Molecular Signatures from Gene Expression Data

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Abstract

Motivation “Molecular signatures” or “gene-expression signatures” are used to predict patients’ characteristics using data from coexpressed genes. Signatures can enhance understanding about biological mechanisms and have diagnostic use. However, available methods to search for signatures fail to address key requirements of signatures, especially the discovery of sets of tightly coexpressed genes.

Results After suggesting an operational definition of signature, we develop a method that fulfills these requirements, returning sets of tightly coexpressed genes with good predictive performance. This method can also identify when the data are inconsistent with the hypothesis of a few, stable, easily interpretable sets of coexpressed genes. Identification of molecular signatures in some widely used data sets is questionable under this simple model, which emphasizes the needed for further work on the operationalization of the biological model and the assessment of the stability of putative signatures.

Availability The code (R with C++) is available from http://www.ligarto.org/rdiaz/Software/Software.html under the GNU GPL.

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Supplementary information http://ligarto.org/rdiaz/Papers/signatures-supl.mat.pdf

1 Introduction

“Molecular signatures” or “gene-expression signatures” are a key feature in many studies that use microarray data in cancer research (e.g., Alizadeh et al. 2000, Golub et al. 1999, Pomeroy et al. 2002, Rosenwald et al. 2002, Shipp et al. 2002, Shaffer et al. 2001, p. 375) refer to signatures as “(...) genes that are coordinately expressed in samples related by some identifiable criterion such as cell type, differentiation state, or signaling response” (emphasis is ours). Molecular signatures are often used to model patients’ clinically relevant information (e.g., prognosis, survival time, etc) as a function of the gene expression data, but instead of using individual genes as predictors, the predictors are the signature components or “metagenes”.

If we are successful searching for a signature, then we will be able to model, for instance, the probability of developing a metastasis as a function of a few signature components or metagenes where each signature component is made of genes that show strong coexpression. Thus, molecular or gene expression signatures can be important both for diagnostic purposes and for providing information about the biological mechanisms underlying certain conditions by highlighting genes that both coexpress and are related to that condition.

In spite of the widespread use of the term “molecular signature”, no explicit definition is available. Following the conventions of the literature (e.g., Alizadeh et al. 2000, Golub et al. 1999, Pomeroy et al. 2002, Rosenwald et al. 2002, Shipp et al. 2002, West et al. 2001), and building upon the definition above (Shaffer et al. 2001, p. 375), we will consider a signature to be composed of one or more signature components or metagenes, where each signature component is a weighted combination of one or more coexpressed genes, and such that statistical models that use signatures both have good predictive performance and are easy to interpret biologically. Interpretation is made easier because the prediction is based on signature components that are weighted averages of subsets of tightly coexpressed genes, which can help when attempting to relate specific biological features to, for example, particular alterations on a metabolic pathway. Based upon the above references, we can try to formalize these goals by requiring that signatures and signature components satisfy the conditions shown in Figure 1

The conditions in Figure 1 reflect a very specific biological model. Our objective is to develop a statistical method appropriate for this biological model. By using a method that tries to fulfill these conditions we can also provide evidence that, for any particular case, the underlying biological assumptions behind this attempt are inconsistent with the data or, in other words, the the assumptions embodied in Figure 1 are inappropriate. As will be discussed later, our method is an attempt to map a particular biological model into a statistical method, but other statistical approaches would be more appropriate if more complex biological models are regarded as appropriate.

1.1 Limitations of alternative methods

A variety of approaches have been used to identify molecular signatures. A review is provided in the supplementary material. Briefly, most methods return either a single signature component (e.g., Golub et al. 1999, Hedenfalk et al. 2001, van Belle 2002) which is a weighted average of a set of genes, or several signature components (e.g., Antoniadis et al. 2003, Hastie et al. 2001a, Huang et al. 2003b, Ramaswamy et al. 2001).
which are often obtained using dimension reduction techniques (e.g., principal component analysis [PCA], partial least squares [PLS], sufficient dimension reduction) either on the complete set of genes or on a preselected subset.

The most common problems of available methods are:

- Genes within signature components do not necessarily show tight coexpression: no method makes tight coexpression a requirement to be fulfilled.
- The interpretation of components is very difficult for most methods that use PCA or PLS, since all the genes to which PCA or PLS is applied have loadings on each component.
- The search for components in many PCA or clustering of genes methods is carried out without incorporating information from the dependent variable.
- Most methods are designed for a specific type of task (e.g., classification or survival, but not both) and would be difficult to extend to other types of dependent variables.

Our objective in this paper is to propose a method that overcomes these problems. Specifically, our method returns signature components of tight coexpression (and thus, signature components that should ease interpretation) with good predictive performance. Although in this paper we focus on class prediction, our method can be used with different types of dependent data (continuous, categorical, survival), and thus sets up a general framework for finding gene expression signatures regardless of the type of dependent variable.

Based on the proposed operational definition of signatures (see figure 1), we first discuss the key elements of our proposed method. Next, we evaluate its predictive performance and finally with discuss it relation with other methods and problems of biological interpretability. Further details about the algorithm, evaluation of recovery of signatures, and a longer review of alternative approaches are provided in the supplementary material.

2 Methods

2.1 Key elements of the proposed method

Our objective is to directly fulfill the conditions in figure 1. We start our search with a seed gene that will be the skeleton of a signature component; this first signature component is found so that genes within the component show tight coexpression and the prediction error is acceptable. We repeat this process (find seed gene for a component and then obtain the whole component) greedily, until no further components are needed. The main steps of the algorithm are shown in Figure 2. In this section we explain how the conditions in figure 1 can be fulfilled and provide a geometrical interpretation of the algorithm.

2.1.1 Fulfilling signature requirements

A common and simple way of characterizing a signature component is to use linear combinations (weighted averages) of the genes that belong to that signature component (e.g., Ramaswamy et al. 2002, Rosenwald et al. 2002, Shipp et al. 2002, West et al. 2001). Although we could characterize a signature component using several different linear combinations of the genes of that component, most methods (but see Liu et al. 2002) characterize a signature component or metagene using only one linear combination. A single metagene per signature component simplifies interpretation, and is implicit in the requirement that each gene of a signature component should show a strong correlation with the signature component.

Thus, to fulfill requirement one in figure 1 we can use Principal Component Analysis (PCA — which is closely related to Singular Value Decomposition [SVD]). PCA yields “the best” representation (or “least distorting” representation, in the least squares sense) of the original data (e.g., Jolliffe 2002, Krzanowski 1998, Morrison 1990) in a subspace of reduced dimensions. The first PC is the best 1-dimensional representation of the original genes of this signature component. If the genes of the signature component are tightly coexpressed, then each of these genes should show a high correlation with the signature component, as we required above (this will also mean that the percentage of variance in the original gene expression data explained by this first PC will be high — see supplementary material). After running the procedure, each signature component will be made of tightly coexpressed genes (we require that all the genes in a component show a correlation larger than a pre-specified threshold of $r_{\min}$.)
In contrast to some previous methods which use PCA or PLS over the complete set of genes, there is no need for our method to return components which are uncorrelated or orthogonal: there is no biological argument that requires that signature components be orthogonal, uncorrelated, or independent (see discussion). For ease of interpretation we will additionally require that no gene belongs to more than one signature component. (In other words, each gene in the original data matrix belongs to either one, and only one, signature component or to none.)

The second and third requirements of figure 1 can be incorporated by adding new signature components only if they result in a relevant reduction of prediction error, and retaining genes in a signature unless they produce large increases in prediction error. In other words, we will penalize adding signature components, but will try to obtain large signature components. The reason is that the search for molecular signatures is often pursued to provide biological insights into coexpressed genes related to conditions and, therefore, minimization of prediction error is not the only goal. Thus, if there are potential trade-offs between prediction error and “biologically interpretable signatures”, the researcher should have the option of modifying the terms of this trade-off flexibly.

2.1.2 Searching for signature components and a geometrical interpretation

Our objective is, thus, to maximize predictive performance using signature components that satisfy that the correlation of each gene in a signature component with the signature component is larger than a given threshold. However, the discussion so far does not indicate how to find the signature components and, given the dimensionality of the problem, an exhaustive search for the optimal solution is not possible. Since we require that each component be highly correlated with the genes of that component, we can start the search with genes that have good predictive abilities on their own. Once we find an initial “seed gene”, we build an initial candidate signature component by including all “promising genes” (e.g., all those with a minimum correlation with the seed gene), and later reduce the signature component eliminating genes until the conditions of minimum correlation with 1st PC (all genes have a correlation with the 1st PC > r_{min}) and predictive performance are met. (If this elimination eliminates all genes except the seed gene, then, of course, the two requirements are met).

The method proposed here is a heuristic search that has an intuitive geometrical interpretation. We require that each component be highly correlated (> r_{min}) with the genes in the component, which is equivalent to saying that the vector of the component must have a similar direction as the vectors of each gene in variable space (the space where subjects are the axes). Therefore, no matter which genes belong to a signature component, the component will have a similar direction as any of its genes. Then, it seems reasonable to start the search with the direction that has the best predictive ability, the seed gene: this seed gene is the single direction in space that most contributes to separation of the groups in a classification problem; analogous for regression or survival analysis. When we form the complete signature component, all other genes of the signature component have directions that are similar to that of the seed gene. Together, all the genes of a signature component move the direction slightly, but this shift is possibly towards directions that contribute more to separation of groups (or that at least do not degrade the separation too much) and never moves us far away from the original seed gene. This process is repeated until the addition of new signature components does not achieve any relevant decrease in prediction rate, or until a maximum pre-specified number of signature components is reached. The algorithm is shown in figure 2. Further details are given in the supplementary material.

2.1.3 Choice of underlying classifier

In this paper we will be dealing with a classification problem. Each signature component is used as a predictor variable for a classifier. Of the available classification methods, we have used DLDA (diagonal linear discriminant analysis), a version of linear discriminant analysis which assumes the same diagonal variance-covariance matrix for all the classes [Dudoit et al. 2002], and NN (k-nearest neighbor, with k = 1), a simple non-parametric rule that assigns a test sample to the class of the closest training sample (where closeness is measured using Euclidean distance in the space whose dimensions are the signature components). KNN and DLDA have been repeatedly shown to perform as well as, or better than, many competing methods with microarray data [Dudoit et al. 2002, Romualdi et al. 2003]. In addition, DLDA and KNN are simple to implement and interpret. Dudoit et al. (2002) used an adaptive procedure to estimate the optimal number of neighbors to use with KNN: that can be time consuming, and we have fixed K = 1, since this is often a successful rule [Hastie et al. 2001; Ripley 1996]. As discussed in the supplementary material, other classifiers can be used.

3 Comparing predictive performance with established methods

Here we compare the predictive performance of our method with that of three well established methods, support vector machines, KNN, and DLDA, using several “real data” sets. The supplementary material reports simulation
studies that show that the suggested method can indeed recover signatures when they are present in the data.

Predictive performance is evaluated using 10-fold cross-validation (i.e., the complete algorithm shown in Figure 2) is applied to each of the 10 “training sets”). This 10-fold cross-validation was repeated 20 times under each condition. The error rates shown are not the CV error rates obtained in steps 1 and 5 of the signature algorithm (see Figure 2), since those are biased down (see supplementary material); the error rates shown are the error rates obtained from cross-validating the complete procedure.

Because an important parameter of our method might be \( r_{min} \), the minimal absolute correlation between each gene in a signature component and the signature component, we have evaluated the performance of the signature method using a set of values of \( r_{min} \) that covers a “biologically interesting” range: \([0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95]\). In addition, we have also examined the differences between using \( c_1 = c_2 = 1 \) compared to \( c_1 = c_2 = 0 \); the first corresponds to the usual “1 se rule” and should lead to more interpretable results (\( c_1 \) and \( c_2 \) are related to how much we penalize adding a new signature component and how much we penalize eliminating genes from signature components; see details in supplementary material).

### 3.1 The data sets

**Leukemia dataset** From [Golub et al. 1999](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt). The original data, from an Affymetrix chip, comprises 6817 genes, but after filtering as done by the authors we are left with 3051 genes. Filtering and preprocessing is described in the original paper and in [Dudoit et al. 2002](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt). We used the training data set of 38 cases (27 ALL and 11 AML) in the original paper (the observations in the “test set” are from a different lab and were collected at different times). This data set is available from [http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt) and also from the Bioconductor package milttest [http://www.bioconductor.org](http://www.bioconductor.org).

**Adenocarcinoma dataset** From [Ramaswamy et al. 2003](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt). We used the data from the 12 metastatic tumors and 64 primary tumors. The original data set included 16063 genes from Affymetrix chips. The data (DatasetA_Tum_vsMet.res), downloaded from [http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt](http://www-genome.wi.mit.edu/cgi/bin/cancer/datasets.cdt), had already been rescaled by the authors. We took the subset of 9376 genes according to the UniGene mapping, thresholded the data, and filtered by variation as explained by the authors. The final data set contains 9868 clones (several genes were represented by more than one clone); of these, 196 had constant values over all individuals.

**NCI 60 dataset** From [Ross et al. 2000](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt). The data, from cDNA arrays, can be obtained from [http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt), is the one in the file “figure3.cdt”. As in [Dettling & Bühlmann 2003](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt); [Dudoit et al. 2002](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt), we filtered out genes with more than two missing observations and we also eliminated, because of small sample size, the two prostate cell line observations and the unknown observation. After filtering, we were left with a 61 x 5244 matrix, corresponding to eight different tumor types (note that, as done by previous authors, we did not average the two observations with triplicate hybridizations). As in [Dudoit et al. 2002](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt) we used 5-nearest neighbor imputation of missing data using the program GEPAS [Herrero et al. 2003](http://gepas.bioinfo.cnio.es/cgi-bin/preprocess); unlike [Dudoit et al. 2002](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt), however, we measured gene similarity using Euclidean distance from the genes with complete data, instead of correlation: [Troyanskaya et al. 2001](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt) found Euclidean distance to be an appropriate metric. Finally, as in [Dudoit et al. 2002](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt), p. 82) gene expression data were standardized so that arrays had mean 0 and variance 1 across variables (genes).

**Breast cancer dataset** From [van Belle 2002](http://www-genome.wi.mit.edu/cgi-bin/cancer/datasets.cdt). The data, from Affymetrix arrays, were downloaded from [http://www-rii.com/publications/2002/vantveer.htm](http://www-rii.com/publications/2002/vantveer.htm) (we used the files ArrayData_less_than_5yr.zip, ArrayData_greater_than_5yr.zip, ArrayData_BRCA1.zip, corresponding to 34 patients that developed distant metastases within 5 years, 44 that remained disease-free for over 5 years, and 18 with BRCA1 germline mutations and 2 with BRCA2 mutations). As did by the authors, we selected only the genes that were “significantly regulated” (see their definition in the paper and supplementary material), which resulted in a total of 4869 clones. Because of the small sample size, we excluded the 2 patients with the BRCA2 mutation. We used 5-nearest neighbor imputation for the missing data, as for the NCI 60 data set. Finally, we excluded from the analyses the 10th subject from the set that developed metastases in less than 5 years (sample 54, IRI000045837, in the original data files), because it had 10896 missing values out of the original 24481 clones, and was an outstanding outlying point both before and after imputation. The breast cancer dataset was used both for two class comparison (those that developed metastases within 5 years vs. those that remain metastases free after 5 years) and for three group comparisons.

Therefore, we use three datasets in which the problem is classification into two classes (leukemia, adenocarcinoma, breast cancer), one dataset with a three class problem (breast cancer) and one dataset with an eight class problem.
3.2 The competing methods

We have used three methods that have shown good performance in reviews of classification methods with microarray data (Dudoit et al., 2002; Romualdi et al., 2004).

**Diagonal Linear Discriminant Analysis (DLDA)** DLDA is the maximum likelihood discriminant rule, for multivariate normal class densities, when the class densities have the same diagonal variance-covariance matrix (i.e., variables are uncorrelated, and for each variable, its variance is the same in all classes). This yields a simple linear rule, where a sample is assigned to the class \( k \) which minimizes \( \sum_{j=1}^{p} (x_j - \bar{x}_{kj})^2 / \hat{\sigma}_j^2 \), where \( p \) is the number of variables, \( x_j \) is the value on variable (gene) \( j \) of the test sample, \( \bar{x}_{kj} \) is the sample mean of class \( k \) and variable (gene) \( j \), and \( \hat{\sigma}_j^2 \) is the (pooled) estimate of the variance of gene \( j \) (Dudoit et al., 2002). In spite of its simplicity and its somewhat unrealistic assumptions (independent multivariate normal class densities), this method has been found to work very well.

**K nearest neighbor (KNN)** KNN is a non-parametric classification method that predicts the sample of a test case as the majority vote among the \( k \) nearest neighbors of the test case (Hastie et al., 2001; Ripley, 1996). To decide on “nearest” here we use, as in Dudoit et al. (2002), the Euclidean distance. The number of neighbors used \( (k) \) is chosen by cross-validation as in Dudoit et al. (2002); for a given training set, the performance of the KNN for values of \( k \) in \( \{1, 3, 5, \ldots, 21\} \) is determined by cross-validation, and the \( k \) that produces the smallest error is used.

**Support Vector Machines (SVM)** SVM are becoming increasingly popular classifiers in many areas, including microarrays (Furey et al., 2000; Lee & Lee, 2003; Ramaswamy et al., 2001). SVM (with linear kernel, as used here) try to find an optimal separating hyperplane between the classes. When the classes are linearly separable, the hyperplane is located so that it has maximal margin (i.e., so that there is maximal distance between the hyperplane and the nearest point of any of the classes) which should lead to better performance on data not yet seen by the SVM. When the data are not separable, there is no separating hyperplane; in this case, we still try to maximize the margin but allow some classification errors subject to the constraint that the total error (distance from the hyperplane in the “wrong side”) is less than a constant. For problems involving more than two classes there are several possible approaches; the one used here is the “one-against-one” approach, as implemented in libsvm Chang & Lin (2003). Reviews and introductions to SVM can be found in Burgues (1998); Hastie et al. (2001).

For each of these three methods we need to decide which of the genes will be used to build the predictor. Based on the results of Dudoit et al. (2002), we have used the 200 genes with the largest \( F \)-ratio of between to within groups sums of squares. Dudoit et al. (2002) found that, for the methods they considered, 200 genes as predictors tended to perform as well as, or better than, smaller numbers (30, 40, 50 depending on data set).

We evaluated predictive performance using 10-fold cross-validation; the results shown are from 20 replications of the 10-fold cv process. In all cases, cross-validation includes gene selection (Ambroise & McLachlan, 2002; Simon et al., 2003); in other words, for the three competing methods and the signature algorithm the selection of genes is carried out within each of the 10 “training sets” of the cross-validation. Thus, we insure that the subjects for which prediction is performed have not been used for the gene selection process.

4 Results

Predictive performance is shown in Figure 3. Predictive performance changes very little from using DLDA vs. NN, or setting \( c_1 = c_2 = 1 \) vs. \( c_1 = c_2 = 0 \); figures for four combinations of classifier and values of \( c_1, c_2 \) are shown in the supplementary material. Comparing \( c_1 = c_2 = 1 \) with \( c_1 = c_2 = 0 \) (see Figures in supplementary material) does not show any relevant differences in predictive performance; of course, there are differences in the outcome because, not surprisingly, using \( c_1 = c_2 = 0 \) tends to result in more signature components of smaller numbers of genes per component, and higher correlations between components.

[Figure 3 about here]

[Table 1 about here]

With respect to \( r_{\text{min}} \), except for the NCI data set, and slightly for the Breast cancer with 3 classes data set, changes in \( r_{\text{min}} \) have little effects on predictive performance. In Table 1 we show the median number of
components, median total number of genes in a signature, and median average number of genes per component obtained in 200 bootstrap runs using $c_1 = c_2 = 1$ with $r_{\text{min}} = 0.85$ and $r_{\text{min}} = 0.6$, and using NN as the classifier (see also section 4.1). It can be seen that changes in $r_{\text{min}}$ do affect the outcome in terms of number of number of genes per component. These results seem to indicate that choice of $r_{\text{min}}$ can probably be guided more by interpretability concerns (whether we want larger signature components of looser coexpression or smaller signature components of tight coexpression) than by concerns over predictive ability.

Finally, the performance of the signature method is only slightly worse than that of the three competing classifiers, except for the NCI data set. As seen in Table 1, most of the signatures, specially with signature component of tight coexpression) than by concerns over predictive ability. by interpretability concerns (whether we want larger signature components of looser coexpression or smaller classifier (see also section 4.1). It can be seen that changes in $r_{\text{min}}$ do affect the outcome in terms of number of number of genes per component. These results seem to indicate that choice of $r_{\text{min}}$ can probably be guided more by interpretability concerns (whether we want larger signature components of looser coexpression or smaller signature components of tight coexpression) than by concerns over predictive ability.

4.1 Stability of results

To evaluate the stability of results, we rerun the complete procedure on all data sets using the bootstrap (Davison & Hinkley, 1997; Efron & Tibshirani, 1993) with 200 bootstrap samples, similar to what Efron & Gong (1983) do to evaluate a complex fitting procedure. We run the procedure for settings of $c_1 = c_2 = 1$ with $r_{\text{min}} = 0.85$ and $r_{\text{min}} = 0.6$, and using NN as the classifier. The results are shown in Table 2. The bootstrap results indicate that, when using a high value of $r_{\text{min}}$ we rarely obtain similar solutions repeatedly; some data sets, however, seem to yield more stable solutions (e.g., Leukemia data sets) and, not surprisingly, if the $r_{\text{min}}$ criterion is set to less stringent values, results tend to be more repeatable.

5 Discussion

5.1 Similarities and differences with other methods

Our method is unique because it simultaneously searches for sets of genes that are tightly coexpressed and will lead to good predictive performance. The search for the sets of genes is carried out using the information from the dependent variable (at the first stage — when selecting the seed gene —, and at the pruning stage of reducing the signature component — when genes that lead to decreased predictive performance are eliminated from the signature component —; see figure 2).

One important difference between our proposed method and most previous approaches that use PCA is that, by performing PCA only on subsets of genes, our method returns signature components where genes show tight coexpression. Because returned components are not orthogonal, and simple components are an explicit goal, our approach is actually closer to some ideas implemented in SAS’s PROC VARCLUS, which is similar to factor analysis with oblique rotation and can be used to obtain clusters of variables, of relatively simple interpretation, to be further used in model building (Nelson, 2001; SAS Institute, 1999; see also Harrell, 2001).

Using PCA on subsets of genes, instead of the complete set of genes is crucial because it makes interpretation easier and allows for subsets of tight coexpression. Simple PCA and related methods (Jolliffe, 2002; Rousson & Gasser, 2003; Vines, 2000) as well as SAS PROC VARCLUS also try to achieve components of tight coexpression, but many of these approaches cannot be applied with $p \gg n$, and all of them carry out the PCA without using the information from the dependent variable, which in our case is a fundamental requirement since the sets of genes with tightest coexpression would be irrelevant for our purposes if they are not related to the dependent variable we are trying to model. This difference in objectives is also evident because our aim is not to explain the most variance in the genes (as in most simple PCA approaches) nor maximize variable explained across all clusters (such as in PROC VARCLUS). Overall summarization of information is not important for our problem, because we are interested in prediction, and we often have the suspicion that most of the genes in the array are not related to the outcome variable. Finally, our approach can result in signature components which are correlated (sometimes strongly), but this is not inherently a problem because there is no biological reason to suggest that the underlying biological causes or factors ought to be independent or uncorrelated; moreover, if the true underlying causes are not orthogonal, using a method such as PCA can lead to interpretational
and conceptual difficulties because each biological cause will be spread over several orthogonal components (Houle et al., 2002): non-orthogonal biological causes are inconsistent with procedures such as PCA and PLS.

Bayesian classification trees using the 1st PC from gene clusters (Huang et al., 2003a) and block PCA (Liu et al., 2002) also used PCA on subsets of genes, instead of the complete set of genes. In both cases their subsets of genes were obtained using criteria that did not make any use of the information from the dependent variable. In addition, in Huang et al. (2003a) the metagenes are not necessarily of subsets of tightly coexpressed genes. In Liu et al. (2002) there is an explicit criterion of % variance accounted for, but often the number of components used to summarize a subset of genes is too large to allow for easy interpretation (11 to 16 principal components).

Supervised harvesting of expression genes (Hastie et al., 2004) also works with clusters of subsets of genes, which are then used in a predictive model. As before, however, the clustering is carried out without using information from the dependent variable; even if the selection of which subsets or clusters to use in the model uses the information from the dependent variable, the very first step of clustering genes does not, and can therefore be unable to recover sets of tightly coexpressed genes that are good predictors. Finally, the “Wilma” and “Pelora” methods (Dettling & Bühlmann, 2002, 2004) do use the information from the dependent variable in the formation of clusters of genes; however, there is no explicit objective of achieving tight gene coexpression within clusters and thus, how tightly coexpressed genes are in each metagene cannot be specified in advance; in addition, Wilma weights each gene equally within a cluster (only possible weights are +1 and -1) whereas we use PCA on unscaled genes, thus allowing genes to play a different role in the specification of the direction of the signature component (genes with larger among-subject variance play a more important role in determining direction).

The rest of the alternative methods differ strongly from our proposed method, either because they do not return subsets of genes but components with loadings from all genes (e.g., PLS based methods), or return subsets of genes where there is not requirement of tight coexpression (e.g., weighted gene voting, Golub et al., 1999).

After gene selection and dimension reduction (i.e., the use of only the 1st PC, that collapses all the information from the genes of a signature component onto one dimension), the predictive model of our choice is fitted. In this sense, the method as presented here is “just” a DLDA or NN that uses signature components instead of genes as the predictors. The choice of DLDA and NN was made based on published results that showed their excellent performance with microarray data. In particular, Dudoit et al. (2002) showed that other forms of discriminant analysis tended to perform much worse because of the small ratio sample size/parameters needed to estimate covariances and different variances per group; however, since in most cases our method returns just a few signature components, other types of discriminant analysis that use the information from the covariance of the predictors (e.g., linear discriminant analysis and quadratic discriminant analysis) might prove useful.

5.2 Coexpression: across-group and within-group

The algorithm can include in a signature component genes that show no correlation within groups but that show correlation among groups because they are far apart in the multidimensional space, and the correlation coefficient is computed across the whole, pooled, sample. The algorithm might even include in the same signature component genes that have very different patterns of correlation in different groups, if they still show sufficiently strong correlation over the pooled sample.

To our knowledge, this issue has not been explicitly addressed in any other approach to the signature problem (see reviews in supplementary material). However, probably the most biologically relevant components are those where there is strong correlation within groups, because this is a more reliable indicator of coordinated expression.

A possible solution is, for example, to only accept results for a signature component if a principal component analysis over the pooled sample after centering the data with respect to the group means yields a relevant first eigenvalue; for added robustness, we might want to use the trimmed mean. This approach, however, does not directly address if there are different multivariate orientations in different groups of subjects, and how these orientations within-group relate to the across-group orientation. In particular, the case where several groups not only have the same first principal component, but are lined up along a common axis, known as “allometric extension” (Bartoletti et al., 1999; Hills, 1982), might constitute the most natural type of signature component.

Krzanowski (see reviews and summary in Jolliffe, 2002; Krzanowski, 1998) has proposed a method to directly compare the subspaces defined by the principal components of each of the groups. In our case, as we only use the first principal component of a set of genes to define a signature component, for each signature component we can compare the first principal component of each group. An example using the NCI 60 data set is shown in the supplementary material. This method only compares the orientation of the principal components (the eigenvectors) but does not compare the location of the multivariate means. The EDDA (Bensmail & Celeux, 1998) and common principal components (Flury, 1988) approaches provide frameworks to examine differences
5.3 Stability and biological relevance of signatures

Our method follows from an operationalization of signatures and signature components (figure 1). If the ideas embodied in figure 1 have empirical support, then it might be possible to build good predictive models using just a few, very interpretable, signature components strongly correlated with the expression of a few key genes are associated to the clinical outcome of interest. In the context of building predictors from gene expression data, Somorjai et al. (2003) have emphasized that this non-uniqueness leads to interpretational difficulties and should make researchers skeptical about the biological relevance of any set of predictors; moreover, they explain how this non-uniqueness can arise from dataset sparsity. Of course, both of these issues are relevant to the present proposed methodology. None of the data sets examined in this paper yield stable signatures when we use stringent criteria of gene coexpression (see 4.1 and Table 2 with \( r_{\min} = 0.85 \)), although some of the data sets are somewhat stable from run to run when the \( r_{\min} \) is set to small values (but other data sets show signatures that vary widely from run to run). These results add to the above references in the sense that biological interpretation should be carried out very cautiously, and emphasize the difference between attempting to build good predictors and attempting interpretation (see also Breiman, 2001). More relevant to the current work, the present results indicate that simple models of molecular signatures warrant further critical scrutiny, and that it might be extremely hard to identify molecular signatures from such sparse data sets (see Rhodes et al., 2004). We must recognize that these results are preliminary for two main reasons. First, establishing that two or more signature components are different probably requires additional information besides the identities of the genes; for instance, information from Gene Ontology, or known participation on certain regulatory networks. Second, we have used two different \( r_{\min} \) thresholds, but it is unclear what constitute “biologically reasonable” patterns of covariation between genes that are to belong to the same signature component. Nevertheless, even if preliminary, these results emphasize the need for further work in the operationalization and explicit definition of what we mean by molecular signatures, careful consideration of the stability of results, and critical assessment of the sample sizes need to reliably identify molecular signatures.

5.4 Alternative statistical methods for alternative biological models

As mentioned in the introduction, we started by trying to clarify, conceptually, what is often understood by molecular signature (see Figure 1), and then devised a statistical method to fulfill those requirements. The biological model underlying the suggested method is one where most of the genes are not relevant for prediction, relevant genes are involved in one and only one signature component (i.e., non-overlapping signature components), and the signature components are common, and have similar covariance matrices, in different groups. However, other biological models are plausible, and for those biological models other statistical methods would be more appropriate. The simultaneous clustering and classification approach in Jörnsten & Ylil (2003) could be extended by placing restrictions on the covariance matrix (i.e., require a minimum correlation between genes) but possibly allowing for different covariance matrices among groups; thus, we could address directly issues of different across vs. within-group correlations (see section 5.2), within a formal inferential framework. Biological models where signature components are not common and/or do not behave similarly in different groups could be investigated using modifications of the Plaid model of Lazzeroni & Owen (2002) (see also Turner et al., 2004). In addition, genes with the highest correlation need not be the best candidates for being in the same biological pathway; activity in a pathway might just require that precursor genes get activated, but once a threshold is reached, it might not be very important by how much the threshold is exceeded. This
type of behavior could preclude strong correlation between genes that belong to the same pathway. This can be modelled building upon the latent class methods of Parmigiani and colleagues (Garrett & Parmigiani, 2003; Parmigiani et al., 2002; Scharpf et al., 2003), where signature components are based on under-, over- or baseline expression (instead of expression levels). Work along these lines is currently in progress in our group.

5.5 “Just” dimensional reduction?

Even if the current method fails to identify stable features that can be associated to molecular signatures, it can be a useful dimension reduction tool. Difficulties associated with a simple mapping of the returned “signature components” to pathways, and problems derived from instability of the found components also affect any other of the existing alternative methods. Thus, in the presence of instability of results, it is more appropriate to regard this method as dimension reduction tool that could lead to simple biological interpretation. The simple biological interpretation could be helped not only because of the coexpression of the genes that make a signature component, but also because the dimension reduction performed is quite remarkable compared to other methods (the number of signature components and genes returned is very small for the five data sets examined; see Results) with, at most, only a slight decrease in predictive performance. Moreover, the user can control the relative trade-offs between predictive performance and potential interpretability of results (e.g., coexpression of sets of genes) by changing the $r_{\text{min}}$ parameter (note that if $r_{\text{min}} = 1$ the method becomes essentially either DLDA or NN with forward addition of genes to the model). This flexible modification of the trade-offs between prediction error and interpretability is of great importance in methods that are largely exploratory and oriented towards providing “biologically interpretable” output; in other words, methods for which minimization of prediction error should not be the only goal.

6 Conclusions

The most common methods for finding signatures present several deficiencies that do not allow them to return signatures and signature components that fulfill basic biological requirements. After suggesting an operational definition of signature and signature components, we have developed a method that follows directly from what are often considered as the biologically relevant signature characteristics. The method developed returns signature components of tightly coexpressed genes and thus can facilitate biological interpretation. In this paper we have applied the method to classification problems, but this approach in fact sets up a framework that allows us to find signatures regardless of the type of dependent variable. Extension to use other classifiers is straightforward and it should also be easy to incorporate other types of dependent variables to allow, for example, survival analysis. We have also shown that the predictive performance of our method is comparable to that of state of the art methods. Finally, our method not only could facilitate mapping pathological alterations to a few, tightly coexpressed sets of genes, but can also provide evidence that the underlying biological assumptions behind this attempt are inconsistent with the data. In the five data sets analyzed, our results suggest that identification of molecular signatures is questionable under this simple model. These results emphasize the needed for further work on the operationalization of the biological model and the necessity of critical assessment of the stability of putative signatures.

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| Data set                  | Total genes $r_{\text{min}} = 0.85$ | # Components | Mean Genes/Component | Total genes $r_{\text{min}} = 0.6$ | # Components | Mean Genes/Component |
|--------------------------|--------------------------------------|--------------|----------------------|--------------------------------------|--------------|----------------------|
| Leukemia                 | 6                                    | 1            | 5                    | 52.5                                 | 1            | 50                   |
| Breast cancer (2 classes)| 2                                    | 2            | 1                    | 10                                   | 2            | 3.875                |
| Breast cancer (3 classes)| 6                                    | 2            | 2.5                  | 63.5                                 | 2            | 33.25                |
| Adenocarcinoma           | 5                                    | 1            | 3                    | 45.5                                 | 1            | 31.5                 |
| NCI 60                   | 4                                    | 2            | 1.67                 | 16                                   | 2            | 7.1                  |

Table 1: Median values from 200 bootstrap runs for total number of genes in signatures, number of signature components and average number of genes per component.
| Data set                  | \( r_{min} = 0.85 \) | \( r_{min} = 0.6 \) |
|--------------------------|-----------------------|----------------------|
|                          | Genes present         | Genes present        |
|                          | at least % runs       | at least % runs      |
|                          | 50 20 10              | 50 20 10             |
| Leukemia                 | 0 7 15                | 7 80 179             |
| Breast cancer (2 classes)| 0 0 9                 | 0 0 43               |
| Breast cancer (3 classes)| 0 0 3                 | 0 51 246             |
| Adenocarcinoma           | 0 0 3                 | 0 45 270             |
| NCI 60                   | 0 0 0                 | 0 0 6                |

Table 2: Stability of results using the bootstrap, with 200 bootstrap iterations. Values shown are the number of genes that are returned, as members of a signature component, in at least those many bootstrap runs.
1. Genes of a signature component should show tight coexpression. We can make this more explicit by requiring that each gene of a signature component should show a strong correlation with the signature component.

2. For a given classification/prediction problem only a few signature components should be needed to obtain reasonable predictive performance.

3. Signature components could have many genes; additionally, it often seems more desirable to include a gene in a signature component even if it does not belong to that signature, than to exclude a gene that does belong to that signature.

4. The same genes are used for a signature component over all samples (i.e., the signature components are the same for all groups).

Figure 1: Requirements of signatures and signature components (see text for details).
1. Find the seed gene for a signature component:
   (a) Seed gene is gene with smallest cross-validated (CV) prediction error among available genes. (The CV prediction error is obtained using as predictive model the chosen predictor [e.g., DLDA], including all previous signatures, if any).
   (b) If CV prediction error < (CV prediction error of the previous signature - $c_1$ standard error), continue; otherwise, terminate signature finding.

2. If signature component = 1, eliminate all genes with (resubstitution) prediction error > prediction error from always betting on the most frequent class.

3. Build an initial signature with all the genes $j$ where $\text{abs}(\text{cor}(\text{gene}_j, \text{seed.gene})) \geq r_{seed}$.

4. Obtain the signature component as the 1st PC of a PCA on the initial signature.

5. Reduce signature component:
   (a) Eliminating, one by one, from the signature the gene with the smallest absolute correlation with the seed gene, until $\text{abs}(\text{correlation}(\mathbf{x}_{pr_i,j}, \mathbf{pr}_i)) > r_{min}$ is met.
   (b) Eliminate, one by one, any gene for which its exclusion from the signature component leads to a CV prediction error < last prediction error - $c_2$ s.e.(prediction error).

6. Exclude from further consideration all genes that belong to the signature component just build.

7. Return to 1. until no further components are needed.

Figure 2: Basic steps of the signature algorithm.
Figure 3: Predictive performance, as a function of $r_{\text{min}}$, of the signature method using NN as classifier and comparison with SVM, KNN, and DLDA. Figures based on 20 replicates of the 10-fold-CV procedure. Results for $c_1 = c_2 = 1$. 