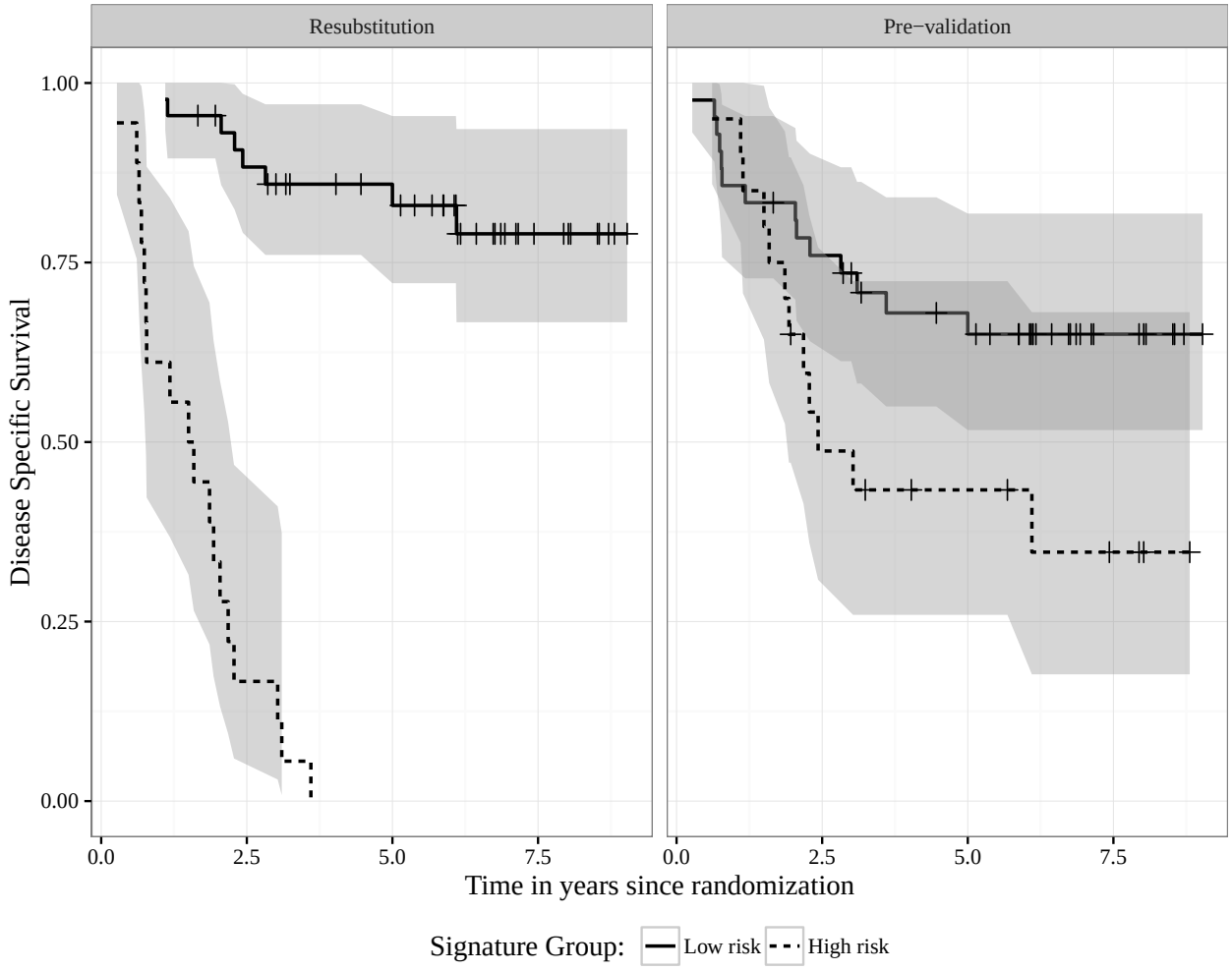

Figure 3: Comparison of survival by gene expression based risk signature. The left plot shows the resubstitution estimate, while the right plot shows the pre-validated estimate.

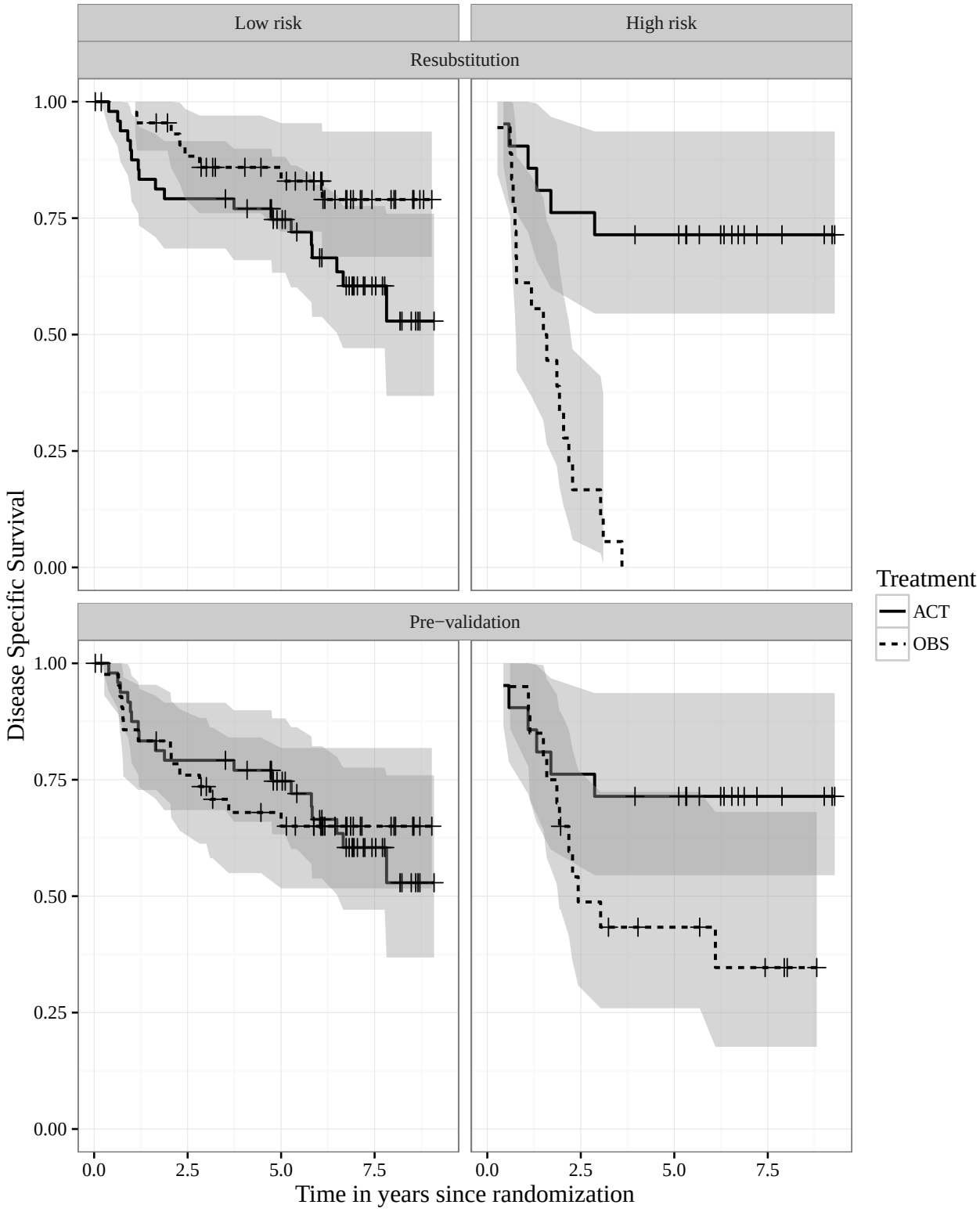

Figure 4: Survival curves comparing the treatment effect by gene expression signature risk group. The top set of plots shows the partial resubstitution based signature and the bottom row shows the pre-validated signature estimates.

Table 3: Hazard ratios and 95% confidence intervals from separate Cox regression models that adjust for tumor histologic subtype, stages, age, and sex. Rows labeled 'High risk vs low risk' show the hazard ratio for the signature-based risk group comparison. The rows labeled 'Trt/Risk interaction' show the hazard ratio for the interaction term of treatment by signature-based risk group. The partial substitution estimates are dramatically optimistically biased.

Method	Comparison	Hazard Ratio	95% CI*	Adjusted p*
Partial Resubstitution	High Risk vs Low Risk	38.9	9.2 to 164.7	< 0.001
	Trt/Risk interaction	14.7	3.2 to 67.0	< 0.001
Prevalidation	High Risk vs Low Risk	1.9	0.8 to 4.3	0.122
	Trt/Risk interaction	1.8	0.5 to 6.5	0.395

CI's and p-values based on incorrect inference.

We also show plots to assess the ability of the signature to be useful for treatment selection (Figure 4). These plots show the survival curves comparing treatment arms grouped in panels by the risk score. As described by Polley et al. [2013], the idea is to determine whether the treatment is beneficial in one group and not benficial or harmful in another group, indicating that different treatment decisions would be made based on the signature. On one hand, the overfit resubstitution signature shows dramatic differences in treatment efficacy between the low risk and high risk groups. In fact it appears that the treatment is harmful in the low risk group, but highly beneficial in the high risk group. The prevalidated signature on the other hand, shows differences that are much less dramatic. It appears that the treatment is mildly beneficial in both groups, possibly to a higher degree in the high risk group. This suggests that the dramatic predictive value of the signature was merely an artifact of overfitting the signature to the OBS arm data.

The Zhu et al. [2010] paper used the approach of identifying a signature using the control arm of the trial, evaluating it using the combined control and treatment arms, and then hoping that the

signature would be useful for treatment selection. This approach has been shown to be invalid [Simon and Freidlin, 2011] for identifying a predictive signature. Indeed we show that the resubstitution aspect of the evaluation likely led to overstatements of the size of the signature's purported predictive effect for therapy selection.

In a multivariable Cox model we observe similar trends when comparing the prevalidated signature to the overfit signature. We fit two regression models. In the first, the aim is to assess the prognostic value of the signature by estimating the hazard ratio for the high risk versus low risk groups, adjusted for tumor histologic subtype, stage, age, and sex. In the second model, the idea is to assess the predictive value of the signature by estimating the treatment by signature interaction effect, adjusting for the same clinical covariates. The results are reported in Table 3. The full model summaries are report in Table 4. For the partial resubstitution approach, we find an extreme hazard ratio of nearly 40 for the prognostic effect, and a strong and significant treatment by signature interaction. Using the prevalidated signature, the effect estimates are much smaller and of small magnitude in comparison to the standard clinical features. Note that the inferences from the pre-validated model (confidence intervals and p-values) are not exactly correct either, because the resampling procedure induces a correlation among the observations (the prevalidated signature values are estimated from heavily overlapping data). Despite this, these results are unimpressive and would not be considered promising for clinical use.

Zhu et al. [2010] should be commended for their commitment to making their data, methods, and analysis code publically available, which allowed us to reanalyze their study data. More often, methods used to derive signatures are vaguely and incompletely presented and a resubstitution analysis or other flawed approach goes undetected or can be discovered only through a very careful scrutiny of the methods and supplementary materials.

Discussion

Molecular signatures are being increasingly integrated into the practice of medicine to personalize clinical care decisions. Unlike new drugs which go through a rigorous development process that includes conduct of prospective clinical trials and review by regulatory authorities, the evidence

Table 4: Complete Results of Multivariable Regression Models. Entries are hazard ratios and 95 percent confidence intervals.

	Dependent variable:			
	Disease-specific Survival			
	Resubstitution	Prevalidation	Resubstitution	Prevalidation
	(1)	(2)	(3)	(4)
High Risk	38.9***	1.9	0.7	1.0
	(9.2 to 164.7)	(0.8 to 4.3)	(0.3 to 2.0)	(0.4 to 2.7)
Trt: OBS			0.5	1.3
			(0.2 to 1.3)	(0.6 to 2.8)
Male Gender	1.3	1.4	1.2	1.4
	(0.4 to 3.8)	(0.5 to 4.2)	(0.6 to 2.4)	(0.7 to 2.8)
Stage > I	2.1*	2.7**	2.0**	2.3***
	(0.9 to 4.7)	(1.2 to 6.3)	(1.1 to 3.6)	(1.3 to 4.1)
Age in Years	1.0	1.1**	1.0*	1.1***
	(0.9 to 1.1)	(1.0 to 1.1)	(1.0 to 1.1)	(1.0 to 1.1)
Histology: LCUC	1.7	2.7	1.5	1.9
	(0.5 to 6.3)	(0.8 to 10.0)	(0.6 to 3.7)	(0.7 to 4.7)
Histology: SQCC	3.4*	0.4**	0.8	0.4***
	(0.9 to 13.3)	(0.2 to 1.0)	(0.3 to 1.7)	(0.2 to 0.8)
High Risk * Trt:OBS			14.7***	1.8
			(3.2 to 67.0)	(0.5 to 6.5)
Observations	62	62	133	133
Score (Logrank) Test	69.6*** (df = 6)	21.5*** (df = 6)	84.5*** (df = 8)	32.0*** (df = 8)

Note: *p<0.1; **p<0.05; ***p<0.01
CI's and p-values based on incorrect inference.

base for molecular signatures is most often derived from retrospectively conducted studies using stored specimens and published literature is relied upon for evidence to assess the worth of a signature. More than a decade after many high-dimensional molecular assays were first introduced, there are many publications reporting signatures that have not been adequately evaluated for their performance. Many published reports contain highly exaggerated claims of signature performance due to use of inappropriate methods to develop them and assess their performance. It is important that use of inappropriate methods is recognized so that flawed or poor performing signatures are not adopted into clinical practice [McShane et al., 2013a,b].

As an aid to researchers, journal editors, journal reviewers, regulatory and funding agencies, we have attempted to describe in this paper some of the flawed signature development and validation approaches that we have commonly encountered and to explain their impact on reported performance metrics. Many researchers have embarked on studies intended to develop and assess performance of molecular signatures, but many have not had much prior experience in this analytical area. The problematic approaches highlighted in the examples and simulations included naïve resubstitution, partial resubstitution, partial holdout, and partial CV. The simulations demonstrated how even in a simple null situation, the over-optimism bias in performance estimates is quite substantial, possibly enough to persuade some to adopt these signatures for clinical care prematurely. The magnitude of the bias could be even greater with more modest samples sizes or more convoluted signature development methods as are typical in the published literature. In our simulations and data example, we used a simple variable selection procedure follow by multivariable regression. More complex statistical methods for developing signatures such as the lasso, random forests, and others are not immune to this type of bias. In fact, more complex methods often involve tuning parameters, which give more opportunities for bias due to overfitting. Strategies to avoid this type of bias are well-studied in the statistical literature and here we demonstrated how they can be implemented in a real clinical example.

The ability to perform the kind of in-depth data reanalysis we performed on the example in this paper is usually limited by restrictions on data access and insufficient description of analysis methods. It is imperative that investigators and other stakeholders such as journal editors, funders, and regulators take overfitting bias seriously to ensure that signature performance claims are valid.

This will require a concerted effort to ensure that reviewers are sufficiently informed of acceptable approaches to signature development and evaluation, data are made available for independent verification, and methods are reported in complete and transparent fashion.

Note

All analysis code and the source files for this manuscript are available from the authors' webpage: https://github.com/sachsmc/signature-tutorial

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