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Analyze Multivariate Phenotypes in Genetic Association Studies by Combining Univariate Association Tests

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Abstract

Multivariate phenotypes are frequently encountered in genome-wide association studies(GWAS). Such phenotypes contain more information than univariate phenotypes, but how to best exploit the information to increase the chance of detecting genetic variant of pleiotropic effect is not always clear. Moreover, when multivariate phenotypes contain a mixture of quantitative and qualitative measures, limited methods are applicable. In this paper, we first evaluated the approach originally proposed by O'Brien and by Wei and Johnson that combines the univariate test statistics and then we proposed two extensions to that approach. The original and proposed approaches are applicable to a multivariate phenotype containing any type of components including continuous, categorical and survival phenotypes, and applicable to samples consisting of families or unrelated samples. Simulation results suggested that all methods had valid type I error rates. Our extensions had a better power than O'Brien's method with heterogeneous means among univariate test statistics, but were less powerful than O'Brien's with homogeneous means among individual test statistics. All approaches have shown considerable increase in power compared to testing each component of a multivariate phenotype individually in some cases. We apply all the methods to GWAS of serum uric acid levels and gout with 550,000 SNPs in the Framingham Heart Study.

Keywords

multivariate phenotype; O'Brien's Statistic; genome-wide association study; genetic association study; categorical data

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Web Resources

Softwares written in R for O'Brien's method, sampling splitting and cross-validation method is available by email to Dr. Qiong Yang (qyang@bu.edu)

Introduction

Association studies, where the correlation between a genetic variant and a phenotype is assessed to identify disease susceptible loci, have better power and precision than the traditional linkage studies [Risch and Merikangas, 1996]. Historically, genetic association studies have focused on fine mapping the causal variants in candidate gene regions, which limits the possibility of discovering previously unsuspected loci and revealing novel genetic mechanism of disease etiology. With reduction of genotyping cost, and advances of Interational HapMap Project and with nearly 3 million of single nucleotide polymorphisms (SNPs) genome-wide identified across four major human populations, genome-wide association studies have become a reality. Latest commercial marker arrays such as Affymetrix GeneChip 6.0 and Illumina HumanHap550 are estimated to cover 80%-90% of all heterozygous SNPs genome-wide by including or having a high LD ($r2 \geq 0.8$) [International HapMap Consortium et al., 2007]. The recent efforts of genome-wide association studies have been fruitful [Manolio, Brooks and Collins, 2008] in the identification of novel genetic variants predisposing to a variety of complex traits.

In the study of a complex disease, several correlated traits may be measured for a disorder or its risk factors. For example, hyperuricemia usually presents in patients with gout; cytokine interleukin-6 (IL-6), C-reactive protein (CRP), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and fibrinogen are circulating inflammatory markers correlated with each other and predict CHD [Yudkin et al., 2000, Rifai and Ridker, 2002]. There may be genetic variants affecting several of these traits. Analyzing each trait separately will suffer penalties from the multiple testing and result in a reduced power especially for GWAS. It has been shown that modeling multivariate phenotypes may increase the power over analyzing individual phenotypes separately in genetic association studies [Klei et al., 2008, Lange et al., 2004].

Assuming all traits are of the same type (e.g. all continuous, or all binary), classic multivariate methods can be readily applied to genetic association studies of multivariate phenotypes including likelihood based mixed effects model (LME) [Laird and Ware, 1982, Fitzmaurice and Laird, 1993], and generalize estimating equations (GEE)[Liang and Zeger, 1986]. Extensions of these methods to a mixture of continuous and binary responses have been proposed [Liu et al., 2009, Fitzmaurice and Laird, 1997]. Liu et al [Liu et al., 2009] recently proposed an extension of the GEE to perform bivariate association analyses for a mixture of continuous and binary phenotypes. However, only bivariate phenotypes were implemented. In addition, their method is only applicable to samples of unrelated individuals. Fitzmaurice et al. [Fitzmaurice and Laird, 1997] proposed a likelihood method that may require considerable effort to implement for high dimensional multivariate phenotypes. Alternative to likelihood or GEE models, O'Brien [O'Brien, 1984] and Wei and Johnson [Wei and Johnson, 1985] described a much simpler approach that combines dependent test statistics, which can be used to integrate the results from linkage or association tests of individual traits of a multivariate phenotype[Xu, Tian and Wei, 2003] (We refer it to as O'Brien's method thereafter). With a homogeneous mean among individual statistics, O'Brien's method provides the most powerful test statistic among those that linearly combine these statistics. However, this method may not be as powerful when means among individual statistics are heterogeneous. To overcome this limitation, Xu et al. [Xu, Tian and Wei, 2003] proposed an extension of O'Brien's method. However their approach may not improve the power when the effects are not in the same direction and involves permutation that assumes unrelated individuals.

In this article, we have proposed using O'Brien's method that combines univariate GWAS results of multivariate phenotypes as a screening tool for detecting pleiotropic effects. In

addition, we propose two extensions of O'Brien's method to deal with heterogeneous mean among individual test statistics. The first extension is a sample splitting method. The second extension is a cross-validation method averaging multiple sample splitting results.

Methods

O'Brien's method

We first describe the existing method by O'Brien [O'Brien, 1984] and Wei and Johnson [Wei and Johnson, 1985] that can be used to combine dependent test statistics from testing association between each of the correlated traits and a genetic marker [Xu et al 2003].

Assume that there are K correlated traits, either quantitative or qualitative. Let $\mathbf{T} = (T_1,T_2,....,T_k)^T$ be a vector of K test statistics obtained from association analyses of each individual trait against the genetic marker. Assume $\boldsymbol{T}$ follows a multivariate normal distribution with mean $\boldsymbol{\beta} = (\beta_1,\beta_2,....,\beta_K)^T$ and known or estimated covariance matrix Σ.

Suppose the null hypothesis of no association is $H_0 : \boldsymbol{\beta}= (\beta_1,\beta_2,....,\beta_K)^T = \mathbf{0}$, and the alternative hypothesis H_1: at least one $\beta_k \geq 0$, $k = 1, \ldots, K$. O'Brien suggested that the following linear combination of $T_1,T_2,....,T_k$, with weight $e = (1,1,\ldots,1)^T$ of length K,

$$U=e^T \sum^{-1} \mathbf{T} \tag{1}$$

When $\beta_1=\beta_2 = ...=\beta_k > 0$, (1) is the most powerful test statistic among a class of tests statistics that are linear combination of $T_1,T_2,....,T_k$. Under the null hypothesis, U follows the normal distribution with variance $e^T \Sigma^{-1} e$.

This approach can be readily used to combine univariate GWAS test statistics (beta coefficients or $\boldsymbol{Z}$ statistics) of multivariate phenotypes to create a test of pleiotropic effects. The Σ matrix can be approximated by the sample (co)variance matrix of the test statistics of all SNPs. For each SNP, U is obtained as a test for the SNP affecting at least one of the phenotypes

This approach does not require all traits to be observed for an individual in order to contribute to the analyses, and thus can better handle missing data than methods that only use data on individuals with all traits observed. In addition, this method can be used to analyze multivariate phenotypes obtained on unrelated individuals or families, and any types (continuous, dichotomous or survival) of phenotype, as long as $T_1,T_2,....,T_k$ are obtained using a valid method. However, the power of this method may be less optimal when β's are heterogeneous.

To overcome such limitation of O'Brien's method, in what follows, we propose two extensions of O'Brien's method to deal with heterogeneous β's.

Sample splitting method

We propose using non-uniform weights in (1) to reflect the potential heterogeneity of β's. This strategy first splits the sample into two subsets, one for estimating weights and the other for constructing final testing statistic. Suppose the test statistic obtained using the estimation set is $\hat{T}_w$ and using the testing set is T. The final statistic is

$$S = \widehat{\mathbf{T}}_w^T \sum^{-1} \mathbf{T} \tag{2}$$

, approximately normal distributed with variance $\mathbf{T_W}^T \Sigma^{-1} \mathbf{T_W}$

If the data consist of families, each family will be treated as a single unit in the splitting step thus all members will be in the same subset.

Nevertheless, arbitrarily dividing the samples may be an "unfortunate" split. To overcome this limitation, we further propose a cross-validation scheme.

Cross-validation method

We consider cross-validation strategies [Hastie, T., Tibshirani, R., Friedman, J.H., 2003] [Klei et al., 2008] that overcome to a large degree the limitations of the simple one-time splitting. The trade-off, of course, is that these strategies always involve heavier computation. The family of cross-validation methods includes random subsampling, *K – fold* cross-validation, and leave-one-out (LOO) cross-validation. We choose random subsampling that can be considered as execution of random sample splitting method repeatedly. It randomly divides the dataset into training and testing data of a fixed size partition multiple times and averages the resulting statistics from all splits. Below is a description of the algorithm of the proposed cross-validation method:

1. Perform random sample splitting *n* times to obtain *n* realizations of *S* statistic based on (2). Let $\bar{S}_{obs}$ be the average of the *n* S statistics.

   Since the *n* samples are partially overlapped, the S statistics calculated based on the samples are not independent. Thus the distribution of $\bar{S}_{obs}$ is intractable. We hereby propose using permutation and resampling approaches to estimate the variance empirically in the following steps:

2. For data consisting of unrelated individuals, generate *m* random permutations of the multivariate phenotype (treating all components as a single unit) among all the individuals.

3. For each permutation sample generated in step 2, calculate, $\bar{S}_i$ *i*= 1, …, *m* same as in step 1 to form a reference distribution of $\bar{S}$ under the null hypothesis of no association between any of the traits and the genetic marker.

4. The *P – value* can be obtained by

$$P - value = \frac{\sum_{i=1}^{m} I_{\{|\bar{S}_i| > |\bar{S}_{obs}|\}}}{m}$$

   where ***I*** is an indicator function that takes value 1 if $|\bar{S}_i| > |\bar{S}_{obs}|$ and 0 otherwise.

If the data consist of families, simple permutation of phenotypes is not appropriate because the correlation of the phenotypes among related individuals is not maintained. When the sample is not ascertained based on genotype, we can randomize genotype instead of phenotype to obtain the null distribution of $\bar{S}$. Specifically, the second step of the algorithm is modified as follows: Randomly generate genotypes for founders according to the observed genotype frequencies in founders, and then generate offspring genotypes according

to Mendelian transmission. Repeat this procedure *m* times to obtain *m* samples. The third and fourth steps are unchanged.

Simulations

Validity and power of O'Brien's method, the proposed random splitting and cross-validation methods were compared using Monte Carlo simulations.

In the simulation studies, multivariate continuous phenotypes and genotypes on members of independent nuclear families were firstly generated. Correlated dichotomous phenotypes were generated using a threshold model based on correlated continuous phenotypes. We assume each family has four individuals, two parents and two children. Each individual has five phenotypes. The methods were evaluated for the association with a single SNP with allele frequency 0.5 (different allele frequencies yield similar results, data not shown). Parental genotypes were first generated assuming Hardy-Weinberg equilibrium and random mating. Children's genotypes are simulated conditional on parental genotypes assuming Mendelian law of transmission.

Multivariate continuous phenotypes were generated conditional on family relationships and genotypes. Assuming the observations between families are independent, five measurements on the four individuals within the i^{th} family are randomly drawn from a multivariate normal distribution (for convenience, subscript *i* is omitted; y_{kl} is the k^{th} measurement on the l^{th} person):

$$Y=\begin{bmatrix} y_{11} \\ y_{12} \\ \vdots \\ y_{44} \\ y_{45} \end{bmatrix} \sim MVN\left(\begin{bmatrix} \beta_1 X(g_1) \\ \beta_2 X(g_1) \\ \vdots \\ \beta_4 X(g_4) \\ \beta_5 X(g_4) \end{bmatrix}, \Psi\right)$$

where β is additive genetic effect size, with X(.) being the additive score of the coded allele. Ψ is the residual covariance matrix as the follows:

$$\Psi=\begin{cases} \sigma^2_{Gk}+\sigma^2_{ek}+\sigma^2_M & \text{within same person, phenotype } k \text{ and } k \\ \sigma_{Gk}\cdot\sigma_{Gl}+\rho_{kl}\cdot\sigma^2_M & \text{within same person, phenotypes } k \text{ and } l \\ 0 & \text{between parents, phenotypes } k \text{ and } l \\ \sigma_{Gk}\cdot\sigma_{Gl}\cdot 2\Phi & \text{between parent and child, phenotypes } k \text{ and } l \\ \sigma_{Gk}\cdot\sigma_{Gl}\cdot 2\Phi & \text{between 2 siblings, phenotype } k \text{ and } l \end{cases}$$

where σ^2_{Gk} and σ^2_{ek} are the polygenic and error variances of the k^{th} phenotype respectively, σ^2_M the multivariate phenotype variance within a individual, ρ_{kl} is the correlation between phenotype *k* and *l* attributable to multivariate phenotype variance, and Φ is the kinship coefficient between the two person (in this case, ¼), and $\sigma^2_{Gk}+\sigma^2_m+\sigma^2_{ek}=1$.

Under this model, the effect size of the SNP on the k^{th} measurement is

$$\beta_k=\sqrt{\frac{\sigma^2_{gk}}{2p(1-p)}}$$

where p is the modeled allele frequencies at the locus and σ^2_{gk} is the total phenotypic variance explained by the SNP. For the sake of simplicity, we kept total heritability at 40% for all scenarios.

We first considered several scenarios where the multivariate phenotypes contained continuous and dichotomous phenotypes. After generating five continuous multivariate variables for each individual, we converted the second and the fourth variables into dichotomous phenotypes using a threshold of 0.5 Briefly, if the original trait value was above the threshold, 1 was assigned as the new dichotomous phenotype value and 0 otherwise, which resulted in about 30% individuals assigned 1. These scenarios are summarized in Table 1 and named as Scenario 1, 2 and 3 respectively, which range from a setting with homogeneous means among individual test statistics to the one with very different means among individual test statistics.

We next studied the performance of all methods when the genotype effects were in different directions among the individual phenotypes where the pairwise phenotypic correlations were all in the same direction. We simulated negative effects for the SNP on phenotypes 2 and 3 and positive effects on the remaining phenotypes. This is referred to as scenario 4 in Table 1.

Finally, since these combining statistics approaches can be applied to all-continuous multivariate phenotypes, we further compared them with a score of general multivariate methods including linear mixed effects model with a common fixed effect coefficient for the SNP genotype with all phenotypes, multivariate analysis of variance (MANOVA), summary statistics (SS, specifically, taking average of the phenotype), and principal components of phenotypes (PCP, using the first component from principle component analyses of the phenotypes). We consider one scenario with concordant effect direction and the other with discordant effect direction. They were referred to as scenario 5 and 6 respectively in Table 1.

Application to GWAS of Uric Acid and Gout in the Framingham Heart Study

Uric acid (UA) is a final product from purine oxidation. Because human beings lack uricase to further break down uric acid into solutable form, most uric acid dissolves in blood and is excreted to urine through kidney. Hyperuricemia is often found in patients with gout, a common form of inflammatory arthritis caused by deposition of mono-sodium urate or uric acid crystals on the joints and surrounding tissues[Choi et al., 2005][Lawrence et al., 2008]. Gout affects more than 3 million people in the United States and the trend is increasing [Lawrence et al., 2008]. There has been renewed interest in the controversial roles of hyperuricema and gout in cardiovascular disease, renal function, diabebtes and hypertension [Feig, Kang and Johnson, 2008]. Traditional risk factors of hyperuricemia and gout include age, gender, body mass index, alcohol consumption and diuretics use. Uric acid is also highly heritable with heritability previously estimated at 0.63[Yang et al., 2005] in the Framingham Heart Study suggesting potential genetic determinants. Recent GWAS have identified many novel genes, most are urate transporters, associated with UA or gout [Li et al., 2007, Wallace et al., 2008, Kolz et al., 2009, Dehghan et al., 2008, Woodward et al., 2009]. Extending our previous GWAS that focused on UA and gout as individual phenotypes [Dehghan et al., 2008], we applied the combining statistics approaches proposed in this article to joint analyze uric acid and gout in more than 7,000 Framingham Heart Study participants to further elucidate genetic etiology of uric acid metabolism and gout.

The Framingham Heart Study (FHS) is an ongoing prospective longitudinal study aimed to research cardiovascular disease, stroke and their risk factors. Initiated in 1948, 5209 individuals were enrolled from the town of Framingham, Massachusetts [DAWBER,

KANNEL and LYELL, 1963] and underwent biannual examinations. Beginning in 1971, 5124 adult children of original cohort and spouses of these children were enrolled and examined approximately every 4 years, consisting of the offspring cohort [Feinleib et al., 1975]. In 2002, 4095 third generation individuals were enrolled and have been examined [Splansky et al., 2007]. Most FHS participants are self-identified as Caucasian (white).

In 2007, genotyping of Affymetrix 500K mapping array and the Affymetrix 50K supplemental array was performed on 9274 participants from FHS all three generations (SNP Health Association Resource; www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000007.v2.p1). Genotyping was considered successful for 8481 individuals (sample call rate ≥97%). SNPs were excluded from our analyses if call rate was less than 95%, MAF<0.05 or Hardy–Weinberg equilibrium $p<10^{-6}$, making the total final number of 391,059 SNPs. Each participant has provided written informed consent, and study protocols were approved by the Institutional Review Boards of Boston University Medical Campus.

UA was measured at the first examination cycle of each cohort using an autoanalyzer with a phosphotungstic acid reagent [CROWLEY, 1964]. Gout was ascertained via self-report in the offspring subjects during exam cycles 3–7, and the first exam of the third generation. The final sample for analyses consists of 7699 individuals for uric acid and 7386 individuals for gout (n cases=197, 2.7%), who were successfully genotyped and have phenotype and covariates including age, gender, body-mass index, alcohol consumption, and hypertension treatment.

In current study, we applied the three combining statistical approaches to joint analyze UA and gout to examine their association with the 391,059 SNPs. The threshold for genome-wide significance is 1.28×10^{-7}, i.e. Bonferroni corrected alpha level of 0.05 for the total number of 391,059 SNPs (tests) for each method. We also considered a less stringent threshold 2.56×10^{-6} for observing one false positive out of all tests [Gordon et al., 2007]. For O'Brien's method, we used the Z statistics from previous individual UA and gout GWAS using multivariable adjusted linear mixed effects model and generalized estimating equations respectively that accounted for family relationship [Dehghan et al., 2008]. The covariance between the two Z statistics was estimated using the sample covariance of the two Z statistics of all SNPs. Thus for each SNP, the test statistic (1) takes the following form:

$$(1,1)\begin{bmatrix} \sigma^2_{Z_{UA}} & \rho\cdot\sigma_{Z_{UA}}\sigma_{Z_{gout}} \\ \rho\cdot\sigma_{Z_{UA}}\sigma_{Z_{gout}} & \sigma^2_{Z_{gout}} \end{bmatrix}\begin{pmatrix} Z_{UA} \\ Z_{gout} \end{pmatrix}$$

where Z_{UA} and Z_{gout} are the Z statistics for association test of the SNP with UA and gout respectively; $\sigma^2_{Z_{UA}}=1.00$ is the sample variance of Z_{UA} of all SNPs; $\sigma^2_{Z_{gout}}=1.07$ is the sample variance of Z_{gout} of all SNPs; and ρ=0.17 is the sample correlation coefficient of Z_{UA} and Z_{gout} of all SNPs.

For random sample splitting method, we randomly selected 30% of the 1,399 families (n=420) as estimation sample, and the rest 979 families as testing sample. Separate GWAS of UA and gout were carried out using estimating and testing samples respectively. For each SNP, the final test statistic (2) is

$$(Z_{UA}^{(w)}, Z_{gout}^{(w)}) \begin{bmatrix} \sigma^2_{Z_{UA}} & \rho \cdot \sigma_{Z_{UA}} \sigma_{Z_{gout}} \\ \rho \cdot \sigma_{Z_{UA}} \sigma_{Z_{gout}} & \sigma^2_{Z_{gout}} \end{bmatrix} \begin{pmatrix} Z_{UA}^{(t)} \\ Z_{gout}^{(t)} \end{pmatrix} \quad (3)$$

where $Z_{UA}^{(w)}, Z_{gout}^{(w)}$ are Z statistics for UA and gout calculated using the estimating sample, and $Z_{UA}^{(t)}, Z_{gout}^{(t)}$ are those estimated using the testing sample; $\sigma^2_{Z_{UA}}, \sigma^2_{Z_{gout}}, \rho$ are sample variances and correlations coefficient estimated using the testing sample. For cross-validation method, 10 replicates of random sample splitting were performed, and for each SNP, tests statistics (3) from the 10 replicates were averaged to form the final test statistic. The null distribution of the final test statistic was obtained by randomizing the genotypes outlined at the end of the cross-validation method. We generated 2 million replicates at minor allele frequency 0.25 (null distributions at other MAFs are similar to this based on initial comparison of 10,000 replicates) and the null distribution was estimated using kernel smoothing method.

Although our example used family data, the process of constructing each of the statistics (O'Brien's, sample splitting and cross-validation) would be nearly identical had the sample contained only unrelated individuals. With unrelated individuals, the only difference would be that all the Z statistics (Z_{UA}, Z_{gout}, $Z_{UA}^{(w)}, Z_{gout}^{(w)}, Z_{UA}^{(t)}, Z_{gout}^{(t)}$) should be obtained using methods valid for unrelated individuals. Similarly, had the types of phenotypes were different from our example, the only difference would be that these Z statistics should be obtained using methods valid for those phenotypes.

Results

Splitting ratio for sample splitting method

For random splitting, we evaluated power and type I error for different ratios of the estimation to testing sample sizes. The results reveal that using 30% sample for estimating weights and the remaining 70% for calculating test statistic is slightly more powerful than other splitting ratio in all the scenarios (Supplementary Figure 1s). Thus, we used the 30% to 70% ratio for sample splitting and cross-validation methods throughout the simulations

Type I error and power of combining statistics approaches

Valid type I error were observed for O'Brien, random splitting and cross-validation method (Table 2) for all scenarios. The power of the three methods at alpha=0.05 is compared in Figure 1 for scenarios 1–4. Cross-validation method outperformed the sample splitting method consistently. Generally speaking, when the means among the individual test statistics were similar (Figure 1–Scenario 1), O'Brien's method was more powerful than sample splitting and cross-validation approach and showed the largest improvement in power over using any individual test statistics, with or without adjusting for multiple testing for the latter. When the means among the individual test statistics were very different but in the same direction (Figure 1–Scenarios 2, 3), O'Brien's method may be inferior to sample splitting and cross-validation approach, and the power of all considered combining statistics approaches was similar to the most powerful individual test statistic after multiple testing adjustment. When the means among individual test statistics had opposite directions (Figure 1–Scenario 4), O'Brien's method may suffer huge power loss, while sampling splitting and cross-validation methods had notable increase in power, and outperformed any test of individual phenotypes with or without any multiple testing correction. The relative power among methods was similar for alpha=0.01. Power for all scenarios at alpha=0.05 and 0.01 are included in Supplementary Table 1.

Comparing combining statistics methods with other multivariate methods

The combining statistics approaches have valid type I error when applied to all-continuous multivariate phenotypes (Table 2). When comparing combing statistics approaches with multivariate methods for continuous traits with effects in the same direction, we found that combining statistics approaches were just slightly less powerful than all other approaches (Figure 2, Scenario 5) for the scenarios considered in our simulations. When the effects were of different directions, methods (O'Brien, LME-common effect for all phenotypes, SS and PCP) that assumed the same effect direction show the largest power loss and the proposed cross-validation method had a similar power to MANOVA (Figure 2, Scenario 6).

GWAS of uric acid and gout in FHS

A comparison of individual GWAS of UA and Gout with approaches combining the GWAS results of UA and gout is presented in Figure 3. Loci identified at the suggestive threshold are presented in Table 3. The genomic control parameter [Devlin and Roeder, 1999] that measures inflation due to population admixture or other confounders was 1.04, 1.02, for GWAS of UA and gout respectively, and <1 for all three combining statistics approaches. Combing statistics approaches detected more genes compared to GWAS of individual phenotypes at the suggestive threshold but genome-wide significant findings (*SLC2A9*, *ABCG2*, *SCL17A1*, *SCL17A2*, *SCL17A3*) are almost identical across all approaches. O'Brien's method detected more loci at suggestive level than sample splitting and cross-validation methods, but failed to detect *SCL17A1/SCL17A2/SCL17A3* with genome-wide significance as the other two methods.

Some of genes detected only by O'Brien's method and our cross-validation approaches have not been previously implied in UA or Gout, but may be potential candidates: The product of *P2RX2* belongs to the family of purinoceptors for ATP[Lynch et al., 1999]; *FAM92A1* is a new tumor-related gene with oncogenic potentials to probably play roles in renal carcinogenesis[Liang et al., 2009]; *INHBC* encodes the beta C chain of inhibin, a member of the TGF-beta superfamily*; KYNU en*codes kynureninase, a pyridoxal-5′-phosphate (pyridoxal-P) dependent enzyme that catalyzes the cleavage of L-kynurenine and L-3-hydroxykynurenine into anthranilic and 3-hydroxyanthranilic acids, respectively. The kynurenine pathway has been proposed as a drug target for hypertension [Schwarcz, 2004], and polymorphism in KYNU has been found associated with essential hypertension[Zhang et al., 2005]. Further confirmation of these findings in independent cohorts is warranted.

Discussion

In this work, we have proposed using O'Brien's method that combines univariate GWAS results of multivariate phenotypes as a screening tool for detecting pleiotropic effects. In addition, we have proposed two new extensions of O'Brien's method: a sample splitting approach and a cross-validation approach.

Compared to multivariate approaches that model all outcomes simultaneously, combining statistics approaches have the advantages of involving less knowledge or assumptions about the relationship among the individual phenotypes. The combining statistical approaches considered here can be easily applied to data consisting of unrelated individuals or families, and to individual phenotypes that are not of the same type. Although we have used family data in our simulations and applications, the methods and conclusions about the methods are applicable to studies of multivariate phenotypes obtained on unrelated individuals.

When the means of individual test statistics are homogeneous, O'Brien's method provides the highest power among all methods that linearly combine test statistics, and shows substantial power gain over tests of individual phenotypes, with or without multiple testing

corrections. Our newly proposed approaches (sample splitting and cross-validation) outperform O'Brien's method only when the effects are very heterogeneous. When the effects are in different directions, O'Brien's method may have a very low power while the new methods gain additional power.

Currently huge efforts have been put into GWAS to search for genetic determinations of hundreds of phenotypes related to human health. But most of the time, phenotypes were considered separately. While such efforts have been fruitful, it is apparent from our simulation studies that combining the association from multiple phenotypes may greatly increase the power to detect genetic variant affecting multiple phenotypes. The existing O'Brien approach and our newly proposed approaches may help discover previously unknown mechanism of pleiotropic effects of genetic variants.

Furthermore, we found combining test statistics methods have comparable power to general multivariate methods for continuous traits. Since O'Brien's method is generally easier than fitting a multivariate model, especially when individual phenotypes may have already been analyzed in a first round of GWAS, it can serve as a convenient tool for data mining of pleiotropic effects in GWAS result databases for all types of phenotypes. We considered only complete data and a fixed number of phenotypes in our simulations. The relative efficiency between O'Brien's method and general multivariate methods may be different from our simulation results when these conditions are changed. We randomly assigned 25% and 50% observations of each individual phenotype to be missing for data generated under scenario 5. With the incomplete data, the power of O'Brien's method was still slightly below linear mixed effects model (Supplementary Figure). Further studies of the relative efficiency between O'Brien's method and general multivariate methods are warranted.

Our sample splitting and cross-validation approaches, which are more powerful than O'Brien's method to detect SNPs affecting phenotypes in opposite directions, are especially useful to detect such pleiotropic effects usually previously unsuspected for phenotypes having a positive correlation. A good example is SNP rs780094 in glucokinase regulatory protein (GCKR) gene. The less common C allele of this SNP is associated with increased fasting plasma triglyceride and CRP levels but decreased fasting glucose concentrations[Orho-Melander et al., 2008], although the three phenotypes are positively correlated. We have provided a theoretic justification (Appendix) of why the power would increase for sample splitting but decrease for O'Brien's method for bivariate phenotypes. Basically when the effects are not consistent, they may cancel each other in O'Brien statistics but not in the statistics of sample splitting or cross-validation method.

When the effects are not all in the same direction, we saw a large power increase in methods that allow effects being of different directions (sample splitting, cross-validation, and MNOVA), comparing to analyzing effects of the same magnitude that are all in the same direction. This phenomenon has also been noted in the recent study analyzing bivariate phenotypes consisting of continuous and dichotomous traits [Liu et al., 2009]. Theoretic justification (Appendix) for bivariate phenotype suggests that O'Brien statistics can lose power if the two individual statistics are of opposite directions, while sample splitting and cross-validation can gain power because the denominator decreases with a decreased correlation between the test statistics.

O'Brien's method and the newly proposed sampling splitting used similar amount of time to run (3.5 minutes for analyzing 550,000 SNPs for two phenotypes on a 2.6 GHz 64-bit AMD Opteron CPU and 12 GB of RAM using R). Not surprising, cross-validation method was more time consuming due to the need to infer the null distribution but is still realistic with

today's computing power: 10,000 replicates of 10-fold cross-validation for a single MAF took about 75 hours to complete on a single computer node.

In our simulations, we assume that the correlations of the individual statistics are known. For statistics calculated from linear regression or other simple models, the correlation of the statistics can be estimated using the data. However, for more sophisticated models such as linear mixed effects model, the correlation is intractable. In this case, bootstrap resampling may be used to estimate the correlation. In genome-wide association analyses, correlation of individual test statistics can be approximated by the sample correlation of the test statistics of a large number of SNPs.

In summary, we proposed combining statistics approaches for testing genetic association with multivariate phenotypes in genome-wide association analyses. Combining statistics approaches are powerful tools for screening genetic loci of pleiotropy effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendices

O'Brien and Sample Splitting methods for bivariate phenotypes

The univariate test statistics from analyzing individual phenotypes of the a bivariate phenotype are denoted as $\hat{T} = [\hat{T}_1, \hat{T}_2]^T$ and the covariance of individual statistics are denoted as

$$\sum = \mathrm{cov}(\widehat{T}_1, \widehat{T}_2) = \begin{bmatrix} 1 & \rho \\ \rho & 1 \end{bmatrix}$$

The O'Brien statistic takes the following form:

$$\begin{aligned} \frac{e^T \sum^{-1} \widehat{T}}{\sqrt{e^T \sum^{-1} e}} &= \frac{[1,1] \begin{bmatrix} 1 & \rho \\ \rho & 1 \end{bmatrix}^{-1} \begin{bmatrix} \widehat{T}_1 \\ \widehat{T}_2 \end{bmatrix}}{\sqrt{[1,1] \begin{bmatrix} 1 & \rho \\ \rho & 1 \end{bmatrix}^{-1} \begin{bmatrix} 1 \\ 1 \end{bmatrix}}} \\ &= \frac{[1,1] \frac{1}{1-\rho^2} \begin{bmatrix} 1 & -\rho \\ -\rho & 1 \end{bmatrix} \begin{bmatrix} \widehat{T}_1 \\ \widehat{T}_2 \end{bmatrix}}{\sqrt{[1,1] \frac{1}{1-\rho^2} \begin{bmatrix} 1 & -\rho \\ -\rho & 1 \end{bmatrix} \begin{bmatrix} 1 \\ 1 \end{bmatrix}}} \\ &= \frac{\frac{1}{1+\rho}(\widehat{T}_1 + \widehat{T}_2)}{\sqrt{\frac{1}{1+\rho} \cdot 2}} \\ &= \frac{1}{\sqrt{1+\rho}} \cdot \frac{(\widehat{T}_1 + \widehat{T}_2)}{\sqrt{2}} \end{aligned}$$

For sample splitting method, denote $\widehat{T}_W = \begin{bmatrix} \widehat{T}_{1W} & \widehat{T}_{2W} \end{bmatrix}^T$ as statistics obtained using estimation sample, and $\hat{T} = [\hat{T}_1, \hat{T}_2]^T$ as statistics obtained using testing sample. The final test statistic is

$$\frac{\widehat{T}_W^T \Sigma^{-1} \widehat{T}}{\sqrt{\widehat{T}_W^T \Sigma^{-1} \widehat{T}_W}} = \frac{\frac{1}{1+\rho}(\widehat{T}_{W1}\widehat{T}_1 + \widehat{T}_{W2}\widehat{T}_2)}{\sqrt{\frac{1}{1+\rho}\cdot(\widehat{T}_{W1}^2 + \widehat{T}_{W2}^2)}} = \frac{1}{\sqrt{1+\rho}} \cdot \frac{(\widehat{T}_{W1}\widehat{T}_1 + \widehat{T}_{W2}\widehat{T}_2)}{\sqrt{\widehat{T}_{W1}^2 + \widehat{T}_{W2}^2}}$$

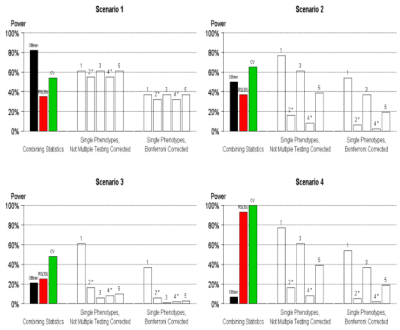

Figure 1. Power comparison among combining statistics methods, univariate tests of each individual phenotypes (scenarios 1–4)

Note:

Color bars present the power of the combining statistics approaches: The bar with Obrien on top represents O'Brien's method, with RS(30) presents random splitting method that uses 30% families to estimate T_W, with CV represents cross-validation method Power for each approach was calculated at alpha=0.05.

White bars present power of univariate test of individual phenotypes with phenotype number on the top; * indicates the phenotype is dichotomous. The group of bars in the middle corresponds alpha=0.05 for each phenotype, not corrected for multiple testing; the group of bars on the right corresponds an alpha level with Bonferroni correction for 5 tests (alpha=0.01 for each individual phenotype)

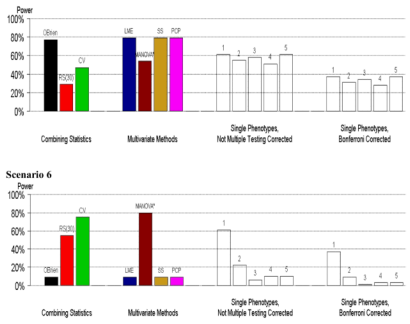

Figure 2. Power comparisons between combining statistics methods, general multivariate methods and single phenotype analysis for all continuous traits (scenarios 5 and 6) Scenario 5

Note:

Color bars present the power of the combining statistics approaches: Obrien on top represents O'Brien's method; RS(30), random splitting method that uses 30% families to estimate T_W; CV, cross-validation method; LME, linear mixed effects model assuming the same effects across all phenotypes; SS, summary statistics (mean of the statistics); PCP first principal component of the phenotypes. Power for each approach was calculated at alpha=0.05.

White bars present power of univariate test of individual phenotypes with phenotype number on the top; * indicates the phenotype is dichotomous. The group of bars in the middle right corresponds alpha=0.05 for each phenotype, not corrected for multiple testing; the group of bars on the far right corresponds an alpha level with Bonferroni correction for 5 tests (alpha=0.01 for each individual phenotype)

*The power of MANOVA method was adjusted to empirical 5% level.

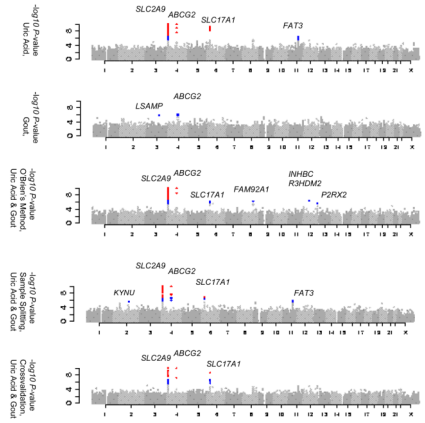

Figure 3.

Comparison of individual GWAS of uric acid (UA) and gout with combining statistics approaches. The top two panels are GWAS of UA and gout respectively; 3rd–5th panels are results of O'Brien's, random sample splitting and cross-validation methods respectively. The x-axis is chromosome locations, y-axis is the minus log base 10 of the p-value truncated at 10. Points highlighted in red are those exceeding the genome-wide significant threshold (1.28×10^{-7}) and in blue are those exceeding a threshold (2.56×10^{-6}) of suggestive association.

Table 1

Scenarios of multivariate phenotypes.

Scenario	Phenotype Number (effect direction)	Effect Size/Relative Risk†	Phenotype Correlation Coefficients				
1	1 (+)	0.167	1				
	2*(+)	1.298	0.4	1			
	3 (+)	0.167	0.5	0.4	1		
	4*(+)	1.289	0.4	0.3	0.4	1	
	5 (+)	0.167	0.5	0.4	0.5	0.4	1
2	1 (+)	0.2	1				
	2*(+)	1.114	0.4	1			
	3 (+)	0.167	0.5	0.4	1		
	4*(+)	1.057	0.4	0.3	0.4	1	
	5 (+)	0.126	0.5	0.4	0.5	0.4	1
3	1 (+)	0.167	1				
	2*(+)	1.114	0.4	1			
	3 (+)	0.014	0.5	0.4	1		
	4*(+)	1.057	0.4	0.3	0.4	1	
	5 (+)	0.045	0.5	0.4	0.5	0.4	1
4	1 (+)	0.2	1				
	2*(−)	0.903	0.4	1			
	3 (−)	−0.167	0.5	0.4	1		

Scenario	Phenotype Number (effect direction)	Effect Size/Relative Risk†	Phenotype Correlation Coefficients				
	4*(+)	1.057	0.4	0.3	0.4	1	
	5 (+)	0.124	0.5	0.4	0.5	0.4	1
5	1 (+)	0.167	1				
	2 (+)	0.155	0.5	1			
	3 (+)	0.161	0.5	0.5	1		
	4 (+)	0.148	0.5	0.5	0.5	1	
	5 (+)	0.167	0.5	0.5	0.5	0.5	1
6	1 (+)	0.167	1				
	2 (−)	−0.089	0.5	1			
	3 (−)	−0.014	0.5	0.5	1		
	4 (+)	0.045	0.5	0.5	0.5	1	
	5 (+)	0.045	0.5	0.5	0.5	0.5	1

Note:

Scenarios 1–4 represent multivariate phenotypes that contain both continuous and dichotomous traits; scenarios 5–6 represent multivariate phenotypes that contain only continuous traits

*indicates the phenotype has been transformed into dichotomous variable.

†Effect size (for continuous variable with variance=1) or relative risk (for dichotomous) per allele assuming an additive model estimated using 10,000 replicates.

Table 2

Estimated type I error rates for combining statistics methods with mixed continuous and dichotomous phenotypes based on 10,000 replicates (100 for CV).

Scenario*	Methods†	Type I Error	
		α = 0.05	α = 0.01
1–4	O'Brien	0.048	0.0096
	RS(30)	0.052	0.0102
	CV	0.05	<0.01
5–6	O'Brien	0.0486	0.0091
	RS(30)	0.0536	0.012
	CV	0.06	0.02
	LME	0.0507	0.0094
	MANOVA	0.0729	0.018
	SS	0.0502	0.0094
	PCP	0.0498	0.0097

Note:

*Scenarios use in type I error estimation correspond to those in Table 1 except the effect size (risk ratio) are set to 0 (1). As a result, scenarios 1–4 in table 1 corresponds to the same null model.

†O'Brien, O'Brien's method; RS(30): random splitting method (30% to estimate T_W); CV: cross-validation method

Table 3

Loci identified ($P<2.56\times10^{-6}$) from individual GWAS of uric acid levels and gout, and from applying multivariate methods.

Chr	rs Number	PhysPos	MA[a]	MAF[a]	In Reference Gene	Gout		Uric Acid		Obrien's Method		Sample Splitting		Cross-Validation	
						Z	P-value	Z	P-Value	Z	P-Value	Z	P-Value	Z	P-Value
2	rs16858223	143367241	T	0.07	*KYNU*	2.93	3.30E-03	−5.07	3.9E-07	−3.65	2.6E-04	−4.76	1.9E-06	1.65	2.4E-02
3	rs6788787	118353538	T	0.16	*LSAMP* [b]	4.81	1.53E-06	−0.60	5.5E-01	2.78	5.4E-03	3.54	4.0E-04	3.25	3.7E-04
4	rs16890979	9531265	T	0.23	*SLC2A9*	−3.21	1.33E-03	−0.36	1.6E-76	−13.65	2.0E-42	15.51	3.0E-54	15.04	<3E-20
4	rs2231142	89271347	T	0.11	*ABCG2*	4.81	1.50E-06	0.25	9.0E-20	8.81	1.3E-18	7.51	6.0E-14	7.75	3.2E-18
6	rs11754288	25884928	A	0.43	*SLC17A4*	−1.24	2.14E-01	−5.81	6.0E-09	−4.43	9.5E-06	4.61	4.1E-06	4.42	1.9E-06
6	rs1165196	25921129	G	0.45	*SLC17A1*	−1.57	1.10E-01	−6.18	6.2E-10	−4.87	1.1E-06	5.05	4.4E-07	4.71	2.7E-07
6	rs1165205	25978521	T	0.46	*SLC17A3*	−1.63	1.00E-01	−6.21	5.6E-10	−4.93	8.4E-07	5.33	1.0E-07	4.81	2.4E-09
8	rs278600	94258095	T	0.18	*FAM92A1*[b]	3.30	9.74E-04	4.54	5.7E-06	4.98	6.3E-07	3.38	7.3E-04	3.80	2.4E-05
11	rs7116144	91539784	C	0.48	*FAT3*[b]	−0.76	4.40E-01	1.36	1.7E-01	2.77	5.6E-03	4.82	1.4E-06	3.92	1.2E-05
12	rs4760254	56052659	C	0.20	*INHBC, R3HDM2*[b]	−3.33	0.0009	−4.63	3.6E-06	−5.06	4.2E-07	3.92	8.8E-05	3.90	1.2E-05
12	rs4242909	131689174	C	0.44	*P2RX2*[b]	3.35	0.0008	4.06	5.0E-05	4.72	2.4E-06	4.11	4.0E-05	3.80	1.6E-05

[a] MA stands minor allele (less common allele) that is the coded allele in our analyses. MAF is minor allele frequency.

[b] SNP is located 706Kb out of LSAMP; 523 Kb out of FAM92A1; 185 Kb out of FAT; 62 Kb out of INHBC and 75Kb out of R3HDM2; 16Kb out of P2RX2.