

# Issues in developing multivariable models for treatment selection

*Michael C Sachs and Lisa M McShane*

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## Abstract

Omics technologies that generate a large amount of molecular data about biospecimens have the potential to provide information about a patient’s disease characteristics above and beyond standard clinical and pathological features. By combining the information from a large amount of molecular 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. A biomarker signature can guide the decisions to treat or not to treat and help identify the patients who are most likely to survive. The key challenge is to combine features from a high dimensional molecular assay to derive a signature with good clinical performance and appropriately characterize its performance. The inappropriate practice of using overlapping data to both build a signature and evaluate its performance can lead to severe over-optimism bias in performance estimates. We summarize the key statistical issues and methods for developing and validating biomarker signatures, using examples from the literature to illustrate them.

## Introduction

Omics technologies that generate a large amount of molecular data about biospecimens have the potential to provide information about a patient’s disease characteristics above and beyond standard clinical and pathological features. By combining the information from a large amount 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 treatment decisions can be personalized. A multivariable biomarker can guide the decisions to treat or not to treat and help identify the patients who are most likely to survive. The key challenge we address in this paper is how to combine features from a high dimensional molecular assay to derive a signature that is fit for a specified clinical use and to provide valid estimates of performance characteristics.

## Terminology and Notation

A **biomarker signature** is a transformation of multiple individual features, typically molecular characteristics measured on a multiplex assay, to a one-dimensional space. Specifically,

let  $X$  denote the set of  $p$  features 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.

Let  $S$  denote the development dataset which includes, for each of  $n$  represented individuals, a feature vector  $X$ , an outcome  $Y$ , a treatment  $Z$ , and possibly other variables.  $S$  is a sample of size  $n$  from distribution  $\mathcal{P}$  with domain  $\mathcal{S}$ . Let  $\mathcal{F}$  be a mapping from  $\mathcal{X}$  to the space of continuous functions with domain  $\mathbb{R}^p$  and range  $\mathbb{R}$ ,  $\mathcal{D}$ . Thus  $\mathcal{F} : \mathcal{X} \mapsto \mathcal{D}$  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  $\phi : \mathcal{D} \times \mathcal{X} \mapsto \mathbb{R}$  denote the statistic that quantifies the performance of the function  $f$ , such as predictive accuracy, mean squared error, or area under the receiver operating characteristic (ROC) curve (AUC). This could also be a measure of association, such as an odds ratio, hazard ratio, or log-rank statistic. This is a function of both  $f$  and  $S$ . We are interested in estimating  $E_{\mathcal{P}}[\phi_{f^*}(S)]$ , which is the expected error under the data generation mechanism, for a particular  $f^* \in \mathcal{F}$ . This allows us to understand how the signature will perform on future observations generated from  $\mathcal{P}$ . We may also be interested in estimating  $E_{\mathcal{P}}[\phi_f(S)]$  for all  $f \in \mathcal{F}$ , which is the generalization error 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. As shorthand we will write this as  $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$

A signature that **reliably predicts** an outcome  $Y$  is one that has generalization error small enough for the clinical context. Such a signature may be useful for treatment selection, prognosis, or other type of clinical management.

## Overview of biomarker signature development

The main goal is to produce a good signature. To establish this requires a valid estimate of the signature's performance. Assessment of the worth of a biomarker signature requires providing a valid estimate of the performance of  $f \in \mathcal{F}$ . Additionally, one can provide a specification of  $f$  for others to use. Typically, a specific  $f$  is estimated using  $\mathcal{F}$  based on some training data. This can be done using a variety of different methods. In recent years there have 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 of such approaches are [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, determining how to combine the feature measurements, and whether to apply thresholds/cutoffs to the resulting signature value.

A biomarker signature can inform clinical practice in a number of ways. In early-stage disease, a highly prognostic signature may identify a subpopulation of patients that has such a good chance of long term 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 to treat or not.

Signatures can be estimated by identifying naturally occurring clusters, or intrinsic subtypes using  $X$  alone without regard to the outcome  $Y$ . The PAM-50 gene signature was developed using clustering methods in an attempt to identify intrinsic subtypes with different prognosis [Parker et al., 2009]. Later, the intrinsic subtypes were shown to be strongly associated with clinical outcomes [Nielsen et al., 2010]. Supervised learning techniques can also be used to identify prognostic signatures, as was the case with Oncotype DX [Paik et al., 2004], another signature developed for clinical decision making in early stage breast cancer. In this case, regression-based methods are used to estimate a model that is highly predictive for the outcome  $Y$ . In the case of Oncotype DX, the outcome in question was distant recurrence of breast cancer.

An approach to identifying treatment-selection signatures is to use regression techniques to estimate a signature that has a strong interaction with a particular treatment. It is possible, and quite common in high-dimensional settings, to combine multiple approaches to estimating  $f$ . For instance, a data-reduction step by variable selection or clustering may be performed before doing regression analysis on the resulting components.

No matter what the particular model building method is, our main concern and focus of this paper is with obtaining a valid estimate of its performance, that is, a good estimate of  $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$ . This depends on the true signal in the data and the specific algorithm  $\mathcal{F}$  used. An optional component of the development phase is to provide a specification of  $f$  for others to use on independent data or in clinical practice.

## Biomarker signatures in clinical practice

Biomarker signatures are useful if they can correctly and reliably classify patients into distinct subgroups for which different treatment decisions would be made. There are two distinct but related statistical concepts involved here: calibration and discrimination. Signatures are often optimized to be well-calibrated, that is, accurate for predicting outcomes. However if the signature does not separate a population into distinct subgroups with therapeutic relevance, then it is unlikely to be informative enough to change clinical practice.

In the development process it is important to evaluate both calibration and discrimination. It is not trivial to assess each of these in a valid manner when the data are used to define the signature itself. We illustrate the potential for bias through a series of examples, and we review some strategies to avoid the pitfalls that lead to bias.

## Data analysis example

Throughout this paper, we reanalyze data from Zhu et al. [2010]. Briefly, the data of interest are from the JBR.10 trial, which was a randomized controlled trial of adjuvant

vinorelbine/cisplatin (ACT) versus observation alone (OBS) in 482 participants with non small cell lung cancer (NSCLC). Of those 482 participants, 169 had frozen tissue collected, and of those 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 compared to the good prognosis subgroup. The signature was trained to predict disease specific survival. The annotated gene expression data and clinical information are available from the Gene Expression Omnibus (identifier: GSE14814, [Edgar et al. \[2002\]](#)).

[Zhu et al. \[2010\]](#) present results that mainly focus on the discrimination ability of their estimated signature. They do that by demonstrating 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. \[2010\]](#), as 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 features of the original. 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.

Following the processing steps 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 selected for further analysis. Then, each gene was weighted by its univariate Cox regression coefficient, and the resulting weighted gene expression values summed to form risk scores. Genes were selected in a forward selection manner, starting with the most significant genes, the gene that improved the concordance between survival times and the risk score was selected. If no gene improved the concordance, the process was stopped. The final list of selected genes were all included in a multivariable Cox regression model to fit the final risk score. The cutoff that yielded the smallest log-rank statistic p-value was used to dichotomize into two risk groups.

## Issues

Recall that the main goal is to estimate  $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$ , the expected value of a given statistic on future observations for  $f \in \mathcal{F}$ . This 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. This bias results from the fact that  $\phi$  depends on  $f$ , and thus the statistic  $\phi$  is being adaptively

defined based on the observed data  $S$ . 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.

In many cases during signature development, the performance metric  $\phi$  in question is a statistic that relates to calibration. While a biomarker signature may accurately predict a clinical outcome, that does not necessarily imply that the signature is clinically useful. To assess discrimination, a different statistic  $\phi$  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 examples of performance metrics that are commonly used, although their value for biomarker signatures has been debated [Pepe et al., 2004]. Evaluation of  $\phi$  is subject to bias due to overfitting regardless of what  $\phi$  is used, a fact that is commonly overlooked in the medical literature.

In Zhu et al. [2010],  $\phi$  was the hazard ratio comparing the high risk and low risk groups as defined by the JBL10 signature  $f$ . The risk groups were determined by the signature  $f$ , which was estimated using  $S$ , the same data that was then used to produce the hazard ratio estimate of 15. They go on to assess the performance of the JBL10 signature in a series of independent data sets, in which they estimate hazard ratios of approximately 2. Here we reanalyze the dataset and illustrate some remedies to avoid bias in estimating signature performance.

## Avoiding Overfitting

A traditional method to avoid overfitting is the split sample 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 both  $E_{\mathcal{P}}[\phi_{f_t}(S)]$  and  $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$ . The specific form of  $f_t$  that is fixed using the  $S_t$  partition can be reported as the function for others to use, therefore the aforementioned estimator is also an estimate of the error for that specific  $f_t$ . The drawback of the split-sample 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 [2011] investigate how to optimally split a dataset into training and holdout partitions.

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 select a partition of  $k$  observations from  $S$ , denoted  $S_k$ . Then  $f_{-k}$  is estimated and fixed by applying  $\mathcal{F}$  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 unbiased estimate of  $E_{\mathcal{P}}[\phi_{\mathcal{F}}(S)]$ . This process is repeated  $K$  times to yield  $K$  estimates. Each of these estimates is unbiased, but noisy, because typically  $k$  is very small relative to  $n$ . Thus, we average over  $K$  to get a less noisy estimate. This process is called “leave  $k$  out” or “ $n/k$  fold” cross-validation. Note that for each partition that is selected, 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.

Table 1: Description of various commonly used but biased approaches to signature performance evaluation. These 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.
Naive Resubstitution	Select features on full dataset $S$ and build model on $S$ using features pre-selected from $S$ . Then test that model on $S_h$ .
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 $S$ .

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  $\phi$  is calculated on the subset of  $S$  that is disjoint from  $S_b$ :  $S_{-b}$  to yield an estimate of  $E[\phi_{f_b}(S_{-b})]$ . This process is repeated  $K$  times to yield  $K$  estimates. These  $K$  estimates of the performance metrics 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 naive estimate of  $\phi_f$  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  $\phi$  for each of the held-out subsets ( $S_{-b}$  for the bootstrap or  $S_k$  for cross-validation), the fitted predictor  $\hat{f}(X_i)$  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 predictor estimates  $\hat{f}$  which are then used to calculate  $\phi$ . For single-step holdout, this process is equivalent to what is described above. For cross-validation and the bootstrap, this process avoids the problem of having too few cases to estimate the statistic  $\phi$  on the held-out dataset.

## Simulation Study

To illustrate the different properties of these estimates and how they deal with overfitting, we conduct a simulation study. Data were generated with 1000 observations, each with a binary outcome  $Y$  with prevalence 0.3, and 500 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 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. All of the approaches are described in 1. Two of the biased approaches use the full sample to select the features, followed by fitting the multivariable model on the holdout subset. 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 naive resubstitution approach, wherein the model is trained and evaluated on the same dataset, and the partial resubstitution approach wherein the model is trained on a holdout set and then evaluated on the combined complete dataset. 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 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.

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
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

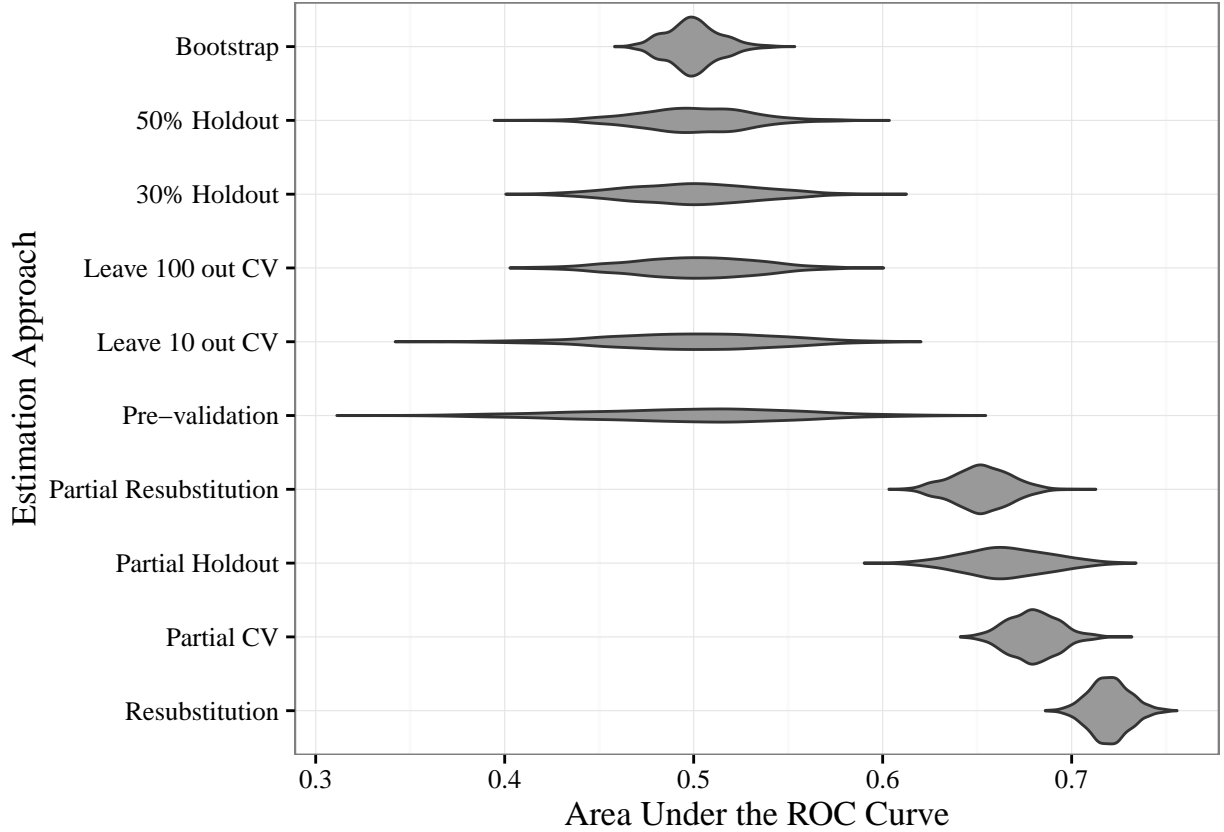


Figure 1: Comparison of different approaches to estimating the Area Under the ROC Curve (AUC) in the setting where a dataset is used to both develop the signature and evaluate its performance. The violin plots show mirrored density estimates for the AUC for 1000 replicates of the numerical experiment. In each replicate, there are 1000 observations and 500 features. The true value of the AUC is 0.5. CV = Cross validation.



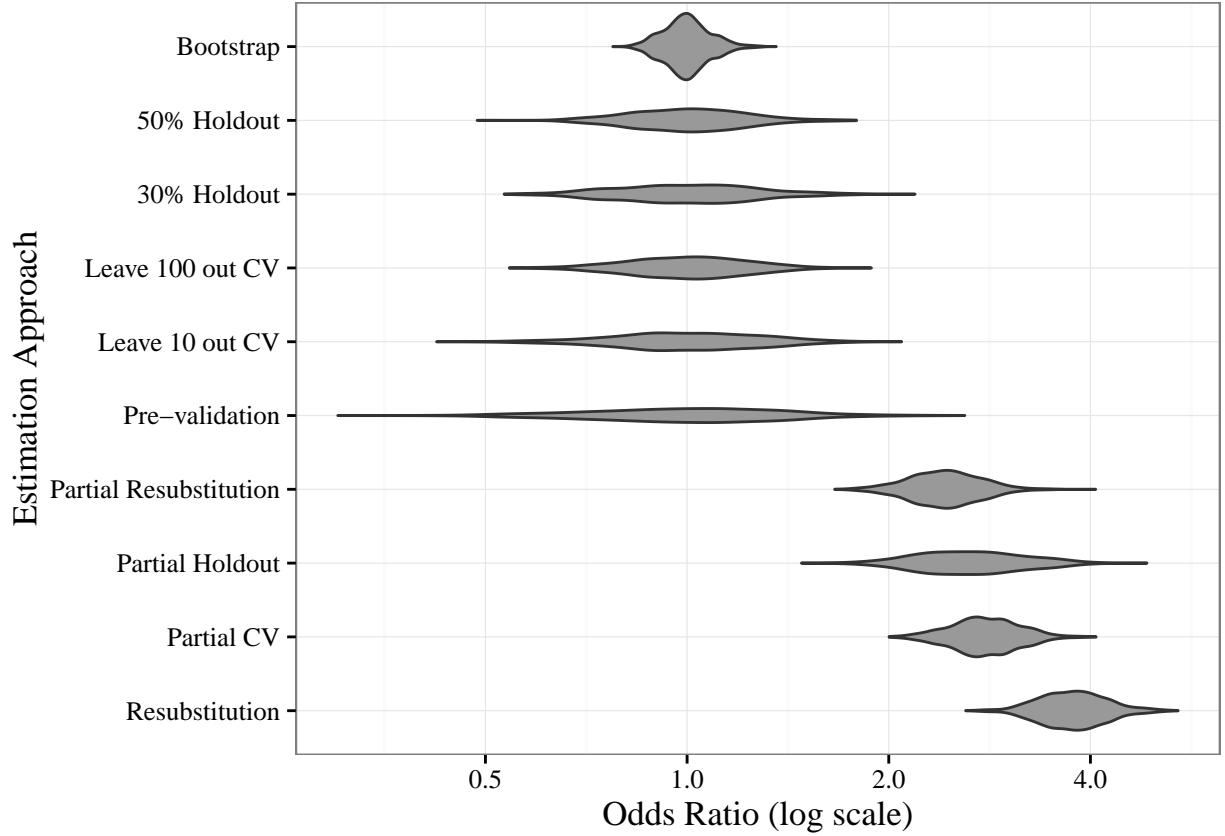


Figure 2: Comparison of different approaches to estimating the odds ratio (OR) in the setting where a dataset is used to both develop the signature and evaluate its performance. The violin plots show mirrored density estimates for the log OR for 1000 replicates of the numerical experiment. In each replicate, there are 1000 observations and 500 features. The true value of the OR is 1.0. CV = Cross validation.

Not surprisingly, the resubstitution estimates are optimistically biased: the naive resubstitution estimate of the AUC is 44% larger than it should be and the 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 often feel as though partial holdout estimates are 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.

All of the proposed remedies to avoid these biases are successful and are unbiased, 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 differences in precision of the estimates. The bootstrap approach appears to be the most precise of the unbiased estimates, 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, 3). It provides the best balance between allocating data to precisely train the signature and having independent data remaining to precisely estimate the statistic  $\phi$ .

## Data Analysis

The signature development procedure 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: this 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 cohort ( $n = 62$ ). 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.

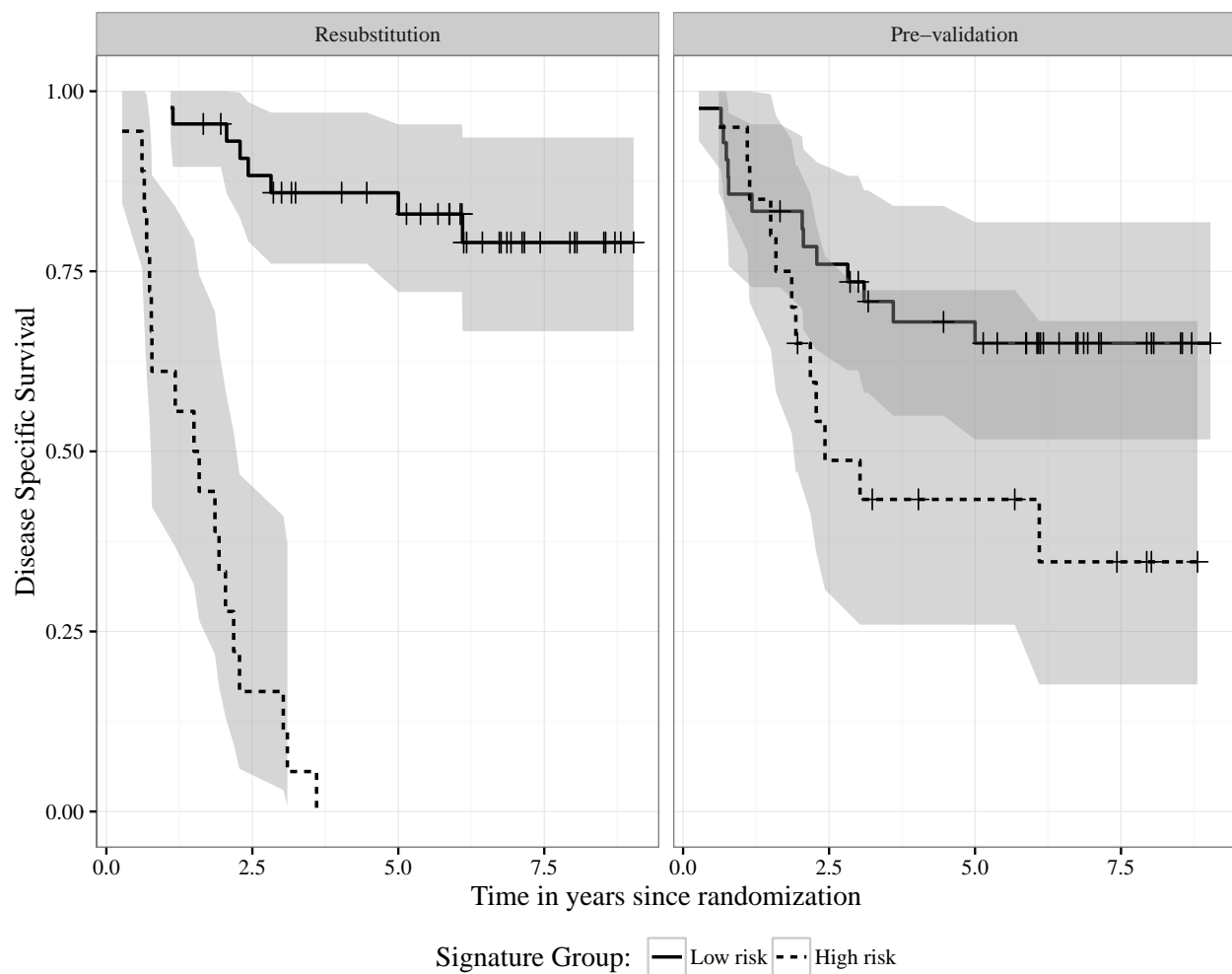


Figure 3: Comparison of survival by gene expression based risk signature. The left plot shows the resubstitution estimate, while the right plot shows the pre-validated estimate.

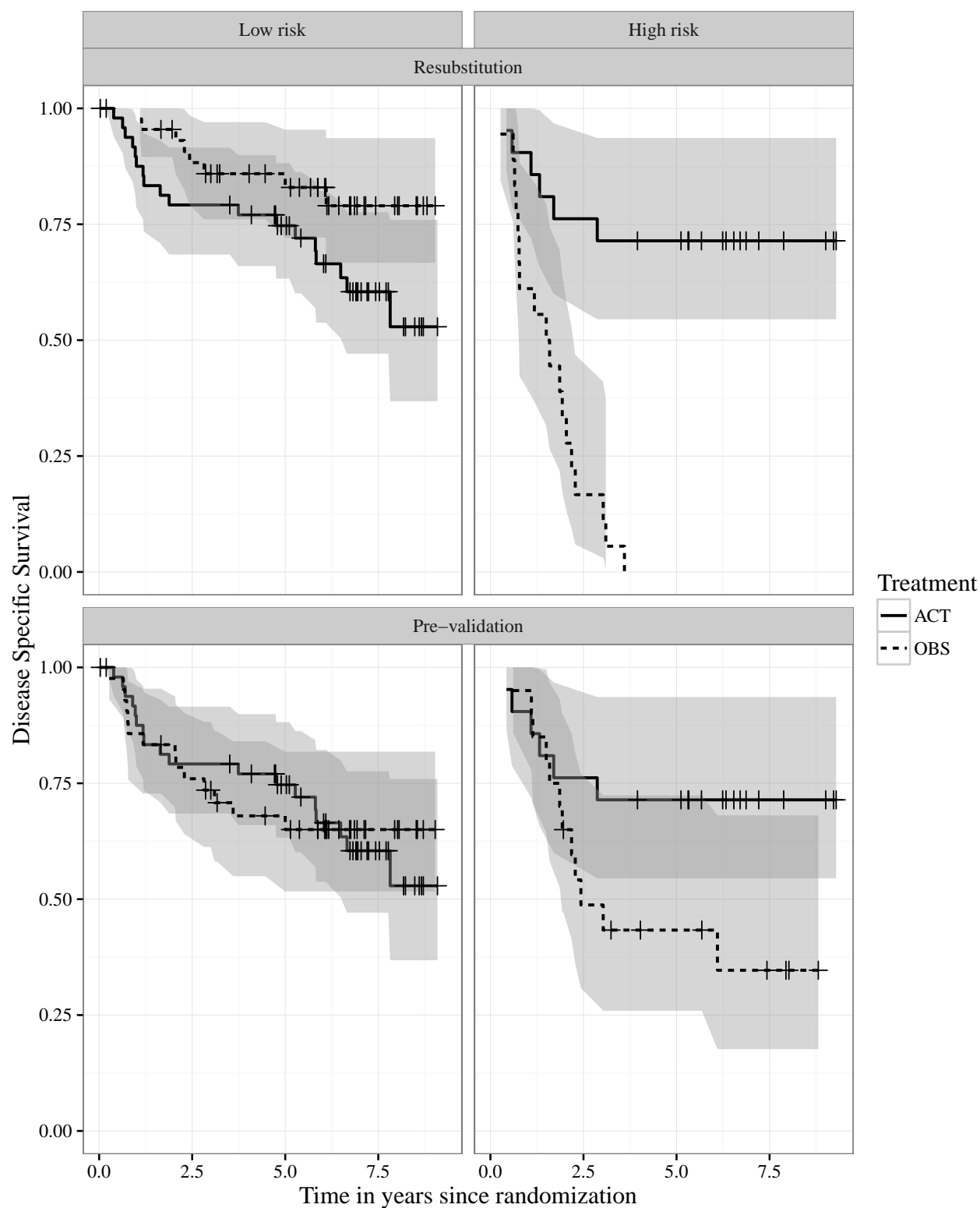


Figure 4: Survival curves comparing the treatment effect by gene expression signature risk group. The top set of plots shows the partial resubstitution based signature and the bottom row shows the pre-validated signature estimates.

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
<b>Partial Resubstitution</b>	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
<b>Prevalidation</b>	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

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 beneficial or harmful in another group, indicating that different treatment decisions would be made based on the signature. On one hand, the overfit 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, a very rare finding. 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 the overfitting process on the OBS arm, as pointed out by Simon and Freidlin [2011].

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. 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 insignificant in comparison to the standard clinical features. Note that the inference from the pre-validated model is not exactly correct either, because the procedure induces a correlation among the observations. Despite this, these results are unimpressive and would not be considered promising for clinical use.

## Additional Examples

Examples of resubstitution estimates of performance are highly prevalent in the literature. [Zhu et al. \[2010\]](#) was not unique in that sense, but rather they should be commended for their commitment to making their data, methods, and analysis code publically available, which allowed us to reproduce and reanalyze their study. More often, the methods are obfuscated through vague and non-standard descriptions, short methods sections, and hidden data analysis code. Most of the time, a resubstitution analysis can be discovered only through a very careful scrutiny of the methods and supplementary materials, if at all. Here we give some additional examples.

[Van't Veer et al. \[2002\]](#) used gene expression measurements to develop a signature and predict clinical outcome of patients with axillary lymph node-negative breast cancer (metastatic disease within 5 years versus disease-free at 5 years). The signature was developed using a stepwise selection and classification procedure, in which a subset of the genes was first selected using all of the subjects, then further subsets were selected using cross-validation until they arrived at a 70 gene signature. This is the classic form of the partial cross-validation as we've described above. The resubstitution estimate of the odds ratio to develop distant metastasis within 5-years was estimated to be 28, with  $p < 0.0001$ . A later study of a similar population used similar gene expression profiling approach, but with a valid split sample holdout validation approach [[Wang et al., 2005](#)]. A univariate analysis of the locked-down signature evaluated on the holdout set gave a much more conservative odds ratio estimate of 5.7 for distant metastasis within 5 years.

[Zhang et al. \[2001\]](#) describe a tree-based partitioning approach to use gene expression data to classify specimens as either cancer or normal tissue. Again, from a large number of candidate genes, a subset of three genes is identified using the entire set of 48 tissue specimens. Then, the cutoff values for the classifications are re-estimated using cross validation. This is yet again the classic form of partial cross validation that we have shown to be biased, contrary to their reports of "unbiased" estimates of 6-8% missclassification rates. This key detail of the analysis is only briefly described in the results section.

## Discussion

All statistics, whether they assess calibration or discrimination or something else, are subject to bias due to overfitting. Remedies to this type of bias are well-studied in the statistical literature and here we have demonstrated how they can be implemented in a real scenario. Sadly, reports of strong associations with overfit biomarker signatures are all too common in the medical literature. The amount of bias that is possible is not known and can be difficult to decipher based on study reports. It is imperative that investigators and journal editors take overfitting bias seriously to ensure that signature estimates are valid. Unfortunately this often results in study reports that are far less optimistic than usual.

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, and indeed we show that the resubstitution aspect of the evaluation likely led to overstatements of the size of the signature effect. Properly doing cross-validation for estimating the calibration of the signature and doing pre-validation for assessment of the discrimination of the signature show that the associations are much more modest.

When we assess the signature on the independent treatment arm, we see that there is no significant difference between the risk groups. This raises the question of whether it is appropriate to use non-random splits of the dataset in order to obtain valid estimates of the calibration or discrimination. Instead of treatment arms, we could imagine a large multi-center study, and we could split the data into disjoint subgroups based on the center. Specifically, suppose we split the development dataset  $S$  into  $S_1$  and  $S_2$  according to a discrete covariate  $X$  that takes on levels 1 or 2. Then we develop a signature  $f_{S_1}$  using  $S_1$  and evaluate it on  $S_2$  by estimating  $\hat{E}[\phi_{f_{S_1}}(S_2)]$ . This is an estimate not of  $E_{\mathcal{P}}[\phi_f(S)]$ , but rather  $E_{\mathcal{P}_2}[\phi_f(S)]$ , where  $\mathcal{P}_2$  is the distribution for the sub-population with  $X = 2$  that  $S_2$  is a sample from. This estimate would only be recommended if the signature is intended for use in that specific subpopulation, and if that were the case, then it doesn't make much sense to develop the signature using the subpopulation  $\mathcal{P}_1$ . If these groups differed substantially, then we would not expect the signature to perform adequately. The differences in performance may depend on many factors, including how  $X$  is associated with the distribution of the features and the outcome. If a signature becomes broadly used in clinics, these center-to-center differences would be important to assess as a part of signature efficacy surveillance.

## Note

All analysis code and the source files for this manuscript is available from the authors' webpage.

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