Testing for differentially expressed genetic pathways with single-subject N-of-1 data in the presence of inter-gene correlation

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Abstract

Modern precision medicine increasingly relies on molecular data analytics, wherein development of interpretable single-subject ("N-of-1") signals is a challenging goal. A previously developed global framework, N-of-1-*pathways*, employs single-subject gene expression data to identify differentially expressed gene set pathways (DEPs) in an individual patient. Unfortunately, the limited amount of data within the single-subject, N-of-1 setting makes construction of suitable statistical inferences for identifying DEPs difficult, especially when non-trivial inter-gene correlation is present. We propose a method that exploits external information of gene expression correlations to cluster positively co-expressed genes within pathways, then assesses differential expression across the clusters within a pathway. The clustering can persuasively uncover the genetic patterns of association within pathways. A simulation study illustrates that the cluster-based approach exhibits satisfactory false-positive error control and reasonable power to detect DEPs. An example with a single N-of-1 patient's triple negative breast cancer data illustrates use of the methodology.

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1 Introduction

1.1 Background

Modern precision medicine increasingly relies on molecular data analytics, where standard approaches accumulate large amounts of molecular data from multiple patients.[1;2] Within this context, an experiential paradigm that has garnered recent attention is development of interpretable single-subject signals to truly focus on the individual patient.[3;4] A novel approach to generating single-subject information is known as the *N-of-1* trial[5], where the individual patient is the sole source/unit of observation and any statistical descriptions and inferences are intended to relate only to that patient.

While the broad N-of-1 strategy can possess obvious impact for advancing personalized medicine, in certain settings the approach can exhibit greatly expanded capabilities. Consider, e.g., a molecular N-of-1 analysis where data on a gene's messenger RNA (mRNA) expression are sampled from a patient's diseased organ or tissue. Rather than study expression levels across the patient's entire transcriptome *en masse* we previously proposed a framework, called N-of-1-*pathways*, that applies the N-of-1 strategy to pertinent gene pathways, i.e., to preassigned collections of gene sets within which the genes are assumed to have associated mechanisms or functions.[6–8] Gene set (pathway) membership is typically defined using curated knowledgebases; here, we employ the Gene Ontology (GO)[9] knowledgebase and its corresponding GO-Biological Processes (GO-BP) gene sets.

Our aim in[6–8] was to improve and enhance mechanism-anchored, single-subject, gene expression analysis. In practice, the tactic assumes that the mRNA expressions are collected across all pathways of interest from a single patient under two different paired conditions—e.g., baseline and case, unaffected tissue vs. tumor tissue, before and after treatment, etc. The goal is then to quantify and test for differential pathway expression for that patient using

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Table 1. Paired mRNA (log$_2$) expression data in pathway GO:0003071 (renal system process involved in regulation of systemic arterial blood pressure), from a breast cancer patient. See text for details.

Gene	Case/cancer expression	Normal tissue expression	Difference
CYP4A11	0.00	3.71	-3.71
AGTR1	6.13	7.86	-1.73
OR51E2	2.90	1.54	1.36
CYP11B2	0.00	0.00	0.00
PTPRO	3.72	6.22	-2.50
CYP4F2	0.00	0.40	-0.40
AGT	8.40	7.89	0.52
PCSK5	6.68	6.92	-0.25
PDGFB	8.82	9.56	-0.74
F2RL1	8.25	7.91	0.35
EMP2	12.54	12.50	0.04
GAS6	11.89	12.35	-0.47
CYP4F12	1.43	4.72	-3.29
F2R	9.15	9.77	-0.61
SERPINF2	6.38	9.57	-3.19

the data derived under the two paired transcriptomes from those different conditions. The framework represents a substantial opportunity for a clinically actionable and economically efficient approach to personalized medicine.[10]

1.2 Breast cancer example

To fix the concepts and also motivate our methodological development, consider the following example. Table 1 gives mRNA expression data from a female subject exhibiting triple negative breast cancer, or TNBC. (TNBC is often more difficult to treat than other breast cancers and thus demands a poorer prognosis; it also disproportionately affects minorities.[11] The data in Table 1 are from an African-American patient.) The table presents matched normal-tumor pairs of mRNA expression outcomes from a sample of the patient's healthy breast tissue (left column) and of her cancerous breast tissue (middle column) in a specific gene pathway, GO:0003071/renal system process involved in regulation of systemic arterial blood pressure, made up of 15 individual genes. The data were taken from a large collection of RNA-seq data sets obtained from the Cancer Genome Atlas (TCGA; see Acknowledgments). The collection yielded 20,051 mRNA counts, as mapped to HUGO gene symbols[12] for both normal and tumor samples derived from this patient. Following standard practice, a stabilizing transformation was applied by adding 1 to each normalized count and then taking a base-2 logarithm.

Of interest here is whether or not the paired observations in this pathway exhibit significant differential expression between normal and tumor tissues *in this single patient*. It is of further

interest to also repeat the calculations across the larger collection of all pathways under study. [A total of 3411 pathways are available with this patient's data; see Sec. 4.1 below. As noted above, we define the pathways using the Gene Ontology (GO)[9] knowledgebase via GO-Biological Processes/GO-BP gene sets.] By isolating significant, differentially expressed pathways (DEPs) for this patient, the N-of-1-*pathways* approach offers a glimpse into the individual, dysregulated, cellular mechanisms between her tumor and normal breast tissue control.

1.3   Identifying differentially expressed pathways (DEPs)

Generically, for this N-of-1-*pathways* setting we assume the matched-pair data are collected as expression levels from the gth gene ($g = 1, \ldots, G$) in some pre-specified pathway with G genes. Denote each gene's (log$_2$-transformed) expression under the baseline condition as B_g and its matched case expression as C_g. The paired differences become $D_g = C_g - B_g$, with sample mean $\overline{D}$ and standard error $s_{\overline{D}} = s_D/\sqrt{G}$, where s_D is the sample standard deviation of the D_gs.

The sample mean $\overline{D}$ of the difference in log-expression between case and baseline conditions is a natural statistic to use for quantifying any observed dysregulation in a given pathway. We have found that for sufficiently large pathways the sampling distribution of $\overline{D}$ presents as roughly symmetric and bell-shaped, consistent with standard central limit results on sums of random variables.[13] Thus one might consider testing for no difference between baseline and case conditions using the differences D_g and applying the usual paired, $G - 1$ d.f. *t*-test[14] via the test statistic $t = \overline{D}/(s_D/\sqrt{G})$. If questions exist regarding the *t*-test's reliance on normal (Gaussian) distribution sampling, appeal can be made instead to its well-established nonparametric analog, the Wilcoxon signed-rank test for two-sample matched-pairs data.[14] Applied over many different, preselected pathways, corrections for multiple false discoveries to these simple per-pathway inferences may also be included.[15]

Additional complexities occur with matched-pair RNA-seq N-of-1 data, however. It is important to recognize that expressions across genes within a given pathway are likely to be both heterogeneous and, more critically, correlated. When testing for differences between the two conditions, inter-gene heterogeneity may not detrimentally affect the null distribution of the test statistic; however, experience with cohort-based gene set testing has shown that inter-gene correlation is non-trivial, and should be accounted for in order to maintain nominal operating characteristics of any inferential procedure.[16;17] Essentially, both the naïve *t*-test and the signed-rank test assume independence among the pathway's gene expressions and as we will see below, in the presence of non-zero inter-gene correlation they generally fail to contain the test's false-positive error rate at nominal levels.

Indeed, an unanswered problem when testing for DEPs with single-patient N-of-1 data is how to incorporate non-zero inter-gene correlation(s) from a single N-of-1 sample of G gene expressions. Herein, we propose use of external information to identify clusters of correlated genes within a pathway, and then manipulate that information to build a correlation-adjusted, cluster-based test statistic for assessing differential N-of-1 expression in that pathway. (Mention of external information naturally leads one to consider some form of hierarchical Bayesian model. While we do not dismiss this possibility, it is not our goal in the methods we present below to employ a Bayesian approach. Our attention focuses instead on developing frequentist strategies for the DEP testing scenario.) We begin in Sec. 2 with a description of our model and clustering strategy, along with a suggested algorithm for its implementation. Sec. 3 follows with a short simulation study of the method's operating characteristics, with focus on its performance in the presence of inter-gene correlation. Sec. 4 illustrates the approach by returning to the TNBC data from Sec. 1.2, while Sec. 5 ends with a brief discussion. Note that all calculations we present below are performed in the **R** statistical programming environment. [18]

2   Methodological strategy and clustering algorithm

Our motivating goal is to identify differentially expressed pathways (DEPs) within a single "N-of-1" subject while recognizing that the genes' expressions within a pathway are likely correlated. We restrict attention in this section to a single target pathway, studied under two paired conditions—generically listed as "Case" and "Baseline"—as is typical in N-of-1-*pathways* applications. (Consideration of more than two conditions is certainly possible, but we have not seen any instances of such in practice.)

Our experience indicates that without some form of external information on the within-pathway correlation(s), it is difficult to develop a test of differential pathway expression for a single, N-of-1 sample. Incorporating external gene expression data in testing procedures for pathway dysregulation in a single sample is rare, but not unexplored. [19] To address the issue, we appeal to the growing amount of transcriptomic information being uploaded to modern data storehouses/knowledgebases, and in particular aim to extract from these sources relevant biological-context input to aid in the test's construction. Our approach has a bioinformatic flavor, and it attempts to turn the problem on its head: rather than allow the inter-gene correlations to stymie DEP assessment, we *use* the external correlation information to aggregate non-negatively correlated genes (assumed strictly co-expressed, not anti-expressed) into correlated clusters within a pathway. We propose the following strategy: (a) identify an existing knowledgebase from which all $\binom{G}{2}$ inter-gene correlations can be determined/calculated for the G genes within

our target gene set (pathway), (b) apply an existing clustering algorithm to group the G genes into clusters based on the external inter-gene correlation data, then (c) incorporate the derived cluster information into a test for differential expression. The latter effort is facilitated by a useful cluster-adjusted t-test given by Williams.[20] The following sub-sections provide details for each step.

2.1 Correlation-based clustering of genes within a pathway

To define gene clusters within a pathway, we devised a two-stage algorithm: (i) estimate inter-gene correlation via independent source(s), then (ii) conduct unsupervised clustering of those genes using the external correlations to define gene-gene similarity.

In the first stage, we identify biological context-relevant mRNA expression data from some external knowledgebase(s). Many different possibilities exist and users can choose any external database that suits their needs. Some caution may be necessary, however: many sources provide a wide selection of gene-pair correlations, derived from multiple inputs. When these are collected to define inter-gene correlations for the G genes within a specific pathway, the consequent correlation matrix need not be positive definite, as the original observations likely did not come from a single, coherent sample. This can lead to computational (and interpretational) problems. To avoid this concern, we recommend use where possible of knowledgebases that contain multiple gene-expression samples from independent individuals. We give an example of such in Sec. 4.2, below.

In general, suppose mRNA expression measurements from P independent patients are taken on the G genes under study. Form the corresponding matrix of ($\log_2$-transformed) gene expression data $\mathbf{X}_{G \times P} = \{x_{ih}\}$ and use it to compute the usual Pearson product-moment correlation coefficients

$$r_{ih} = \frac{1}{P-1} \sum_{\ell=1}^{P} \left(\frac{X_{i\ell} - \overline{X}_i}{s_i} \right) \left(\frac{X_{h\ell} - \overline{X}_h}{s_h} \right) \tag{1}$$

for each $(i, h)^{\text{th}}$ pair of the G genes ($i \neq h$), where $\overline{X}_i$, s_i, $\overline{X}_h$, and s_h are the sample means and standard deviations across patients for the ith and h^{th} genes, respectively. For convention, we define any gene with zero standard deviation (likely to be caused by a gene with null expression across all samples) as uncorrelated with every other gene.

2.2 Gene clustering via Affinity Propagation (AP)

We employ the information from the externally derived inter-gene correlations r_{ih} to define a set of gene clusters for our target pathway. To perform the clustering, we favor use of Affinity

Propagation (AP).[21] AP clustering exhibits similarities with the well-known k-means clustering approach: it forms clusters around multiple center points, called *exemplars*, that are assessed for cluster similarity in an iterative fashion; see Frey and Dueck[21] for details. Our strategy shares features with a widely used framework known as correlation network analysis, whose applications in biology include clustering of genes into densely connected sets.[22]

To cluster via AP, we require a *similarity* metric $s(g_i, g_h)$ to define the suitability of a data point (gene) g_h to serve as the cluster exemplar for another data point (gene) g_i ($h \neq i$). Since our goal is to cluster on correlations, we use $s(g_i, g_h) = r_{ih}$, i.e., the recorded correlation between genes g_i and g_h in the external knowledgebase from Sec. 2.1. Rather than pre-specify the number of clusters, AP allows for as many (or as few) clusters as the similarity values encourage: higher input similarities increase the likelihood that a gene serves as a cluster exemplar. To add a level of performance stability, however, we recommend that each final cluster should contain at least four genes, unless AP requires smaller cluster sizes in order to converge. We impose that requirement here.

We enlist the **R** package *apcluster* to implement the AP algorithm. We generally accept most of the package defaults, although we manipulate one selected "input preference" parameter, q: assuming all genes are equally valid candidates for the cluster exemplars, the program uses the qth sample similarity quantile as the shared input preference that a gene is chosen as such an exemplar. Informally speaking, this input preference is the propensity of a gene to become an exemplar for itself, i.e., $q = s(g_i, g_i)$. (See the *apcluster* documentation at `https://cran.r-project.org/web/packages/apcluster/index.html` for further details.) In effect, q acts as a tuning parameter that affects the number of clusters in the final AP solution: $q = 0$ tends to produce fewer numbers of clusters, while $q = 1$ tends towards increasing numbers of clusters. The *apcluster* default is the median at $q = \frac{1}{2}$.

To select the tuning parameter q we appeal to the external correlation data. We begin with a definition for the separation distance between any two points (genes), where greater "distance" implies poorer rationale for assigning two genes to the same cluster. Here, we take the distance metric as simple Pearson distance[23] scaled to the unit interval: $\Delta(g_i, g_h) = \frac{1}{2}(1 - r_{ih})$. For a given input q, suppose AP has converged to a solution of m_q clusters, with $n_j \geq 4$ genes assigned to each jth cluster among the $G = \sum_{j=1}^{m_q} n_j$ genes in the target pathway. Denote the corresponding collection of clusters as $\{\mathcal{C}_1, \ldots, \mathcal{C}_{m_q}\}$. To keep the calculations manageable we vary q over some range of values such as $q = 0, 0.05, 0.10, \ldots, 1.0$, and borrow from methods of partitioned cluster

analysis[24]: first, calculate the cumulative within-cluster variation

$$\omega_q(\mathcal{C}_1, \ldots, \mathcal{C}_{m_q}) = \sum_{j=1}^{m_q} \sum_{\substack{i \in \mathcal{C}_j \\ h \in \mathcal{C}_j}} \Delta(g_i, g_h). \tag{2}$$

and then the corresponding between-cluster variation

$$\beta_q(\mathcal{C}_1, \ldots, \mathcal{C}_{m_q}) = \sum_{j=1}^{m_q} \sum_{\substack{i \in \mathcal{C}_j \\ h \notin \mathcal{C}_j}} \Delta(g_i, g_h) . \tag{3}$$

A popular measure for exploring the quality of different clustering solutions is the "pseudo-F" statistic[25]

$$F_q(\mathcal{C}_1, \ldots, \mathcal{C}_{m_q}) = \frac{(\tau_q - \omega_q)/(m_q - 1)}{\omega_q/(G - m_q)} \tag{4}$$

where $\tau_q = \omega_q + \beta_q$. Since it mimics the F-ratio from an analysis of variance, large values of (4) suggest higher pertinence for the given cluster assignment, or translated here, for the input value of the corresponding q. Thus, we maximize (4) to help select an operable value for q.

With all these components in place, our correlation-based clustering algorithm takes the following steps:

Step 1. Set $q = 0$, $q_{\text{step}} = 0.05$, and define `min.clust.size` $= 4$.

Step 2. Cluster the G genes within the target pathway using AP, supplying the similarity matrix of correlations, $\mathbf{R} = \{r_{ih}\}$, and current input preference q. To begin, start with $q = 0$.

Step 3. Compute the pseudo-F statistic from (4) for the given cluster assignment.

Step 4. Check that the `min.clust.size` restriction was met. If so, increment q by $q_{\text{step}} = 0.05$ and repeat Steps 2–4. Find the largest value of F_q and select that cluster solution; let m be the corresponding number of clusters.

Step 5. If the `min.clust.size` restriction fails, or if $q = 1$, then select the cluster solution from the largest value of F_q where `min.clust.size` is met or $q = 0$. Let m be the corresponding number of clusters.

Step 6. Return the gene list $\mathcal{G}$ with cluster assignments $\{\mathcal{C}_1, \ldots, \mathcal{C}_m\}$.

2.3 Assessing pathway differential expression

Given the external, correlation-based cluster assignments from Sec. 2.2, we next return to the current N-of-1 data set of gene expressions from our single subject. Let $D_{jk} = C_{jk} - B_{jk}$ be the kth gene-wise case-to-baseline difference ($k = 1, 2, \ldots, n_j$) in the jth cluster ($j = 1, 2, \ldots, m$) from a total of $G = \sum_j n_j$ genes in the target pathway. Also let $D_{++} = \sum_j \sum_k D_{jk}$ be the grand sum,

$D_{j+} = \sum_k D_{jk}$ be the jth cluster sum, $\overline{D} = \sum_j D_{j+}/m$ be the mean difference across clusters, and $\overline{\overline{D}} = D_{++}/G$ be the grand mean. Lastly, take S_D^2 as the sample variance $\frac{1}{m-1}\sum_j (D_{j+} - \overline{D})^2$ across clusters. (If desired, these expressions translate in a straightforward fashion when pre-determined, cluster-specific weights, w_j, are available to differentially weight the clusters; e.g., use $\overline{D}_w = \sum_j w_j D_{j+} / \sum_j w_j$, etc.)

We take the parameter $\mu_{DEP} = E(\overline{\overline{D}})$ to represent the true magnitude of pathway differential expression. The statistical hypotheses of interest are

$$
\begin{aligned}
H_0 : \quad & \mu_{DEP} = 0 \\
H_a : \quad & \mu_{DEP} \neq 0 \ .
\end{aligned}
\tag{5}
$$

Our aim is to employ the externally derived cluster assignments to construct a test statistic from which we can assess if our N-of-1 data can identify the target pathway as a significant DEP. The grand mean $\overline{\overline{D}}$ serves as an obvious starting point, since it measures the extent of differential expression in the pathway. We prefer, however, to base our test statistic's construction on the mean cluster difference $\overline{D}$: although less interpretable than $\overline{\overline{D}}$, operating with $\overline{D}$ provides for some useful computational simplification; e.g., testing H_0 in (5) reduces to calculations only involving $\overline{D}$.

To estimate $\text{Var}[\overline{D}]$, we turn to a robust, unbiased variance estimator offered by Williams[20] for cluster-correlated data. Given externally defined clusters $\{\mathcal{C}_1, \ldots, \mathcal{C}_m\}$, Williams showed that under our notation the variance of the grand sum can be estimated as

$$
\widehat{\text{Var}}[D_{++}] = \frac{m}{m-1} \sum_{j=1}^m (D_j - \overline{D})^2
\tag{6}
$$

where $\overline{D} = \sum_j D_{j+}/m$ was defined above as the mean difference across the m clusters. Notice that (6) is simply $\widehat{\text{Var}}[D_{++}] = m S_D^2$. With it, the standard error of $\overline{D}$ becomes $S_D/\sqrt{m}$.

Now, suppose under H_0 that the within-pathway cluster sums are distributed as $D_{j+} \sim N(0, \sigma^2)$, with variances σ^2 taken as constant across clusters. The use of a stabilizing log-transform on the original read counts supports the homogeneous-variance normal/Gaussian assumption here, at least approximately, when clusters are sufficiently large and roughly equal in size.[26] The hyperbolic arc-sine can provide an even-more-stable transformation with read counts such as these, although the improved stability it provides may not be sufficient to support its additional complexity.[27] We do not generally see it applied to RNA-seq read counts in practice.

Conditional on the external definitions for the clusters it is straightforward then to show that the t-statistic

$$T = \frac{\overline{D}}{S_D/\sqrt{m}} \tag{7}$$

is distributed as $T \sim t(m-1)$ under H_0. A (two-sided) P-value for testing H_0 against H_a is simply $P = 2\mathrm{P}[t(m-1) \geq |T|]$. Reject H_0 and report the target pathway as differentially expressed when P is smaller than some pre-specified α-level.

3 Simulation study

3.1 Simulation design and generation

The test statistic for DEP assessment constructed in Sec. 2.3 relies on approximating arguments to be valid with N-of-1-*pathways* gene expression data. To investigate the performance of our test methodology in practice, we conducted a series of Monte Carlo evaluations.

We examined how four different inputs affect the test's operating characteristics: (1) pathway size; (2) proportion, say, p, of differentially expressed genes (DEGs) within the pathway; (3) the fold-change increase, say, ψ, in mean differential expression from the Case to the Baseline sample; and (4) the inter-gene dependence structure as quantified by a within-pathway correlation matrix $\mathbf{R}$. We explain each of these components in detail below.

Our focal setting for building the simulation configurations is the pathway size G. We studied five different sizes: $G = 15, 30, 50, 100, 200, 400$. In order to represent true practical settings, we associated each different pathway size with an existing pathway annotation from the GO-BP knowledgebase[9] (downloaded 1 September 2015). We focused on matched-pair breast cancer outcomes, following on our example from Sec. 1.2. We we able to identify 110 patients in RNA-sequencing from TCGA's breast adenocarcinoma (BRCA) database and modified existing GO-BP gene set definitions to include gene symbols that were measured in their BRCA data sets (20,501 genes for the 110 subjects' matched pairs; downloaded 27 Jul 2016). This resulted in 634 distinct mRNAs being filtered out from GO-BP (11,530 down to 10,896 genes). Next, we randomly selected a pathway meeting these requirements at each of the six sample sizes. Table 2 displays the selected pathways and five-number summary statistics for the $\binom{G}{2}$ inter-gene correlations within each. We note from the summary data that these pathways exhibit a broad range of inter-gene correlations, centered at or near zero and often with a slight positive skew. Indeed, every correlation matrix $\mathbf{R}$ for the six pathways departs significantly from a diagonal matrix: using a nonparametric sphericity test from[28], none of the six corresponding (pointwise)

Table 2. Description of the six pathways selected for our simulation study. G is the total number of genes for which the TCGA BRCA data have measurements for each pathway; m is the number of clusters in each particular pathway determined by the correlation clustering algorithm (see text). The remaining columns contain the five-number summary and mean for the inter-gene correlations, r, observed in TCGA BRCA normal tissue RNA-seq data on an available sample of 110 patients.

Gene set identifier	Description	G	m	r_{min}	r_{Q1}	r_{Q2}	$\bar{r}$	r_{Q3}	r_{max}
GO:0060350	endochondral bone morphogenesis	15	2	-0.66	-0.14	-0.02	0.0030	0.120	0.60
GO:0016925	protein sumoylation	30	6	-0.75	-0.18	-0.00	0.0269	0.259	0.89
GO:0048565	digestive tract development	50	4	-0.73	-0.15	-0.01	0.0175	0.174	0.87
GO:0045185	maintenance of protein location	100	9	-0.81	-0.20	0.00	0.0179	0.228	0.95
GO:0002683	negative regulation of immune system process	200	12	-0.79	-0.12	0.00	0.0310	0.169	0.99
GO:0002520	immune system development	400	28	-0.86	-0.14	0.00	0.0220	0.172	0.98

P-values from the test is larger than 10^{-4}. Thus we feel confident that each chosen pathway exhibits non-trivial inter-gene correlation.

Table 2 also contains a column reporting the number of separate clusters, m, for each pathway determined by our correlation clustering algorithm from Sec. 2.2. Not surprisingly, the number of clusters is low for small pathway sizes and increases as G grows very large. We give the complete list of gene cluster assignments in a supplementary document: see Supplemental Table S1. Supplemental Fig. S1 also gives histograms for each set of $\binom{G}{2}$ correlations.

To model gene expression in the gth gene in any of the six selected pathways ($g = 1, \ldots, G$), we followed established practice[29;30] and assumed a negative binomial (NB) distribution for the frequency of reads, Y_g, with mean expression μ_g and dispersion parameter δ_g such that $\text{Var}[Y_g] = \mu_g + \delta_g \mu_g^2$. To determine rough estimates of both parameters for each gth gene, we returned to the TCGA BRCA database and retrieved RNA-seq read counts on normal breast tissue samples from the same 110 independent patients. For each gene in the specified pathways from Table 2, we found corresponding estimates $\hat{\mu}_g$ and $\hat{\delta}_g$ via the method of moments. (If $\hat{\delta}_g$ indicated under-dispersion compared to a Poisson random variable, we conservatively deemed the gene's read count to be Poisson distributed with mean $\hat{\mu}_g$.) These values defined the NB distribution for Baseline responses of gene g in our simulations.

To generate the corresponding, affected, Case responses in gene g, we imposed a ψ-fold increase in its mean response, and assigned that gene a differentially expressed mean of $\psi \hat{\mu}_g$. We varied this fold-change over the range $\psi = 1.5, 2, 4$. We combined the fold-change setting with a "DEG

proportion" setting in order to study how changes in a pathway's differential expression are identified by our proposed methodology. That is, we allowed the proportion of DEGs in a pathway to vary over the range $p = 0, 0.3, 0.6, 0.9$. When $p = 0$, no DEGs were present, and both the Baseline and Case mean responses were taken as $\hat{\mu}_g$. This studied the false-positive error rate of our cluster-based test procedure. When $p > 0$, however, that proportion of the G genes in the pathway were assigned a differential mean response equal to $\psi \hat{\mu}_g$, as described above. (We rounded pG to the nearest integer.) This latter case was designed to study the power our test procedure exhibits to identify DEPs.

Lastly, we studied the effects of inter-gene dependence by manipulating the inter-gene correlations. This was quantified by a within-pathway correlation matrix $\mathbf{R}$ consisting of all pairwise correlations r_{ih} between genes g_i and g_h in the given pathway. We used the externally determined correlations calculated from the TCGA database (summarized in Table 2) and constructed a $G \times G$ $\mathbf{R}$ matrix for the pathway's G genes using the corresponding r_{ih} values. For each pathway, we considered three forms for the correlation structure:

(i) Cluster-correlated data that imposes the AP cluster assignments from Sec. 2.2, such that pairs of genes within each cluster receive their corresponding correlation of r_{ih} from the full set of pairwise correlations, and genes between clusters (within a pathway) receive a correlation of zero. This is intended to reproduce the clustered correlation structure assumed by our testing methodology. We refer to this as "block" correlation, since $\mathbf{R}$ takes on a block-diagonal form.

(ii) Cluster-correlated data where gene pairings receive their corresponding correlations of r_{ih} irrespective of their imposed, within-pathway, cluster assignments. This is intended to study our method's robustness when the cluster assignments are not correctly specified. We refer to this as the "all" correlation case.

(iii) An "independence" assumption among all genes, such that $\mathbf{R}$ equals the $G \times G$ identity matrix $\mathbf{I}$. This studies the operating characteristics of our method under the supposition of no within-pathway correlation.

Correlated RNA-seq gene expressions were then simulated under our negative binomial model using the correlations in $\mathbf{R}$. The negative binomial read counts were simulated via $\mathbf{R}$'s `rnbinom` pseudo-random variate generator. We employed copulas[31;32] in order to 'tie' together the marginal negative binomial distributions and form a multivariate construction. We found that a few simulation replicates could result in poor performance in the implementation[32] if generated serially, so we conducted all 2000 simulations at once: an entire non-DEG simulated data set of size $G \times 2000$ was first simulated. Then, an entire DEG simulated data set of size $G \times 2000$ was

generated with every marginal gene mean μ multiplied by the current fold change ψ. The data sets were merged based on the selected DEG genes (see above) to form the final simulation data set for the particular configuration with the appropriate proportion of DEGs, p. This ensured that the desired correlation structures were properly represented.

Combining the six different pathway sizes with three types of fold change, four different proportions of DEGs/pathways, and three different correlation structures produced 216 separate simulation configurations for study. (When $p = 0$ the value of ψ is irrelevant, but for completeness' sake we ran separate simulations at each value of $\psi = 1.5, 2, 4$ even for $p = 0$. We expect the results to show essentially similar performance in all three cases.) For each simulation configuration, 2000 N-of-1 samples were generated to produce paired pseudo-random NB read counts $Y_{Bg} \sim \mathrm{NB}(\hat{\mu}_g, \hat{\delta}_g)$ and $Y_{Cg} \sim \mathrm{NB}(\hat{\psi}\mu_g, \hat{\delta}_g)$ for the specified pathway of size G. To impose $\mathbf{R}$, we used a standard Gaussian copula given the clustering solution for the specified pathway (Supplemental Table S1) and the simulation correlation setting ("block", "all", or "independent"). Then we transformed the individual NB counts, Y, to the stabilized values $X = \log_2(Y + 1)$. Lastly, we tested for differential expression using the cluster-based T-statistic in (7). For comparison purposes, we also applied the Wilcoxon signed-rank test and the naïve t-test discussed in Sec. 1.3 to assess their operating performances.

For each test statistic, we set our pointwise false-positive rate to $\alpha = 0.05$ and recorded the proportion of cases where the test rejected the null hypothesis of no differential gene expression. Notice that with 2000 simulations per configuration, the approximate standard error of our empirical Monte Carlo rejection rates at the nominal 5% level is $\sqrt{(0.05)(0.95)/2000} = 0.005$ and it never exceeds $\sqrt{(0.5)(0.5)/2000} = 0.011$.

3.2   Simulation results

3.2.1   False-positive rates   Empirical false-positive error rates corresponding to $p = 0$ from our simulations are graphed in Figure 1. The Figure presents results for $\psi = 1.5$ at $\alpha = 0.05$. We note that results with $\psi = 2, 4$ were essentially similar, as was expected. The display cross-classifies the results by correlation structure $\mathbf{R}$ and pathway size G. We see that for the independence case ($\mathbf{R} = \mathbf{I}$; far left column of graphs), all three methods control error rates at or slightly below the nominal 5% level within Monte Carlo sampling variability. Since we argue that genes within pathways should not be assumed independent, however, results from this independence case are primarily valid only as a proof of concept.

For the more practical case with non-zero inter-gene correlations, Figure 1 (right two columns) shows that our cluster-based approach using (7) exhibits reasonable false-positive

error performance. Under the cluster-correlated ("block") structure for which it is designed, its empirical error rates are essentially at or slightly below the nominal rate for all pathway sizes. Interestingly, under the "all" correlation structure our cluster-based test also shows good performance, either maintaining the nominal false-positive rate or becoming slightly conservative. We view this as a form of robustness to misspecified clustering, at least of the form we impose in our "all" correlation setting. The feature might be explained by the propensity of positively correlated genes to cluster together in the clustering algorithm (Sec. 2.2), which would tend to drive inter-cluster correlations to be negative (depending on the assumed correlation distribution) rather than close to zero. This could then lead to over-estimation of the standard errors and produce slight drops in sensitivity in the test statistic.

By contrast, both the signed-rank and naïve t-test exhibit substandard false-positive error performance when the data incorporate either form of inter-gene correlation. Error rates run upwards of 60% in some cases, although for the $G = 15$ pathway under the all-correlation structure all methods appear to control false-positive error. This may be due to that pathway's relatively symmetric and tighter pattern of inter-gene correlations (see Table 2). The result is not reproduced with the block correlation structure, however, so we do not place much value in this indication.

3.2.2 Simulated power Empirical powers, as rejection rates, from our simulations are graphed in Figure 2. The Figure presents the results as a function of the DEG proportion p, and stratifies power curves by the fold change parameter ψ. (To provide a baseline, the figure also includes the false-positive results at $p = 0$.) As in Figure 1, the display cross-classifies the results by correlation structure $\mathbf{R}$ and pathway size G, and the nominal significance level was again set to $\alpha = 0.05$. Only results for our cluster-based T-statistic in (7) are presented, since the other, independence-based methods were seen in Figure 1 to be substandard.

The patterns in Figure 2 show a trend towards increasing power with increasing DEG proportion p (i.e., greater departure from H_0). The effect is more pronounced as the fold-change increases from $\psi = 1.5$ (dot-dashes) to $\psi = 4$ (solid lines). Also, faster rises in power are indicated for the independence case ($\mathbf{R} = \mathbf{I}$; far left column of graphs). In all instances, these phenomena are not unexpected. Interestingly, the power curves were all visibly dampened for the smallest pathway at $G = 15$. We attribute this more to the small number of clusters ($m = 2$) than to the small pathway size, since the cluster-based t-test then operates with only $m - 1 = 1$ d.f. The two features are related, of course, since it is difficult to generate a large number of clusters with only 15 separate genes. Indeed, for the larger pathways with $G \geq 100$, the power curves rise far more
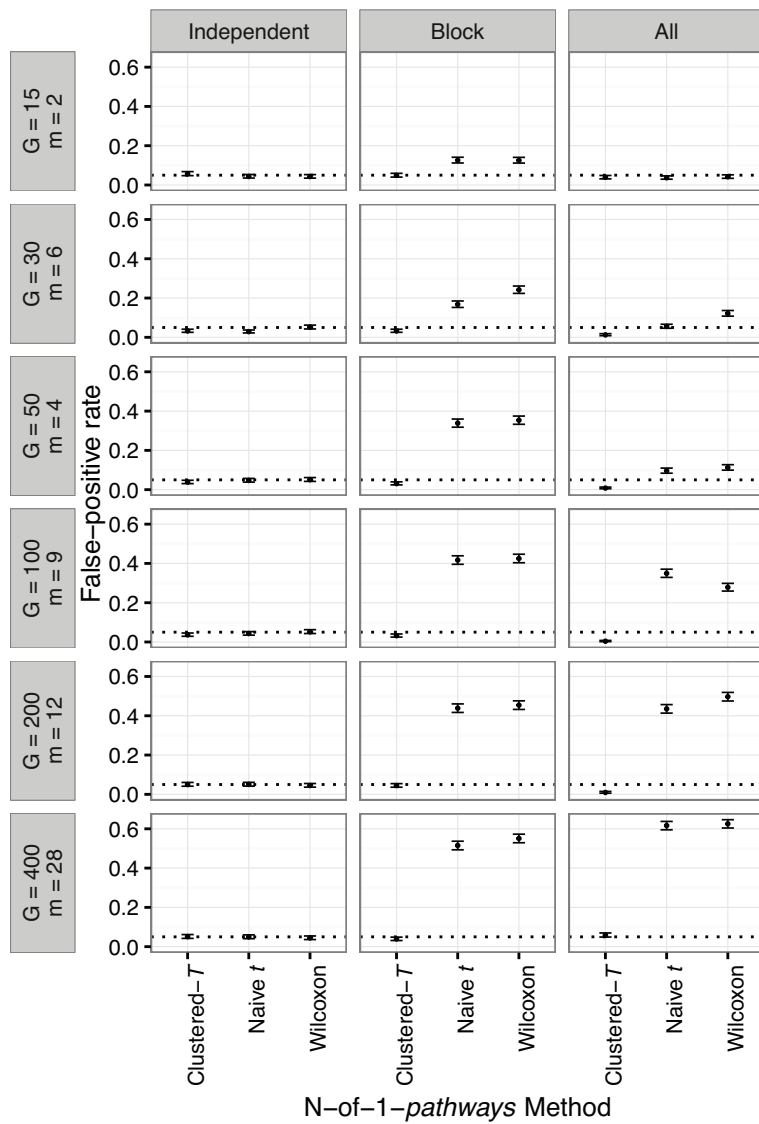
Figure 1. Empirical false-positive rates (dots) based on 2000 simulated N-of-1-*pathways* data sets for three competing testing procedures (lower horizontal axis: Clustered-T = proposed test, naïve t = standard t test, Wilcoxon = signed-rank test), cross-classified by correlation structure (top: Independent = uncorrelated mRNA expression, Block = cluster-correlated expression, All = unconstrained inter-gene correlation) and pathway size G (left). The corresponding cluster numbers, m, for each pathway are also listed; see Table 2. Nominal significance level is set to $\alpha = 0.05$ (dotted horizontal lines). Results reported for $\psi = 1.5$ (see text). Horizontal bars are pointwise 95% Agresti-Coull[33] confidence intervals for the underlying false-positive rate based on each set of 2000 simulated samples.
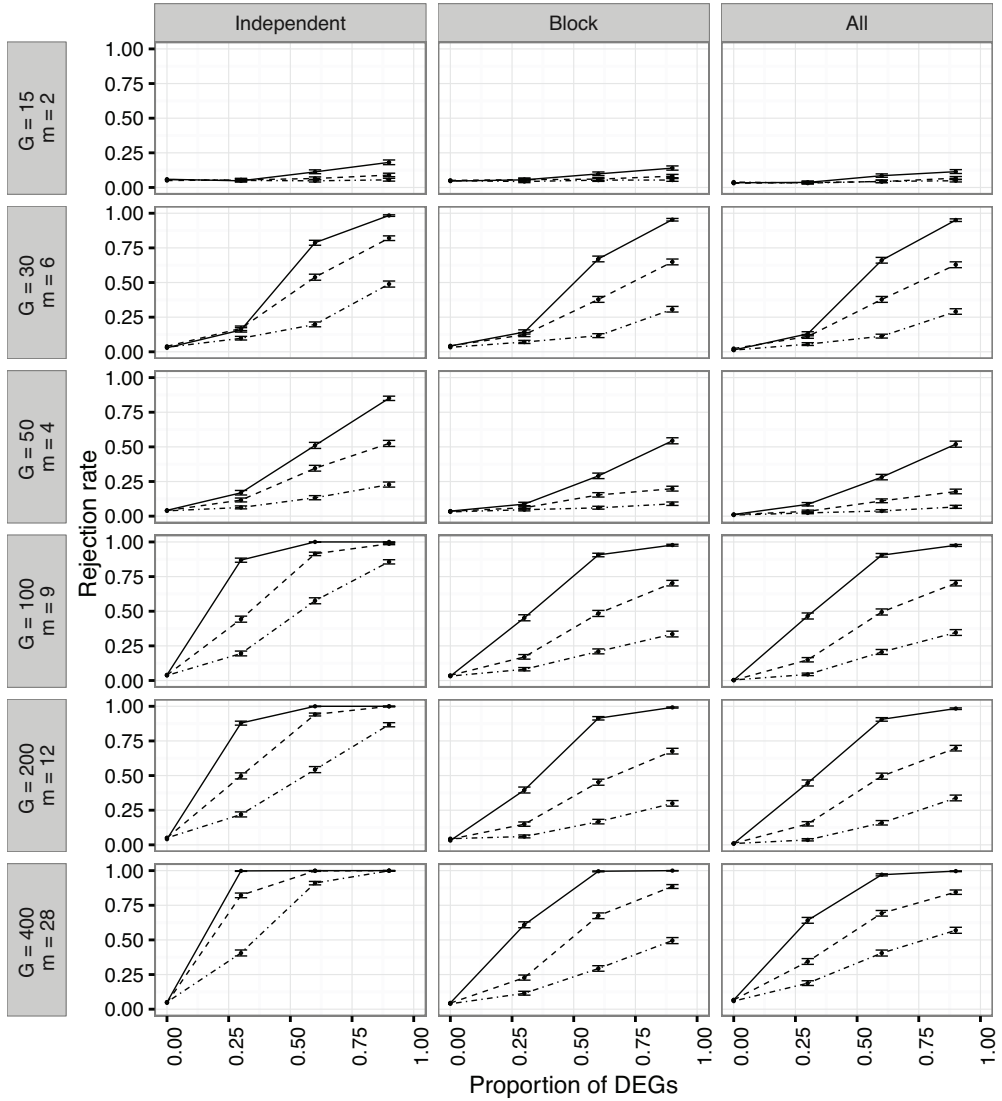
Figure 2. Empirical rejection probabilities ('power') for the cluster-based approach using (7), based on 2000 simulated N-of-1-*pathways* data sets. Results are presented as a function of DEG proportion p (lower horizontal axis). Displays are cross-classified by correlation structure (top: Independent = uncorrelated mRNA expression, Block = cluster-correlated expression, All = unconstrained inter-gene correlation) and pathway size G (left). The corresponding cluster numbers, m, for each pathway are also listed; see Table 2. Simulated fold change is indicated by line styling: $\psi = 4$ (solid lines), $\psi = 2$ (dashes), and $\psi = 1.5$ (dot-dashes). Nominal significance level is set to $\alpha = 0.05$. Horizontal bars are pointwise 95% Agresti-Coull[33] confidence intervals for the underlying rejection rate based on each set of 2000 simulated samples.

impressively; less so for the smaller pathways with fewer clusters. Supplementary Tables S2–S3 contain full numeric summaries of the rejection rates associated with this simulation study.

In general, these results suggest that the cluster-based T-statistic in (7) exhibits good false-positive error control and reasonable power, at least under the settings chosen for these simulations.

4 Breast cancer example revisited

To illustrate use of our cluster-based methodology, we return to the breast cancer example from Sec. 1.2. Recall therein that a female patient exhibiting triple negative breast cancer (TNBC) provided matched samples from both her healthy breast tissue and her cancerous breast tissue. (The complete data for this patient—identifiable only as "TCGA-A7-A0CE"—were quarried from the Cancer Genome Atlas/TGCA.)

Our goal is not to present a comprehensive investigation for further medical treatment(s) with this particular patient, rather, simply to exhibit how our cluster-based statistics are calculated in practice and to suggest possible avenues for their further use. To start, gene expressions on all available genes for the patient were normalized to account for library size (via transcripts per million) and then transformed via $\log_2(y_g + 1)$ for each gth gene's expression y_g in both the Baseline (normal tissue) and Case (cancerous tissue) groups. As above, we defined our pathways from a standard gene set knowledgebase, chosen here to be the GO-BP[9] ontology. Recall that example data from one such pathway, GO:0003071 (renal system process involved in regulation of systemic arterial blood pressure), were presented in Table 1. To make the process manageable and results more interpretable, we retained the pathways that had no fewer than 15 and no more than 500 genes annotated and provided a measured RNA-seq data set. This produced 3411 different GO-BP pathways.

4.1 Clustering of Gene Ontology-Biological Processes pathways

To construct our inter-gene, correlation-based clusters to correspond with this patient's breast cancer data, we returned to the same 110 independent, external subjects (these excluded TCGA-A7-A0CE's expression data) in the TGCA BRCA database employed in our simulation study (Sec. 3.1). Gene expressions in normal breast tissue from these women were available across the same genes listed in the 3411 pathways chosen above. Within each of these pathways, inter-gene correlations r_{ih} for the expressions were calculated from the 110 samples. We verified that each pathway's associated correlation matrix represented non-trivial inter-gene correlation, using the sphericity test from [28]. Four of the 3411 pathways yielded P-values above 0.05, and so we applied

our DEP test under the assumption of inter-gene independence to these four pathways. [That is, in these independence cases we applied the T-test via (7) using $m = G$.] For the remaining pathways, using the calculated correlations r_{ih}, within-pathway gene clusters were then assigned via our AP clustering algorithm as described in Sec. 2.2.

The external clustering proceeded in a manageable fashion. Employing **R** without parallelization the algorithm processed all pathways in slightly under 30 minutes on a MacBook Pro carrying a 2.5 GHz Intel Core i7 processor and 16 GB of 1600 MHz DDR3 RAM. Note that the external clustering need be completed only once for a given ontology and biological context/target tissue.

Most pathways yielded numbers of clusters m from the high single-digits to upwards of 30. For five of the pathways, however, we did experience an anomaly: the algorithm converged to a solution with m equal to 1. (That is, it assigned all genes in the pathway into a single cluster.) Since our cluster-based T-statistic in (7) requires at least $m = 2$ clusters to operate, single-cluster pathways cannot be assessed with our method. Thus we chose to remove these five pathways from consideration and not report a P-value. (One might instead manually inspect each single-cluster pathway and reason out some other, plausible, $m \geq 2$ clustering assignment. This would require additional, subjective, domain-specific judgment on a case-by-case basis, however.) The five-number summary for m across the remaining, scored pathways was $\{2, 3, 5, 9, 41\}$ with the average cluster number equaling 6.98. The distribution was strongly skewed to the right.

4.2   Differentially expressed pathways for patient TCGA-A7-A0CE

Conditional on the external cluster assignments we calculated our cluster-based T-statistic from (7) and found its pointwise, two-sided P-value, corresponding to a $t(m-1)$ reference distribution, for each of the scored pathways (including the four pathways exhibiting no inter-gene correlation). Pathways could be classified as either differentially expressed (DEP) or non-dysregulated, depending on whether the null hypothesis was rejected or not, respectively. One goal of the N-of-1-*pathways* strategy is to use such information to isolate possible pathways that affect or associate with the patient's disease outcome, i.e., here with TNBC. For example, a non-dysregulated pathway that is a target of a therapeutic drug could be indicative of a patient's poor response to therapy.

For patient TCGA-A7-A0CE, 602 of the scored pathways gave a pointwise, unadjusted P-value at or below 5%. To correct for multiplicity, we then further applied a Benjamini-Hochberg[15] false-discovery adjustment. This resulted in 80 DEPs with a false discovery rate (FDR) less than 15%.

Table 3. The 10 top-hit (smallest P-value) differentially expressed pathways and corresponding summary statistics for TNBC patient TCGA-A7-A0CE, after application of the cluster-based test procedure. See text for details.

Gene set identifier	Description	$\overline{\overline{D}}$	T-statistic	P-value	G	m
GO:0045785	positive regulation of cell adhesion	-0.75	-4.92	0.00011	226	19
GO:0032101	regulation of response to external stimulus	-0.47	-4.42	0.00015	458	28
GO:0070124	mitochondrial translational initiation	0.28	7.55	0.00028	84	7
GO:0010769	regulation of cell morphogenesis involved in differentiation	-0.51	-4.80	0.00028	168	15
GO:0030155	regulation of cell adhesion	-0.51	-4.24	0.00037	384	22
GO:1902532	negative regulation of intracellular signal transduction	-0.42	-4.37	0.00042	283	18
GO:0009306	protein secretion	-0.65	-4.68	0.00043	272	14
GO:0034142	toll-like receptor 4 signaling pathway	-0.33	-5.35	0.00046	105	10
GO:0051223	regulation of protein transport	-0.56	-3.94	0.00047	452	30
GO:0051092	positive regulation of NF-kappaB transcription factor activity	-0.54	-5.06	0.00049	111	11

(Among these was included the GO:0003071 pathway in Table 1.) Table 3 displays the 10 top-identified dysregulated pathways, ordered by their original, cluster-based P-values. Supplemental Table S4 lists all the 80 DEPs mentioned above.

For TNBC patient TCGA-A7-A0CE, further study of her 80 DEPs may offer insights into disease pathogenesis, progression, and possible therapeutic targets. For example, the dysregulated molecular pathways identified in in Table 3 are known to be associated with breast cancer progression, including cell adhesion[34], signal transduction[35], NF-κB[36], and Toll-like receptors.[37;38] The latter (Toll-like receptor) has particularly promising immunotherapy potential.[39]

5 Discussion

We have introduced a method for scoring and testing differentially expressed pathways (DEPs) from a single-subject, matched-pair collection of gene expression data under the N-of-1-*pathways* paradigm.[6] By focusing on gene sets/pathways, this N-of-1 strategy provides a powerful tool for development of subject-specific precision medicine. Unfortunately, the limited amount of data within the N-of-1, single-subject setting makes construction of suitable statistical inferences difficult. In particular, we illustrated that the presence of inter-gene correlation within a pathway undermines the ability of standard paired-testing methods to control false-positive error. We argue instead for incorporation of external information into the significance test procedure.

Toward this end, we propose a novel correlation-based clustering algorithm that employs external gene expression data to aggregate positively co-expressed genes in clusters within pathways. To our knowledge, this is the first single-subject gene set testing procedure that accounts for inter-gene correlation.

In a short simulation study, the method exhibited satisfactory false-positive error control and robustness against certain modeling assumptions, including the form of inter-cluster correlation. The method also powerfully detected DEPs when the number of clusters and fold-change of differentially expressed genes (DEGs) was large. In addition, it is worth mentioning that our cluster-based approach does not require estimation of inter-gene correlations for the single N-of-1 subject being studied, as we show in an example with a TNBC patient.

While the method presents ample potential, there are, of course, some caveats. One potential drawback is the availability of external, context-relevant, gene expression data. In the cancer literature, there is a growing number of openly available patient data sets and cell lines that could be recruited for use. Other biomedical contexts may prove less accommodating, however. Furthermore, we implicitly assume that co-expression of genes within the same tissue is stable across the external database, but further study is warranted to test the validity of this supposition. Depending on the estimated, external, inter-gene correlations and the chosen gene set ontology, some pathways may exhibit resistance to accurate and effective clustering. This could result in small cluster numbers or large, positive, inter-cluster correlation, and potentially lower power under our cluster-based approach.

With the above limitations in mind, one might consider a number of possible extensions and variations to our cluster-based approach. We propose what is, in effect, a self-contained gene set test[40], meaning that only genes in the pathway impact the score. A test that employs inter-gene correlations across the entire transcriptome could present a competitive alternative approach when many pathways fail to cluster under a chosen ontology. Indeed, correlation-based clustering is an active area of research across many disciplines.[41] And, as noted above, future N-of-1 data may present more than two conditions beyond our Case-vs.-Baseline pairing. The statistical details for identifying DEPs under multiple conditions would make for an interesting extension of our cluster-based approach.

It is also natural when considering incorporation of external information to consider some form of Bayesian methodology.[42] The nature and form of any such model would be highly complex, and it is unclear how much external information or subjective input would be required to implement any eventual construct. Still, the richness of the Bayesian paradigm offers many roads for study and it may provide a useful area for development with N-of-1, precision-medicine inferences. We are exploring all these various issues and hope to report on them in future manuscripts.

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Declaration of conflicting interests

The authors declare no conflicts of interest.

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Supplemental material

Various supplemental tables and graphics, referenced above, are available with this paper at the journal's website.

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