‘Single-subject studies’-derived analyses unveil altered biomechanisms between very small cohorts: implications for rare diseases

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Abstract

Motivation: Identifying altered transcripts between very small human cohorts is particularly challenging and is compounded by the low accrual rate of human subjects in rare diseases or sub-stratified common disorders. Yet, single-subject studies (S3) can compare paired transcriptome samples drawn from the same patient under two conditions (e.g. treated versus pre-treatment) and suggest patient-specific responsive biomechanisms based on the overrepresentation of functionally defined gene sets. These improve statistical power by: (i) reducing the total features tested and (ii) relaxing the requirement of within-cohort uniformity at the transcript level. We propose Inter-N-of-1, a novel method, to identify meaningful differences between very small cohorts by using the effect size of ‘single-subject-study’-derived responsive biological mechanisms.

Results: In each subject, Inter-N-of-1 requires applying previously published S3-type N-of-1-pathways MixEnrich to two paired samples (e.g. diseased versus unaffected tissues) for determining patient-specific enriched genes sets: Odds Ratios (S3-OR) and S3-variance using Gene Ontology Biological Processes. To evaluate small cohorts, we calculated the precision and recall of Inter-N-of-1 and that of a control method (GLM + EGS) when comparing two cohorts of decreasing sizes (from 20 versus 20 to 2 versus 2) in a comprehensive six-parameter simulation and in a proof-of-concept clinical dataset. In simulations, the Inter-N-of-1 median precision and recall are >90% and >75% in cohorts of 3 versus 3 distinct subjects (regardless of the parameter values), whereas conventional methods outperform Inter-N-of-1 at sample sizes 9 versus 9 and larger. Similar results were obtained in the clinical proof-of-concept dataset.

Availability and implementation: R software is available at Lussierlab.net/BSSD.

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1 Introduction

Empirical evidence unveils a methodological gap when comparing transcriptomic differences in biological mechanisms within very small human cohorts due to variations in heterogenicity, uncontrolled biology (age, gender, etc.) and diversity of environmental factors (nutrition, sleep, etc.) (Griggs et al., 2009; Liu et al., 2014; Schurch et al., 2016; Soneson and Delorenzi, 2013). Even in isogenic conditions, studies recommend at least six biological replicates for applying GLMs (Liu et al., 2014; Schurch et al., 2016). These sample size requirements are unfeasible for clinical care of a patient. Paradoxically, rare diseases are common: 8% prevalence in the population (Elliott and Zurynski, 2015) and 26% of children who attend disability clinic (Guillem et al., 2008). As timely and sizeable patient accrual of rare or micro-stratified diseases are prohibitive, there lies an opportunity for empowering clinical researchers with feasible statistical designs that enable smaller cohorts.

On the other hand, well-controlled isogenic studies (e.g. cellular models) can yield differentially expressed genes (DEGs) between two small samples. We and others have applied the power of the
isogenic framework through the comparison of two sample transcriptions from one subject in single-subject studies (S\(^1\)). While transcript-level differences between two-sample remain inaccurate (Vitali et al., 2019; Zaim et al., 2019), gene set-level (pathway/biosystem) S\(^1\) have been shown to accurately discover altered biomechanisms from paired transcriptome samples drawn from the same patient under two conditions (e.g. tumor-normal, treated-untreated) (Ozturk et al., 2018; Vitali et al., 2019). The results of the S\(^1\) gene set analyses have been validated in various contexts such as cellular/tissular models (Balli et al., 2019; Gardoux et al., 2014, 2015), retrospectively in predicting cancer survival (Li et al., 2017a,b; Schissler et al., 2015; Schissler et al., 2018) circulating tumor cells (Schissler et al., 2016), biomarker discovery simulations (Zaim et al., 2018) and therapeutic response (Li et al., 2017a,b). Despite the success of these models to derive effect sizes and statistical significance in S\(^1\) of transcriptions, these samples are isogenic or quasi-isogenic, and thus do not necessarily generalize to a group of subjects (cohort-level signal). To address the latter, we reported that determining single cohort-level significance by combining gene set signal (e.g. pathways) from S\(^1\) analyses can be more accurate than conventional DEG analyses (e.g. GLMs) followed by gene set enrichment analysis (GSEA) (Subramanian et al., 2005) in small cohort simulations (Zaim et al., 2018) and in previously published datasets (Li et al., 2017a,b). However, these methods could only summarize information within a single cohort and were not designed to compare two distinct cohorts to evaluate subgroup interactions.

To address the methodological gap, we therefore hypothesized that single-subject transcriptomic studies of gene sets increase the transcriptomic signal-to-noise ratio within subject and lead to an improved signal between small patient cohorts, as small as 3vs3 subjects per group. While technically different from the analysis of the standard two factor interactions in conventional cohort statistics, the proposed framework is conceptually related to a statistical interaction in that a within-single-subject analysis (subject-specific transcriptome dynamics) is followed by within-group agreement for characterizing Factor 1 (e.g. cancer versus paired normal tissue) and between-group comparisons (Factor 2, e.g. responsive versus unresponsive to therapy). The strategy improves the statistical power by: (i) reducing the total features tested (gene set-level rather than transcript-level), (ii) relaxing the requirement of within-cohort uniformity at the transcript level as the coordination is conducted at the gene set-level and (iii) reducing confounding factors through the paired sample design of S\(^1\)-analyses within subject. The novel bioinformatic method identifies meaningful biomechanisms differences between very small cohorts by using single-subject-study-derived effect sizes for gene sets. Additionally, we show through both extensive simulations and a real data case example using TCGA human breast cancer cohort data that -within cohorts of varying sizes (3 to 7 subjects)- the Inter-N-of-1 method outperforms traditional methods, which are based on generalized linear modeling (GLM) followed by common gene set enrichment or overlap analysis. We then apply this novel method to the effect sizes of two different single-subject analyses to illustrate the flexibility and utility of the proposed method for a variety of inputs.

2 Materials and methods

Figure 1 provides an overview of the proposed new method (Inter-N-of-1). To motivate the development of transcriptome analytics between very small human samples, by nature heterogeneity, we first demonstrate the limitation of a Generalized Linear Model to DEGs between 23 TP53 and 19 PIK3CA breast cancer samples. Next, we describe two new methods Inter-N-of-1 (MixEnrich) and Inter-N-of-1 (NOISeq) that work by combining single subject study results using contingency tables to obtain cohort-level estimates for enrichment of GO terms which are then contrasted to discern differences in enrichment between the two cohorts. We then compare these two methods to a Generalized Linear Model (implemented in LIMMA) (i) in simulation studies with parameters estimated from empirical analyses of real datasets and (ii) in a proof-of-concept study of TCGA breast cancer cohorts. Also, the evaluation of the proposed new methods is conservative as it is conducted against a reference standard built with a distinct Generalized Linear Model (edgeR) using all samples.

2.1 Datasets

We obtained 5179 gene sets from Gene Ontology (Ashburner et al., 2000) Biological Processes (GO-BP) (downloaded on 02/07/2019). The Human breast cancer cohort consists of RNA-seq expression profiles samples of 224 paired breast cancer (BC) tumor and unaffected breast tissue normal (Factor 1) from the same subjects (n = 112) from The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma data collection (Cancer Genome Atlas, 2012; Ciriello et al., 2015) (Obtained 10/22/2015). The proof-of-concept application of the proposed methods (Figs 3 and 4) pertains to the subset of subjects with either TP53 (n = 23) or PIK3CA (n = 19) mutations (Factor 2), but not both. These BC oncogenes have been reported (i) in expression patterns (Cancer Genome Atlas, 2012), (ii) cancer subtypes (Van Keymeulen et al., 2015), (iii) clinical outcomes (Kim et al., 2017) and (iv) responsiveness to specific therapi (Andre et al., 2019). These data were downloaded using the R package TCGA2STAT(n=42 cases; 84 files) (Wan et al., 2016).

Data access and preparation: (A) within each sample pair of a single-subject study, (i) we removed all transcripts with mean expression ≤ 5 counts, (ii) found the union of all genes remaining amongst all pairs, (iii) excluded all genes not included in the union of these two steps (17,923 genes remaining) and (iv) added ‘1’ to expression counts to eliminate ‘zeros’. (B) For GLM analyses, we eliminated all the transcripts with 0 counts for each subject and calculated each transcript’s coefficient of variation (CV). We retained the top 70% of transcripts ranked by CV (13,932 genes remaining).

Simulated datasets production is described in Section 2.4. MCF7 breast cancer cells, for estimating simulation parameters, consist of 7 estrogen-stimulated and 7 unstimulated cells sample replicates (Edgar et al., 2002; Liu et al., 2014) (obtained on 10/14/
Table 1. Variable definitions

| Variable | Definition |
|----------|------------|
| \(g_{k,j}\) | The number of DEGs within gene set \(gs\) for subject \(k_j\) in cohort \(K\) |
| \(\mathbf{k}_{gs,k}\) | The number of genes NOT differentially expressed in gene set \(gs\) for subject \(k_j\) in cohort \(K\) |
| \(P_{10}A\) | Number of genes neither differentially expressed nor in gene set \(gs\) for subject \(k_j\) in cohort \(K\) |
| \(P_{10}A\) | Number of gene sets |
| \(N\) | Probability of Event \(()\) occurring |
| \(\rho_{gs,\Delta}\) | Continuity-corrected log \(\text{S3-OR}\) corresponding to gene set \(gs\) for subject \(k_j\) in cohort \(K\) |
| \(Q_{gs,k}\) | The mean continuity-corrected log \(\text{S3-OR}\) in gene set \(gs\) in cohort \(K\) |
| \(S_{k}\) | The number of subjects in a cohort \(K\) (e.g. those with a PIK3CA or with TP53 somatic mutation) |
| \(V_{gs,k}\) | Expected value of the continuity-corrected log \(\text{S3-OR}\) for the molecular-defined cohort \(K\) |
| \(\text{var}(Q_{gs,k})\) | Variance of continuity-corrected log \(\text{S3-OR}\) corresponding to gene set \(gs\) for subject \(k_j\) in cohort \(K\) |
| \(W_{gs,\Delta}\) | The test statistic for the Inter-N-of-1 for gene set \(gs\) |
| \(Z\) | A standard normal random variable |

A standard normal random variable

Figure 2. Overview of two \(S^2\) methods conducted from one sample per condition without replicate generating effect sizes and variance for each gene set. We apply \(S^2\) to each subject to identify either prioritized transcripts (A) or DEGs (B) between paired tumor-normal samples. We identify patient specific enriched gene sets and associated effect sizes in the form of natural log odds ratios through a FET (C). Each effect size is approximately normally distributed with known variance and mean, simplifying subsequent analyses between cohorts. The gene set-level variance enables the extraction of more information from each individual subject than typical variance estimators that work across subjects and thereby leads to increased statistical power.

The \(S^2\) method was previously described and validated (Li et al., 2017a,b; Zaim et al., 2018). NOISeq is also considered as an alternative method because of its performance in prior \(S^2\) evaluations (Zaim et al., 2019).

We also applied NOISeq-sim to each of the tumor-normal pairs (Tarsazza et al., 2015) as shown in Figure 2B. NOISeq-sim simulates counts for each transcript under both conditions and then estimates the joint noise distribution for the \(\log_2 FC\) and the \(\Delta\) (gene expression) between conditions. The estimated noise distribution provides the probability of a DEG. For these applications of NOISeq with no replicates, the ‘\(p\)’ and ‘\(q\)’ parameters were set to 0.0002 and 0.00002 to prevent the method from producing any errors related to setting the size of the inherent multinomial distributions to an integer too large for R to handle. The criteria for identifying genes as differentially expressed for NOISeq were the same as those used for \(S^2\) methods. As shown in Figure 2C, we subsequently used this information to construct contingency tables and calculate the natural log odds ratio for Inter-N-of-1. This process generated two different applications of Inter-N-of-1, \(S^2\)-MixEnrich and NOISeq, to conduct the single-subject analyses preceding the cohort comparison.

Comparing enriched \(Gene\ Sets\ across\ distinct\ cohorts\): We first combined the data within two distinct cohorts into single statistics whose null reference distributions were at least approximately normal. These within-cohort statistics were contrasted via scaled effect size of responsive pathways in each subject and increase the features signal-to-noise ratio. Table 1 summarizes the variables.

Identification of overrepresented gene sets for each subject: As illustrated in Figure 2A, we applied to each of the tumor-normal pairs the \(S^2\)-pathways MixEnrich method that we had previously developed and validated (Li et al., 2017a,b; Zaim et al., 2018) and extended to account for direction of differential expression (Berghout et al., 2018) and contrasted to other methods (Li et al., 2019). Briefly, this method models the absolute value of the log\(_2\) transformed fold change (FC) for each gene across the two paired transcriptomes being studied and uses a probabilistic Gaussian mixture to assign a posterior probability that the gene is differentially expressed between tumor and normal conditions. Within the simulation, prioritized transcripts were defined as those with a posterior probability of being differentially expressed higher than 0.99. Within the TCGA breast cancer cohort, said definition included having both a posterior probability of being differentially expressed higher than 0.99 and an absolute-value \(\log_2 FC\) higher than \(\log_2(1.2)\), which was determined as optimal for downstream GO terms enrichment in this dataset. Genes were assigned to gene sets using the Gene Ontology (Ashburner et al., 2000) Biological Process (GO-BP) hierarchy, filtered to those terms with gene set size between 15 and 500 genes, with subsumption to maximize interpretability. These DEGs were used to determine the overrepresented, or enriched, gene sets of interest using a two-sided Fisher’s Exact Test (FET) (Fisher, 1935) with a False Discovery Rate (FDR) of 5%.

The output of this analysis generated lists of gene sets, with each list representing a single subject’s tumor-normal pair and comprising GO-BP terms accompanied by contingency table counts which were used to calculate an odds ratio \(\text{S3-OR}\) as the effect size.

2.2 Proposed \(S^2\)-anchored responsive pathway \(Inter-N\)-of-1 methods for comparing very small human cohorts

The following paragraphs will develop the methodology by which we conduct \(S^2\) prior to cross-cohort comparison to discover the
establish the difference in gene set enrichment between the two cohorts. Let \( g_s \in \{1, \ldots, N\} \) index the specific gene set being studied where \( N \) is the total number of gene sets, \( k_s \) indexes a specific subject in cohort \( K \) composed of \( S_k \) individuals with subjects numbered \( j \in \{1, \ldots, S_k\} \), and \( K \in \{A, B\} \) indexes a specific cohort. Let \( A \) signify quantities relating to the difference between the two cohorts.

The Inter-N-of-1 analytics for combining information within a cohort considers the abstract contingency table shown as Table 2 where the cell counts are representative for the gene set indexed by \( g_s \) and the subject indexed by \( k_s \).

We obtain DEGs from the application of a chosen single-subject analysis method (either N-of-1-MixEnrich or N-of-1-NOISeq) for a specific gene set \( g_s \) in individual \( k_s \) of cohort \( K \) to fill out the contingency table with counts in the format shown in Table 2. We apply a continuity correction by adding 0.5 to each of the cells in the contingency table to provide a small-sample adjustment in the odds ratio (Agresti and Kateri, 2011). The natural log \( S^* \) OR, denoted as \( Q_{g_s,k_s} \), Equation 1, is approximately normally distributed with variance \( \text{var}(Q_{g_s,k_s}) \) given in Equation 2 (Woolf, 1955).

\[
Q_{g_s,k_s} = \ln \left( \frac{g_{g_s,k_s} + \frac{1}{2}}{h_{g_s,k_s} + \frac{1}{2}} \cdot \frac{g'_{g_s,k_s} + \frac{1}{2}}{h'_{g_s,k_s} + \frac{1}{2}} \right)
\]

\[
\text{var}(Q_{g_s,k_s}) = \frac{1}{g_{g_s,k_s} + \frac{1}{2}} + \frac{1}{g'_{g_s,k_s} + \frac{1}{2}} + \frac{1}{h_{g_s,k_s} + \frac{1}{2}} + \frac{1}{h'_{g_s,k_s} + \frac{1}{2}}
\]

We average the \( Q_{g_s,k_s} \) values within their respective cohorts to obtain the average ln ORs

\[
-\bar{Q}_{g_s} = \frac{1}{S_k} \sum_{k_s=1}^{S_k} Q_{g_s,k_s} \sim N\left( \theta_g, \sum_{k_s=1}^{S_k} \frac{\text{var}(Q_{g_s,k_s})}{S_k^2} \right)
\]

When the null hypothesis \( H_0: \theta_g = E[\ln(\text{OR}_A)] = E[\ln(\text{OR}_B)] = \theta_g \) is true then

\[
W_{g_s,A} = \frac{-\bar{Q}_{g_s,A} - -\bar{Q}_{g_s,B}}{\sqrt{\text{var}(\bar{Q}_{g_s,A}) + \text{var}(\bar{Q}_{g_s,B})}} \sim N(0,1)
\]

at least approximately. The corresponding two-sided \( P \)-value for gene set \( g_s \) is

\[
\sim p_{g_s} = 2 \cdot P(Z > W_{g_s,A})
\]

where \( Z \) represents a standard normal random variable. An FDR adjustment via the Benjamini–Hochberg method (Benjamini and Hochberg, 1995) is then applied to the \( \sim p_{g_s} \) across all the GO terms tested in the particular application. To ensure that the method positively identifies gene sets that are enriched in at least one of the cohorts, we set all FDR adjusted \( P \)-values to 1.0 if both cohort means of the log odds ratios are negative. This step ensures interpretable results since impoverished GO terms with significantly fewer-than-expected DEGs are not well understood from a biological context.

2.3 Description of the generalized linear models and application of Inter-N-of-1 methods for small cohort comparison and their evaluation in the TCGA human breast cancer cohorts

Generalized Linear Model (GLM) Designs: For the cohort analyses, we applied a generalized linear model as implemented in limma (Smyth et al., 2005). Preceding application of the GLM, we performed trimmed mean of M values (TMM) normalization (Robinson and Oshlack, 2010) on the data pre-processed for cohort analysis. We applied the voom normalization (Law et al., 2014) via the limma function voom/withQualityWeights in R.

We used the three different designs described in Table 3 for these GLM-based analyses, which were called the simple design, the interaction design and GLM+EGS respectively. We blocked by subject for each of these GLM designs and all FDR adjustments of \( P \)-values were done using the Benjamini–Hochberg False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995).

Reference standard construction within TCGA breast cancer cohort data using edgeR generalized linear model followed by gene set enrichment: We chose to construct reference standards using all samples of the human breast cancer cohort to estimate accuracies of analyses of smaller sample size. After pre-processing for cohort analyses, we applied generalized linear models as implemented in the R software package edgeR (Robinson et al., 2010) at FDR < 5% to the entire TCGA human breast cancer cohort data to construct three reference standards corresponding to the three designs discussed in Table 3. Each reference standard evaluated the analyses of the TCGA breast cancer cohorts (TP53 versus PIK3CA) and used the same filter thresholds for classifying transcripts as differentially expressed, which were designed to maximize the number of enriched GO terms in this dataset. In the GLM followed by enrichment of gene set (GLM+EGS), the prioritized interacting transcripts are followed by a FET at FDR < 5%.

Subsampling of the TCGA Breast Cancer Cohort and application of GLM and Inter-N-of-1 methods: For each of the values \( S_k = S \in \{2, 3, 4, 5, 7, 8, 9\} \) we ran 100 subsamples of the total cohorts where we randomly selected without replacement \( S \) subjects with TP53 and \( S \) subjects with PIK3CA, without requiring non-redundancy of the random samplings. We applied the GLM+EGS method and the N-of-1-MixEnrich and NOISeq versions of the Inter-N-of-1 method to each of the selected cohorts (TP53 versus PIK3CA). For each of the three methods, FDR < 5% adjustment of the \( P \)-values was done with respect to all 5179 GO terms tested.

For random subsamples of size \( S_k = S \in \{2, 3, 4, \ldots, 19\} \) of subjects, we applied the two transcript-level analyses using generalized linear models as implemented in limma. The performance of

### Table 2. Notation for 2x2 contingency table cross-classifying DEG status with gene set status

| Gene set \( g_s \) | DEG | Not DEG |
|-------------------|-----|---------|
| \( g_{g_s,k_s} \) | \( g_{g_s,k_s} \) | \( g'_{g_s,k_s} \) |
| \( b_{g_s,k_s} \) | \( b_{g_s,k_s} \) | \( b'_{g_s,k_s} \) |

### Table 3. Three experimental designs used for the generalized linear models

| Name               | Level     | What is compared                                      | Results   |
|--------------------|-----------|-------------------------------------------------------|-----------|
| Simple             | Transcript| TP53_Tumoral—PIK3CA_Tumoral                           | Figure 3A |
| Interaction        | Transcript| (TP53_Tumoral—TP53_Normal) — (PIK3CA_Tumoral—PIK3CA_Normal) | Figure 3B |
| GLM+EGS            | Gene set  | 1. Find DEGs using Interaction Contrast               | Figures 4 and 5 |
|                    |           | 2. Enrichment via FET                                 |           |

Note: In the analysis of subsets of the TCGA human breast cancer cohorts, genes were declared differentially expressed if their \( \frac{\text{abs(log2FC)}}{\text{log2(1.2)}} \) and their FDR-adjusted \( P \)-value < 0.05. Within the simulation, genes were declared differentially expressed if their FDR-adjusted \( P \)-values < 0.05.
these transcript-level applications of limma was assessed and illustrated in Figure 3 to demonstrate the necessity and benefit of transforming from transcript-level to gene set-level analyses.

Accuracy measures within TCGA human breast cancer cohorts: For each method, we calculated the precision and recall using the following functions. When a method produced no positive predictions for the gene sets, we assigned values of zero to the precision and recall of the given method. Otherwise, we calculated the precision and recall using Powers’ calculations with adjustments of adding 0.5 to numerators and 1.0 to denominators to avoid divisions by zero (Powers, 2011). In addition, we have previously published extensions to conventional accuracy scores that we termed ‘similarity Venn Diagrams’ and ‘Similarity Contingency Tables’ (Gardeux et al., 2015). In these approaches true positive results between the prediction set and the reference standard include both identical GO-BP terms and those which are highly similar in terms of Information Theoretic Similarity (ITS) and therefore represent highly related biology (Tao et al., 2007). We calculated the precision and recall of the gene set level analyses using this ITS approach because it unbiasedly compares predicted biological concepts against those of the reference standard. For precision, we included in the intersection those predicted GO-BP terms which had an ITS similarity score of 0.70 or higher with any of the GO terms in the reference standards, while the denominator remained as all predicted GO-BP terms. Similarly, for recall, we included in the intersection the reference standard GO-BP terms which had an ITS similarity score of 0.70 or higher with any of the predicted GO terms, while the denominator remained as the total positive reference standard GO-BP terms. Of note, we previously reported that this ITS > 0.70 similarity criteria is highly conservative since ~0.0056 pairs of GO-BP terms are similar at ITS > 0.7 (58,577 pairs among 10,458,756 non-identical combinations of GO-BP terms) (Gardeux et al., 2015).

### 2.4 Simulation of small cohort comparisons to compare GLMs to Inter-N-of-1 methods

Data generation for simulation: The overall scheme for the simulation began by constructing two cohorts of paired tumor-normal RNA-seq expression profiles. We calculated simulation parameters to most realistically create these expression values as described below (Table 4). To calculate statistical interactions between two

| Parameters | How Estimated | Values |
|------------|---------------|--------|
| Control Samples | Randomly sample without replacement from TCGA breast cancer normal samples | NA |
| log₂FC distribution of non-differentially expressed genes | 1. Calculate log₂FCs of randomly paired MCF7 unstimulated breast cancer samples | NA |
| | 2. Split log₂FCs into deciles by baseline expression | |
| | a. All deciles containing 0 are combined into one category | |
| | 3. Sample with replacement from decile containing transcript name in first random pair | |
| Gamma parameters of log₂FCs of DEGs | 1. Run N-of-1-MixEnrich (Fig. 2) on within-subject tumor-normal pairs in TP53 and PIK3CA cohorts to identify DEGs | |
| | 2. MLEs for gamma parameters fit to absolute log₂FCs of DEGs | |
| | a. Used egamma function in EnvironStats R package (Millard et al., 2013) | |
| Proportion of DEGs in enriched GO-BP terms | 1. Split enriched GO terms from edgeR reference standard into deciles based on size | |
| | 2. Calculated DEGs median proportion for deciles containing GO-BP terms (size: 47, 200) | |
| | • (GO size 200): 0.05, 0.10 | |
| | • (GO size 40): 0.10, 0.19 | |
| Proportion of Subjects with coordinated DEGs | 1. Split log₂FCs of DEGs within edgeR reference standard into categories | 0.25, 0.48, 0.75 |
| | a. >1.3, b) -1.3 or c) neither | |
| | 2. Assign the max proportion of subjects per categories (a) or (b) for each transcript | |
| | 3. Find the median proportion of subjects across all transcripts | |
| Balanced Cohort Size | NA | 2, 3, 7, 10, 30 |
| GO-BP terms | 1. Enriched: GO:0002221 (200 genes) | NA |
| | 2. Enriched: GO:0000096 (47 genes) | |
| | 3. Control: GO:006733 (196 genes) | |
| | 4. Control: GO:0090184 (41 genes) | |

Note: Only cohort size and the proportion of subjects with coordinated DEGs were varied. All other parameters were held constant. 30 datasets were generated for each parameter configuration leading to a total of 450 datasets.
factors, we had to design two cohorts of subjects and each subject with two sample conditions. We sought to recreate the TCGA breast cancer conditions with these parameters, using the observed median values in the TCGA dataset as the medians of the simulation parameters and varying the parameters around said medians. The TCGA dataset did not comprise repeated samples in the same condition, and thus we utilized the unstimulated MCF7 cell lines with seven replicates to estimate the variation expected between two paired normal tissues. In our previous pathway expression studies [Yang et al., 2012] and data not shown] where we compared two cohorts, about two-thirds of the observed responsive gene set patterns—as shown in Figure 2—consisted of a gene set responsive in one subject cohort and unresponsive in the other cohort.

These paired tumor-normal samples represented within-subject samples were constructed to have a proportion of the transcripts with altered expression between the tumor and normal states. Through the use of randomly sampling without replacement, we generated the normal tissue samples for these pairs after filtering out all genes in the 112 TCGA breast cancer normal tissues, which were not present within the MCF7 breast cancer dataset (leaving 17,414 genes).

For each sampled normal breast tissue sample, we generated transcript expression for a paired breast cancer sample of that subject rather than sampling the corresponding breast cancer sample from the TCGA data. To produce a paired tumor expression value for a non-differentially expressed gene, we first followed the steps outlined in Table 4 to randomly generate empirical log Fold Change (log(FC)) and then we set the gene’s expression as the product of the gene’s paired normal expression and 2 raised to the exponent of the log2FC value. To generate the expression value for an altered transcript in a tumor sample, we randomly sampled a log2FC from a gamma distribution with parameters described in Table 4 and set said gene’s expression to the product of the gene’s normal expression and two raised to the exponent of the log2FC value. We generated only positive log2FCs for the DEGs to improve the identification of gene set-level biological mechanisms that differentiate between two cohorts rapidly lose power and yield unreliable results as the sample size decreases below five subjects per cohort.

The transcriptomic analyses of TCGA human breast cancer cohorts in Figure 3 recapitulates that small human cohorts are particularly difficult to analyze using GLMs due to their heterogeneous conditions and lack of controlled environment. Thus, small human cohorts present a stark contrast to isogenic controlled experiment cell lines or animal models where the high signal to noise ratio makes transcriptomic analyses possible for very small sample sizes. Therefore, the surprising results provide the justification for the development of the proposed GLM+EGS and Inter-N-of-1 methods conducted at the gene set level. They also attest to the intrinsic lack of signal within the TCGA human breast cancer cohorts for such transcriptomic analyses.

The performance results for subsets of the TCGA human breast cancer cohort data shown in Figure 4 establish that the two versions of the proposed Inter-N-of-1 method degrade more gracefully in performance with decreasing cohort size than traditional GLM-based methods, thereby allowing them to outperform for smaller cohort sizes. Figure 4 shows that the niche where the Inter-N-of-1 methods outperform in terms of median precision and recall extends to all cohort sizes below 7×2, with the GLM+EGS method achieving higher median performance scores for 9×2 and above. The sizes of the crosses suggest a further boon for the developed methods beyond this better ‘on average’ performance. The Inter-N-of-1 methods tend to have very small tight crosses suggesting low variation in performance and greater consistency. The GLM+EGS method on the other hand possesses very large crosses until cohort size 9×2, suggesting wild swings in performance across the different subsets evaluated. In addition, even the gene set-level GLM+EGS method outperforms transcript-level GLM analyses (Fig. 3 versus Fig. 4). Figure 4 also establishes that the N-of-1-MixEnrich version of the Inter-N-of-1 method outperforms the NOISeq version in terms of consistency and median precision and recall. Although these differences remain small for larger cohort sizes of 7×2 and above, they increase gradually with decreasing cohort sizes.
method to underperform. For cohort sizes of 10vs10 and lower, the GLM+EGS method fails to match the performance of the two versions of the Inter-N-of-1 method and so supports the superiority of Inter-N-of-1 in such small sample sizes for breast cancer-like data.

4 Discussion
As stated in the introduction, empirical evidence suggests the existence of a methodological gap when comparing transcriptomic differences in biological mechanisms within very small human cohorts due to variations of heterogeneity, uncontrolled biology (age, gender, etc.) and diversity of environmental factors (nutrition, sleep, etc.). Yet, even in isogenic conditions, two studies have recommended at least six biological replicates for applying generalized linear models (Liu et al., 2014a; Zenger et al., 2017). Examining two-factor interactions in transcriptomics (cohorts x tumor status) further inflates the required sample size by a factor of 4 (Brookes et al., 2004; Fless, 2004; Leon and Heo, 2009). Traditional cohort-based methods impose sample size requirements which simply cannot be met within the framework imposed by rare diseases, prompting the need to develop new methods. On the other hand, we and others have shown it is possible to obtain statistical significance of gene set-level effect size measures from single samples without replicates taken in two conditions, namely S-anchored Inter-N-of-1 addresses this methodological gap. Their slow decay in performance when contrasted with the abrupt decay of GLM+EGS
establishes the superiority of these methods for sample sizes of \(S_1 = S_0 \in \{2, 3, 4, 5, 6\}\) when applied to our TCGA human breast cancer cohort data. Comparison of the median precision and recall of the three considered techniques shows that on average our methods exhibit greater power and importantly less variable performance than GLM + EGS at these low cohort sizes. Our simulation study confirmed that both versions of the Inter-N-of-1 provide substantially improved recall over the GLM + EGS method at small cohort sizes while still maintaining equivalent levels of precision. The simulation results also establish that the expected proportion of subjects with coordinated DEGs within cohorts plays a critical role in determining the range of cohort sizes in which the developed methods outperform traditional GLM-based techniques. In datasets where the proportion of subjects within cohorts sharing their DEGs is lower than 75%, the Inter-N-of-1 methods continue to outperform the GLM + EGS method until cohort sizes > 30. Furthermore, our applications of Inter-N-of-1 to infrequent oncogenic mutations (TP53 versus PIK3CA) of human breast cancer cohorts (Cancer Genome Atlas, 2012) exemplifies how micro-stratified common disorders present a computational ad biological sampling problem related to those observed in infrequent diseases and a proxy for rare disorders.

Several limitations were observed. (i) This study focuses on parameters related to cancers, where substantial differences exist between paired normal and cancer tissues. While \(S_i\) have been shown to be effective in viral response (Gardeux et al., 2015, 2017) or response to therapy (Li et al., 2017a,b), it remains to be demonstrated that the downstream Inter-N-of-1 methods can outperform transcript-level methods in those biological conditions. (ii) The simulation does present some inconsistencies with observations made within the TCGA human breast cancer subsets. Methods within the simulation attain performances which appear to be highly inflated compared to observations made within the small human cohorts. Part of this points to the need to alter simulation parameters to provide more realistic conditions. Part of these discrepancies can probably also be explained by the fact that the breast cancer analyses used a reference standard that favored GLM + EGS over Inter-N-of-1 methods by design. (iii) We explored only one type of difference within gene set response between cohorts in the simulations: a cohort responsive versus unresponsive. We are thus undertaking the complementary analysis to compare the more general paradigm of gene sets more responsive in one cohort than in the other. (iv) Although the developed methods allow for a more accurate testing of interactions in datasets with small sample sizes, the importance of balancing confounders between the two cohorts cannot be overstated. The small samples used within these analyses prevent randomization from balancing key covariates and confounders between cohorts. Future studies could model unbalanced covariates through data or knowledge fusion with external datasets. (v) Transcript independence assumptions in the calculation of the single-subject odds ratio and its variance (Inter-N-of-1 methods) may be transgressed. However, many such assumptions are routinely overlooked in related analyses, such as BH-FDR (Benjamini and Hochberg, 1995) with similar limitations later rectified by the BY-FDR (Benjamini and Yekutieli, 2001). Other methods for controlling FDR may offer increases in power, although these methods again may not properly control FDR under general dependence structures observed in gene expression data (Storey, 2002; Storey et al., 2004). When viewed under that perspective, computational biology may progress by first proving new models and then addressing their biases in subsequent studies. (vi) Other unbiased approaches to generating gene sets could have been utilized (e.g. co-expression network from independent datasets, protein interaction networks, etc.). (vii) Of Note, few datasets are available with two measures in different conditions per subject and more than one clinical cohort of subjects. Similar to physics where experimentalist and theory influence one another, our work presents improvements on solving an experimental design that is infrequently used and merits more consideration for increasing the signal-to-noise ratio in the study of rare and infrequent diseases. (viii) Prospective biologic validation of results is also required in future studies as we have done with \(S_i^1\) in the past (Gardeux et al., 2014). (ix) The results presented for these methods are only for gene sets of size 15–500 and 40 and 200 (Simulation) and therefore validation of the performance of Inter-N-of-1 in gene sets of larger size requires future work.

Another consideration concerns the fact that GLM + EGS and Inter-N-of-1 evaluate different phenomena. The GLM + EGS method discovers GO terms enriched for transcripts and requires the coordination of signals at the transcript-level across subjects belonging to similar classes before the enrichment. The Inter-N-of-1, on the other hand, assesses whether the proportion of responsive transcripts within a given GO term measured in each subject significantly differs across cohorts. In other words, in the Inter-N-of-1, the transcripts contribution to the gene set signal may be different between subjects, while in the GLM + EGS methods a transcript-level coordination is required. The Inter-N-of-1 favors clinical applications where gene set mechanisms are causal to the disease. Cancer is one such condition where numerous genetic and transcriptomic root causes may differ between subjects and yet converge to comparable cellular and clinical phenotypes.

In conclusion, the proposed \(S_i^1\)-anchored Inter-N-of-1-methods demonstrate the utility of within-subject paired sample designs for better controlling the heterogeneity between subjects in a manner reminiscent of experimental isogenic models (e.g. cell lines or mice models). These results motivate further studies of new experimental designs, where paired within-subject samples allow analyses of data-sets previously considered too small. The new design not only presents opportunities in terms of performance within small cohorts, but also in terms of utility. By examining the single-subject results one can understand the degree of concordance and discordance amongst subjects and answer questions pertaining to whether specific subjects possess the overall observed signal. Thus, the Inter-N-of-1 presented here represents not just a new method that performs better within small sample sizes, but also an example for how to simultaneously conduct analyses on patient variability within and across cohorts. In addition, precision therapies designed for increasingly sub-stratified common disorders can benefit from the proposed methods. The strategies and methods presented here open a new frontier that may greatly enrich our understanding of the genetic foundations of rare diseases.

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References

Agresti, A. and Kateri, M. (2011) Categorical data analysis. Springer, Berlin, Heidelberg.

André, F. et al. (2019) Alpelisib for PIK3CA-mutated, hormone receptor-positive advanced breast cancer. N. Engl. J. Med., 380, 1929–1940.

Ashburner, M. et al. (2000) Gene ontology: tool for the unification of biology. Nat. Genet., 25, 25–29.

Ball, M. et al. (2019) Autologous micrograft accelerates endogenous wound healing response through ERK-induced cell migration. Cell Death Diff., 27, 1520–1538.

Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. J. R. Stat. Soc. B, 57, 289–300.

Benjamini, Y. and Yekutieli, D. (2001) The control of the false discovery rate in multiple testing under dependency. Ann. Stat., 29, 1165–1188.

Berghout, J. et al. (2018) Single subject transcriptome analysis to identify functionally signed gene set or pathway activity. In: Joanne Berghout, Qike Li, Nima Pouladi, Jianrong Li, and Yves A. Lussier (eds.) PSB. Symp Biocomput, World Scientific Publishing Company, pp. 400–411.

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Agresti, A. and Kateri, M. (2011) Categorical data analysis. Springer, Berlin, Heidelberg.

André, F. et al. (2019) Alpelisib for PIK3CA-mutated, hormone receptor-positive advanced breast cancer. N. Engl. J. Med., 380, 1929–1940.

Ashburner, M. et al. (2000) Gene ontology: tool for the unification of biology. Nat. Genet., 25, 25–29.

Ball, M. et al. (2019) Autologous micrograft accelerates endogenous wound healing response through ERK-induced cell migration. Cell Death Diff., 27, 1520–1538.

Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. J. R. Stat. Soc. B, 57, 289–300.

Benjamini, Y. and Yekutieli, D. (2001) The control of the false discovery rate in multiple testing under dependency. Ann. Stat., 29, 1165–1188.

Berghout, J. et al. (2018) Single subject transcriptome analysis to identify functionally signed gene set or pathway activity. In: Joanne Berghout, Qike Li, Nima Pouladi, Jianrong Li, and Yves A. Lussier (eds.) PSB. Symp Biocomput, World Scientific Publishing Company, pp. 400–411.
Brookes,S.T. et al. (2004) Subgroup analyses in randomized trials: risks of subgroup-specific analyses: power and sample size for the interaction test. J. Clin. Epidemiol., 57, 229–236.

Cancer Genome Atlas. (2012) Comprehensive molecular portraits of human breast tumours. Nature, 490, 61–70.

Ciriello,G. et al.; TCGA Research Network. (2015) Comprehensive molecular portraits of invasive lobular breast cancer. Cell, 163, 506–519.

Dreszer,T.R. et al. (2012) The UCSC genome browser database: extensions and updates 2011. Nucleic Acids Res., 40, D918–D923.

Edgar,R. et al. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res., 30, 207–210.

Elliott,E.J. and Zarzynski,Y.A. (2015) Rare diseases are a ‘common’ problem for clinicians. Austral. Fam. Phys., 44, 630–633.

Fisher,R.A. (1935) The logic of inductive inference. J. R. Stat. Soc., 98, 39–82.

Fleiss,J. (2004) The Design and Analysis of Clinical Experiments. John Wiley&Sons, New York.

Gardeux,V. et al. (2016) Concordance of deregulated mechanisms unveiled in underpowered experiments: PTBP1 knockdown case study. BMC Med. Genomics, 7, 81–13.

Gardeux,V. et al. (2017) A genome-by-environment interaction classifier for precision medicine: personal transcriptome response to rhinovirus identifies children prone to asthma exacerbations. J. Am. Med. Inf. Assoc., 24, 1116–1126.

Gardeux,V. et al. (2015) Towards a PBMC “virogram assay” for precision medicine: concordance between ex vivo and in vivo viral infection transcriptomes. J. Biomed. Inf., 55, 94–103.

Griggs,R.C. et al. (2009) Clinical research for rare disease: opportunities, challenges, and solutions. Mol. Genet. Metab., 96, 20–26.

Guillem,P. et al. (2008) Rare diseases in disabled children: an epidemiological survey. Arch. Dis. Child., 93, 113–118.

Kim,J.Y. et al. (2017) Clinical implications of genomic profiles in metastatic breast cancer with a focus on TP53 and PIK3CA, the most frequently mutated genes. Oncotarget, 8, 27997–28007.

Law,C.W. et al. (2014) voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol., 15, R29.

Leon,A.C. and Heo,M. (2009) Sample sizes required to detect interactions between two binary fixed-effects in a mixed-effects linear regression model. Comput. Stat. Data Anal., 53, 603–608.

Li,Q. et al. (2017a) N-of-1-pathways MxEnrich: advancing precision medicine via single-subject analysis in discovering dynamic changes of transcriptomes. BMC Med. Genomics, 10, 27.

Li,Q. et al. (2017b) kMEn: analyzing noisy and bidirectional transcriptional pathway responses in single subjects. J. Biomed. Inform., 66, 32–41.

Li,Q. et al. (2019) Interpretation of ‘Omics dynamics in a single subject using local estimates of dispersion between two transcriptomes. In: QiKe Li, Samir Rachid Zaim (eds.) AMIA Annual Symposium Proceedings. American Med. Informatics Association. pp. 582–591.

Liu,Y. et al. (2014) RNA-seq differential expression studies: more sequence or more replication? Bioinformatics, 30, 301–304.

Millard,S.P. et al. (2013) EmuStats An R Package for Environmental Statistics. Springer, New York, ISBN: 978-1-4614-9455-4.

Ozturk,K. et al. (2018) The emerging potential for network analysis to inform precision cancer medicine. J. Mol. Biol., 430, 2875–2889.

Powers,D.M. (2011) Evaluation: from precision, recall and F-measure to ROC, informedness, markedness and correlation. Inter. J. Mach. Learn Tech., 2, 37–63.

Robinson,M.D. et al. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26, 139–140.

Robinson,M.D. and Oshlack,A. (2010) scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol., 11, R25.

Schissler,A.G. et al. (2015) Dynamic changes of RNA-sequencing expression for precision medicine: N-of-1-pathways Mahalanobis distance within pathway-distant subjects predicts breast cancer survival. Bioinformatics, 31, i293–302.

Schissler,A.G. et al. (2016) Analysis of aggregated cell-cell statistical distances within pathways unveils therapeutic-resistance mechanisms in circulating tumor cells. Bioinformatics, 32, i80–i89.

Soneson,C. and Delorenzi,M. (2013) A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics, 14, 91.

Stoeck,J.D. (2002) A direct approach to false discovery rates. J. R. Stat. Soc. B (Stat. Methodol.), 64, 479–498.

Stoeck,J.D. et al. (2004) Strong control, conservative point estimation and simultaneous conservative consistency of false discovery rates: a unified approach. J. R. Stat. Soc. B (Stat. Methodol.), 66, 187–205.

Subramanian,A. et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA, 102, 15454–15500.

Tao,Y. et al. (2007) Information theory applied to the sparse gene ontology annotation network to predict novel gene function. Bioinformatics, 23, i529–i38.

Tarazona,S. et al. (2015) Data quality aware analysis of differential expression in RNA-seq with NOISeg R/bioc package. Nucleic Acids Res., 43, e140.

Van Keymeulen,A. et al. (2016) TCGA2STAT: simple TCGA data access for integrative analyses. PloS One, 11, e0159234.

Van Keymeulen,A. et al. (2016) Testing for differentially expressed genetic pathways with single-subject N-of-1 data in the presence of inter-gene correlation. Stat. Methods Med. Res., 27, 3797–3813.

Schurch,N.J. et al. (2016) How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? RNA, 22, 839–851.

Ward,C.W. et al. (2016) How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? RNA, 22, 839–851.

Wan,Y.W. et al. (2010) Dynamic changes of RNA-sequencing expression for precision medicine: N-of-1-pathways Mahalanobis distance within pathway-distant subjects predicts breast cancer survival. Bioinformatics, 31, i293–302.