**Bivariate association analysis in selected samples: Application to a GWAS of two Bone Mineral Density phenotypes in males with high or low BMD**

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**Abstract**

Our specific aims were to evaluate the power of SUR-based bivariate analysis and to compare its performance with traditional univariate analysis in samples of unrelated subjects under varying sampling selection designs. The advantage of the SUR model is that it permits all traits to have separate genetic models. We conducted extensive simulations for the case of two correlated quantitative phenotypes, with the QTL making equal or unequal contributions to each phenotype. Our simulation results confirmed that the power of bivariate analysis is affected by the size, direction and source of the phenotypic correlations between traits. Overall, SUR-based bivariate analysis was found as powerful as or better than univariate analysis, even when the QTL does not exerts pleiotropic effects, in both unselected and selected samples. The optimal sampling scheme depends on the size and direction of the induced-QTL correlation. We also demonstrated the efficacy of SUR-based bivariate test by applying it to a real GWAS of Bone Mineral Density values measured at the Lumbar Spine and at the Femoral Neck in a sample of unrelated males with low BMD (LS Zscores <=-2) and with high BMD (LS and FN Zscores >0.5). A substantial amount of top hits in bivariate analysis did not reach nominal significance in any of the two single-trait analyses. Altogether, our studies suggest that bivariate analysis is of practical significance for GWAS of correlated phenotypes.

## INTRODUCTION

With the availability of high-density maps of single nucleotide polymorphisms (SNPs), association studies have become popular tolls for identifying genes underlying complex human traits and diseases. For most current population-based genome-wide association studies (GWAS) statistical power is often limited due to the complex interplay among factors that influence the etiology of diseases [1]. Increasing sample size and multilocus or multivariate statistical analyses can improve the power for detecting association. Sample size is often restricted due to genotyping costs and limited sample resources. Several studies have demonstrated that analyzing samples selected with extreme values can be more powerful than analyzing samples randomly selected from the population[2-4]. In addition to using selected samples, another approach to increasing association test power is to perform joint analysis of multiple correlated phenotypes. For many common multifactorial traits, several correlated phenotypes are usually recorded for each individual during sample collection, but most often the phenotypes are analyzed separately in a univariate framework. Joint analysis of correlated phenotypes can theoretically provide greater power than that provided by analysis of individual phenotypes [3,5-7]. Multivariate analysis can also alleviate the multiple testing problem, caused by testing different traits separately, and thereby improve the ability to detect genetic variants whose effects are too small to be detected in univariate analysis[8]. Several multivariate approaches have been applied to linkage studies of correlated complex phenotypes, as osteoporosis and bone-related phenotypes [9-12]. Similarly, various methods, often based on Generalized Estimating Equations (GEE), have been proposed for performing multivariate association tests on population- or family-based data [13-20]. Of the two studies that have investigated the power of bivariate association test in population-based data, one applied the traditional bivariate model that assumes

same QTL effects on each trait [16,18]. Such constraints in the model may have overestimated or underestimated the relative performance of bivariate over univariate analysis. Finally, GWAS studies using multivariate analysis are rare, specially in samples of subjects selected through their phenotype values, and further investigations using this approach are warranted [4].

To this aim, we evaluated the statistical properties of joint association analysis of two correlated quantitative traits in samples of unrelated subjects through simulation studies, using a bivariate model that allows for different QTL effects on traits. The evaluation was conducted under different situations according to the sample selection design, genetic effects and residual correlation between the traits. We demonstrate the efficacy of SUR-based bivariate test by applying it to simultaneous GWAS analysis of two correlated bone phenotypes, Bone Mineral Density at the Lumbar Spine and at the Femoral Neck, which are major risk factors of osteoporosis.

## METHODS

### **Association analysis**:

Bivariate association analysis for two correlated quantitative traits was performed using the Seemingly Unrelated Regression (SUR) model [21] that allows different genetic models for different traits. The SUR model is an extension of the Ordinary Least Squares estimation which takes into account the residual correlation structure across the univariate linear regressions on each phenotype.

Let's N be the total number of unrelated subjects, each having observations on two correlated quantitative phenotypes $T_j$ (j=1,2). Under the SUR model, the relationship between the phenotypic values $y_{i,1}$ and $y_{i,2}$ and the explanatory variable $g_i$ (i.e., genetic

marker) observed in each individual is given by the following system of two equations:

$y_i = g_i \times \beta + e$

where, $y_i = (y_1, y_2)^t$ is a 2 x 1 vector of random observations (i =1,….,N); $g_i$ is a 2 x 2 block-diagonal matrix of the genotypes ($g_i$ = 0,1, 2 under an additive model) at the SNP; $\beta = (\beta_1, \beta_2)^t$ is a 2 x 1 regression parameters vector and $e = (e_1, e_2)^t$ is a 2 x 1 vector of the residuals errors assumed to be normally distributed with mean 0 and variance matrix,

$$\Omega = \begin{pmatrix} \sigma_1^2 & r\sigma_1\sigma_2 \\ r\sigma_1\sigma_2 & \sigma_2^2 \end{pmatrix} \qquad (1)$$

Where $\sigma_1^2$ and $\sigma_2^2$ are the residual variances of T1 and T2 respectively and r is the residual correlation between T1 and T2.

The goodness of fit of the whole system can be measured by the McElroy's r-square ($R^2$). $R^2$ is the proportion of variance due to the studied SNP which takes into account the residual matrix covariance $\Omega^{22}$.

To compare the null hypothesis of no association to neither phenotypes ($\beta_1 = \beta_2 = 0$) against the alternative hypothesis, i.e., the SNP is associated to either one or both phenotypes ($\beta_1 \neq 0$, $\beta_2 \neq 0$), we employed a Fisher test. Under the null hypothesis, the Fisher statistic is asymptotically distributed with 2 and 2 x (N-2) degrees of freedom.

Separate association analyses of T1 and T2 were conducted using traditional univariate linear regression model: $y_{i,j} = g_i \times \beta_j + e_j$, where $y_{i,j}$, $g_i$, and $\beta_j$ are as described above but now $e_j$ is assumed to follow a normal distribution N (0, $\sigma_j^2$). The null hypothesis of no association ($\beta_j = 0$) can be tested against the alternative ($\beta_j \neq 0$) with a Student statistic with one degree of freedom.

**Simulation study:**

We conducted simulations for the case of two correlated quantitative phenotypes (T1 and T2), in which the quantitative trait locus (QTL) made equal or unequal contributions (size and direction of effects) to each phenotype. We considered genetic models of complex traits and specifically tried to generate correlated data mimicking as much as possible our real BMD GWAS data (see below). Since a strong (~0.5) and positive phenotypic co-variation exists for LS and FN BMD values [23], we generated data for two positively correlated quantitative phenotypes. Further, in real datasets, as causal loci usually contribute a small proportion to the total phenotypic correlation, residual correlation approximate phenotypic correlation between traits. It is also more realistic to assume that the investigator has *a priori* knowledge on the magnitude and sign of the co-variation of the studied phenotypes than on the magnitude and sign of the QTL effect on each phenotype. Thus, in all our scenarios, the sign of the residual correlation was positive. Instead, we varied the sign of the induced QTL correlation. Also, our BMD GWAS study used a novel sampling design, with extreme truncate selection of unrelated males, aiming to improve power. Therefore, we also generated samples of subjects drawn from the extremes of the phenotype(s) population distribution.

We considered samples of N=300 and 1 000 unrelated subjects and two continuous traits (T1 and T2) normally distributed, with mean and variance equal to 0 and 1, respectively. We investigated two correlation structures: moderate (residual correlation, rE=0.20) and strong (rE=0.60). We assumed a bi-allelic QTL having additive effects ($a_j$) on $T_j$ (j=1,2). The frequency of the minor and the major allele is q and p, respectively. The genotypic means of Tj, $m_{jk}$, are equal to $2q \times a_j$, $(q-p) \times a_j$ and $-2p \times a_j$ when k, the number of rare alleles, is equal to 0, 1 and 2 respectively. The QTL contribution to $T_j$ is the trait-specific QTL heritability, $h^2_j = 2 \times q \times p \times a_j^2$. Different values of the minor

allele frequency (q) and size of the QTL effect ($a_j$) were chosen to fit $h^2_j$ values of 0%, 0.5%, 1% and 3%. We also varied the sign of $a_j$: both were of same or opposite sign and the QTL correlation (rG) was, thus, equal to +1 or -1, respectively. The three main scenarios varied according to the QTL contribution on $T_j$: it does not affect T1 and T2 (i.e., $h^2_1=h^2_2=0$); it affects T1 only (i.e., $h^2_1>0$, $h^2_2=0$); it exerts pleiotropic effects (i.e., $h^2_1>0$ and $h^2_2>0$). Within the pleiotropic models we further considered the following main cases (1) the QTL affects similarly T1 and T2 ($h^2_1=h^2_2$ and rG=+1) (2) the QTL affects differently T1 and T2 (either $h^2_1\neq h^2_2$ or $h^2_1=h^2_2$ and rG=-1). Overall, the different parameter settings allowed us to generate data for a QTL having same or different effect on the two positively correlated phenotypes, and the two sources of co-variation (QTL and residual) have same or opposite sign.

For a given combination of parameter values (rE, $h^2_1$, $h^2_2$, rG) we generated samples of N unrelated subjects selected according to their phenotype(s) values. We used a left (Zl) and a right (Zr) threshold to ascertained subjects having low (T1<Zl) or high (T1>Zr) T1 values. The unselected sampling design (denoted as Su) was defined by Zl=Zr=0. We then applied a truncate selection design (denoted as S1) by setting Zl=-2 and Zr=0.5. This corresponds to drawing subjects from the 2.5% and 30% left and right tail of the population distribution of T1. The third sampling selection (S2) included T2 in the selection criteria and subjects having low (T1<-2) or high (T1 and T2 >0.5) phenotypes values were selected. Under all sampling selection designs, we generated samples with equal number of subjects drawn from the left (N/2) and the right (N/2) side of the phenotypes distributions.

The simulated data were generated as follows. First, QTL alleles were drawn from a binomial distribution with parameter q, and genotypes were built under Hardy-Weinberg equilibrium. Then, conditionally on the generated genotype, $g_k$ (k=0,1,2), we

jointly drew the values of T1 and T2 via a bivariate normal distribution with mean $(m_{1k}, m_{2k})^t$, as described above, and variance matrix $\Omega$, given in equation (1). Third, the two generated phenotypic values ($y_{i1}$, $y_{i2}$) were evaluated according to the set of threshold values Zr and Zl. Individuals fulfilling the selection criteria were kept; the others were withdrawn from the sample. Steps 1 to 3 were repeated until reaching the required left and right truncated sample sizes of (N/2) subjects.

We performed joint association analysis of T1 and T2 using the genotypes at the QTL, that is, the SNP is the causal variant. Each replicate was analyzed with SUR-based bivariate and with two separate univariate analyses using the systemfit package of R software (http://www.r-project.org/). The mean and standard deviation of each association statistic (F test and $t_1$, $t_2$ tests) as well as the mean and standard deviation of the association parameters (regression coefficients) were derived from K replicates. Power and type I error rates of the SUR-based bivariate analyses were calculated as the proportion of replicates with an F test statistic exceeding a given theoretical threshold ($R\alpha$) value. The thresholds were derived from an F distribution with 2 df, at nominal significance levels, $\alpha$=5%, 1%, 0.1% and $10^{-5}$. Type 1 errors were estimated in the settings were $h^2_1=h^2_2=0$ with K=20 000 replicates. Power rates were derived with K= 1 000 replicates. Power and type I error rates of each separate univariate analysis were similarly computed using theoretical threshold values derived from a t distribution with 1df. To compare the performance of bivariate and that of univariate association analysis, we computed the proportion of replicates where $t_1$ and $t_2$ were both lower than $R\alpha$. One minus this proportion estimated the probability to detect association to either one of the two phenotypes. To adjust for the two univariate association tests, we applied the Bonferroni correction, that is, we used the theoretical thresholds $R\alpha/2$.

## RESULTS

### SIMULATION STUDY

Tables 1 and 2 present the mean (and sd) association statistic of the SUR-based bivariate (F test) and of the traditional univariate tests (t test), respectively when N=1 000 for all 66 scenarios under the alternative hypothesis, defined above.

**Bivariate association statistics:** In randomly selected samples, the results in Table 1 show several well-established power figures. First, mean F statistics of bivariate association analysis increase with the size of the trait-specific QTL heritability ($h^2_1$ and/or $h^2_2$) irrespective of rG and rE. Second, the power is highest in presence ($rG \neq 0$) than in absence (rG=0) of pleitropic effects: the highest power is achieved when rG=-1, that is, when the correlation induced by the QTL effect and the residual correlation are opposite in sign. Third, the results also confirm that the power of bivariate association test varies with the size of the residual correlation: when rG=0 or rG=-1, the power increases with rE while when rG=+1 it decreases with rE. These general trends are observed irrespective of the sampling selection designs. Applying extreme truncate selection increases the power of bivariate association analysis, but the optimal selection design depends on the true genetic model. When rG=0 or rG=-1, extreme selection on one trait (S1) is more efficient than extreme selection on both traits (S2). Conversely, when rG=+1, S2 is more efficient than S1. Overall, under Su or S1, the highest mean F statistics are obtained when rG=-1, irrespective of rE. Under S2, the highest power is achieved when rG=+1 or when rG=-1, depending on the size of rE: when the traits are moderately (rE=0.20) correlated, mean F statistics have greater values when rG=+1 than when rG=-1.

**Univariate association statistics** Table 2 shows again several well-established power figures. In randomly selected samples, the power of univariate analysis increases with

the QTL heritability ($h^2_1/h^2_2$) and varies little with the size of the residual correlation, rE.

For phenotype T1, under a given QTL heritability ($h^2_1$) value, the mean statistic values of all models are similar in the randomly selected samples. Thus, univariate analysis of T1 has similar power irrespective of the presence or not of pleiotropic effects. Applying extreme truncate selection increases the power of univariate association analysis of T1. Under S1, the power remains similar whichever rG. Under S2, the power is the highest and the lowest for the pleiotropic models rG=+1 and rG=-1, respectively. As already noted for SUR-based association analysis, the optimal sampling design depends on rG. When rG=-1 or rG=0, the power of univariate association analysis is greater under S1 than under S2. The reverse trend is obtained when rG=+1.

For phenotype T2, the power of univariate analysis depends on rG and rE. Further, applying extreme selection does not always lead to a gain in power. Indeed, when rG=-1 the power of univariate analysis is the greatest in the unselected samples (Su). When rG=0 the mean t statistic values in the selected samples are biased and inflated. The magnitude of the bias is greater under S2 than under S1. Under S1, the bias increases with rE.

Overall, applying selection criteria on one or both traits is an optimal sampling design when rG=+1: the power of each separate univariate analysis is improved over that in randomly selected samples. When the direction of the QTL effect on T1 and T2 is opposite (rG=-1), applying extreme truncate selection leads to both a substantial gain and decrease in power for T1 and T2, respectively. For the situations in which the QTL does not exert pleiotropic effects (rG=0), the highest power of univariate analysis of T1 is obtained in the selected samples. However, the mean t statistic values for T2, the trait no associated to the QTL, are also increased. Type I error rates of separate univariate

analyses may thus be inflated, especially in selected samples and when the residual correlation is high.

**Type I error rates:** When the QTL/SNP has no effect on T1 and T2, the values of the mean and standard deviation of both bivariate and univariate association tests are close to the theoretical values, regardless of the residual correlation, minor allele frequency of the studied SNP and of the selection sampling design (Supplementary Table 1.A). Indeed, SUR-based bivariate and each separate univariate association tests have correct type I error rates (Supplementary Table 1.B). However, the false-positive rates of univariate association analyses for detecting association to either or both the two traits are, as expected, inflated: the estimated rates are roughly two times higher than the theoretical rates. Applying a Bonferroni correction (denoted as U_b) leads to slightly conservative significance levels, especially when the residual correlation between the traits is strong.

**Power comparisons :** The power to detect association to either or both of the two traits using SUR-based bivariate analysis was compared to the power of separate univariate analysis of T1 and T2 adjusted for multiple testing by the Bonferroni correction (denoted as U_b). Figure 1.A shows the power curves (at significance of $10^{-5}$) against the QTL heritability ($h^2_1$, $h^2_2$) when N=1 000, for moderately (rE=0.2) or strongly (rE=0.6) correlated traits. Power curves under S1 and S2 are shown in Figure 1.B, when $h^2_1 = h^2_2 = 0.005$, N=1 000 and rE=0.2 or 0.6.

In randomly selected samples (Figure 1.A), SUR-based bivariate analysis outperforms univariate analysis across most scenarios. The relative advantage of SUR-based bivariate over univariate association analysis is more obvious when rG=-1 and/or the traits are strongly correlated (rE=0.6) but also when rG=+1 and the traits are moderately correlated (rE=0.2).

The same trends are observed after applying extreme truncate selection (Figure 1.B). Under S1, SUR-based bivariate analysis outperforms univariate analysis in most cases. It shows slightly lower power than univariate analysis when rG=+1 and rE=0.6 or when rG=0 and rE=0.2. For strongly correlated traits, the power rates are equal to 94.5% (SUR) vs. 29.3% (U_b) when rG=-1; 44.0% (SUR) vs 32.3% (U_b) when rG=0; 36.8% (SUR) vs 39.9% (U_b) when rG=+1. For moderately correlated traits, the power rates are equal to 64.6% (SUR) vs 31.7% (U_b) when rG=-1; 32.9% (SUR) vs 34.9% (U_b) when rG=0; 43.7% (SUR) vs 32.6% (U_b) when rG=+1. Under S2, again SUR-based bivariate analysis outperforms univariate analysis when rG=-1 or when rG=0 and rE=0.6. For all remaining scenarios, SUR-based bivariate analysis shows same or slightly lower power than univariate analysis. As already noted above (Tables 1 & 2), the most efficient design depends on rG and rE. Selecting on T1 (S1) is the most efficient sampling design when rG=-1 or when rG=0 and the traits are strongly correlated (rE=0.6). Selecting on both traits (S2) is the most efficient design when rG=+1. Overall, when rE=0.6, the power of SUR if the greatest (94.5%) when rG=-1 and under S1, but the power of univariate analysis is the greatest (56.8%) when rG=+1 and under S2. When rE=0.2, the power of SUR and univariate analysis are both the greatest (72.5% and 72.9%) when rG=+1 and under S2. As shown in Table 3, all these trends are confirmed under various parameter settings.

## ANALYSES OF EMPIRICAL BMD-GENOME-WIDE ASSOCIATION DATA

**BMD sample:** Subjects were recruited from the Network in Europe on Male Osteoporosis study [24,25]. Subjects selected from this cohort were unrelated males > 18 and < 68 years of age. In addition, the subjects were selected by bone densitometry (measured at the Lumbar Spine and at the Femoral Neck) criteria, having either low

BMD (LS Z-scores ≤-2, n=175) or high BMD (both LS- and FN-Z-scores >0.50, n=155). Further details of the study sample are provided in Supplementary Table 2. All the study subjects gave written, informed consent. This study was approved by the Ethical Committee of both Ghent University Hospital (Belgium) and of Lariboisière Hospital (France).

**Genome-wide genotyping and quality control analyses:** Genotyping was carried out at the Centre National de Génotypage (CNG, Evry, France) using the Illumina 370K platform. Quality control analyses on SNPs and DNA data were performed with PLINK [26] using stringent QC criteria. A total of 30 098 SNPs were discarded based on low (<97.5%) call rate, significant ($p<10^{-5}$) deviation from Hardy-Weinberg equilibrium and/or low (<5%) minor allele frequency (MAF). Therefore, 298 783 autosomal SNPs were included in further analysis. Criteria exclusion for DNA samples included a low (<96%) completion rate and cryptic relatedness (>0.14) between pairs of subjects, derived from genome-wide identity-by-descent estimates. The presence of population stratification was studied by principal component analysis with EIGENSTRAT [27]. After removing genetic outliers (standard deviation > 6), principal component analysis identified just one cluster. The final post-QC sample included 313 individuals.

**Association analysis:** The inter-individual variation in BMD measured at a given skeletal site is largely regulated by genetic factors and a strong and positive (~0.50) phenotypic correlation exists for LS and FN BMD values [23]. Thus, our primary analysis was the joint association analysis of LS-Zscores and FN-Zscores by means of SUR-based bivariate test. For comparison purpose, we also applied separate univariate association analyses of LS and FN Z-scores phenotypes. We used single marker analysis assuming additive genetic effects. The mean F statistic of our SUR-based genome-wide association analysis was equal to 1.018 (sd=1.022, median= 0.70). The

mean t statistic of LS and FN were -0.0167 (sd=1.011, median=-0.0165) and -0.0129 (sd=1.006, median=0.0104), respectively. These results indicated that there was no meaningful inflation of univariate as well as bivariate association analyses.

**Results:** SUR-based bivariate analyses identified a substantial number (35) of SNPs with strong evidence of association (P-value<$10^{-4}$). Interestingly, several of the identified SNPs failed to reach nominal (P-value <5%) significance under separate univariate analyses for either one or the two BMD phenotypes. Bivariate and univariate association results were compared in terms of statistical significance and ranks of the SNPs identified in either one of the two approaches. For each SNP, we kept the lowest P-value (denoted as Best_U) of LS or FN univariate association analysis. Univariate P values were not corrected for multiple testing. We ranked the Best_U P-values from the lowest to the highest. We similarly ranked the P-values from SUR-based bivariate analysis of LS and FN. The overlap in results between the two approaches were compared in terms of statistical significance for the top 100 and 300 most associated SNPs identified in bivariate or in univariate association analyses. Figure 2 plots the significance levels in each procedure for the top 100 most associated SNPs identified from SUR-based (Figure 2.A) or from univariate (Figure 2.B) analyses. We found that a majority (52 out of 100) of the top SNPs in SUR-based bivariate analysis also show strong (P<$3x10^{-4}$) association signal in univariate analyses. For a substantial number (16) of the remaining SNPs, univariate analyses fail to reach nominal (P<5%) significance (Figure 2.A) On the other hand, all of the top 100 SNPs in univariate analyses (Figure 2.B) are also highly significant (P<$8x10^{-4}$) in bivariate analysis. Our rank comparisons showed same trends. The top 300 SNPs in bivariate analysis ranked from 1 to 80 682 (mean rank=7 062) in univariate analysis, while the top 300 SNPs in univariate analysis ranked from 2 to 766 (mean rank=313) in bivariate analysis. Overall,

our analyses showed that univariate analysis did not identify new strongly associated SNPs as compared to those detected in bivariate analysis. Conversely, SUR-based analysis identified strongly associated SNPs that were not detected in univariate analysis.

Table 4 shows details of the association results for the top 10 SNPs ($P \leq 3x10^{-5}$) in SUR-based bivariate analysis. The genetic contributions ($R^2$ values) of the 10 top SNPs are not great, as expected for any relatively common polymorphic locus. The three most associated SNPs span the 6q22.1 ($P=8.42x10^{-6}$), the 15q14-q15 ($P=6.97x10^{-6}$) and the 22q11.2 ($P=5.44x10^{-6}$) genomic regions. The table also shows the raw P-values and ranks in each separate univariate analyses of LS and FN. Three SNPs also rank well (i.e., are in the set of top 300 SNPs) in univariate analyses of LS and/or FN. They are located on 6q25: rank=2, $P=1.3x10^{-5}$ (LS) and rank=1, $P=1.2x10^{-5}$ (FN); on 15q14-q15: rank= 2 635, $P=8.4x10^{-3}$ (LS) and rank=3, $P=1.7x10^{-5}$ (FN); and on 22q13: rank=1, $P=3.5x10^{-6}$ (LS) and rank=8, $P=3x10^{-5}$ (FN). All the remaining 7 SNPs show a much stronger association signal in bivariate than in univariate analyses, including 2 of the 3 best SUR-based association signals. For the most significant result, on 22q11.2 ($P=5.44x10^{-6}$), the QTL explains 3.85% of the joint (co)variance of LS and FN. This value likely over-estimates the contribution in unselected populations. Nonetheless, univariate analyses failed to detect association ($P>0.07$) with this SNP.

Our study used a novel design, with extreme truncate selection of unrelated males, aiming to improve power. The approach of studying samples drawn from the extremes of the population distribution of BMD has been used in several linkage studies of BMD variation [25,28], but rarely in association studies [29], and to our knowledge, never in samples drawn from the population of males. Due to our relatively small GWA sample size, no SNP showed evidence of association to either one or both BMD phenotypes at

genome-wide significance threshold of $1.7x10^{-7}$ (0.05/ 298 783 SNPs). However, we used an extreme truncate selection design that, as shown by our simulation studies, has increased power over unselected samples. Our SUR-based bivariate association analyses identified strong association ($P<8.4x10^{-6}$) with 3 genomic regions (6q22.1, 15q14 and 22q11). These SNPs have not yet been reported to be associated with bone density in previous GWAS [30-32]. Two of them, on 15q14-15 and 22q11, are located in genes that are known to be expressed in skeletal muscle. *GLUT 11* encoded by *SLC2A11* on 22q11 belongs to a family of plasma membrane proteins that mediate transport of sugars across the membrane by facilitative diffusion [33]. *RYR3*, on 15q14-15, encodes for one of the ryaodine receptor expressed in brain and muscle [34,35]. It is required for efficient muscle contraction. Because muscle contraction has a major impact on bone density, this might represent an indirect role of these genes on bone density.These genetic variants, whether they are site-specific or possibly shared (pleiotropic), may warrant further follow-up genetic studies on BMD and other bone-related phenotypes.

## DISCUSSION

We have evaluated the performance of bivariate association analysis based on the Seemingly Unrelated Regression (SUR) model, which allows different genetic models for different traits. We conducted extensive simulations for the case of two correlated quantitative phenotypes, in which the QTL made equal and unequal contributions (size and direction of effects) to each phenotype. Further, we simulated data samples of unrelated subjects either randomly selected or selected from the extremes of the trait(s) distribution(s). So far, the performance of bivariate association analysis has been evaluated in unselected samples of either unrelated or related subjects [13-20]. To our

knowledge, this is the first study to specifically derive the power and the relative performance of bivariate association analysis in selected samples of unrelated subjects.

In randomly selected samples, our main results coincide with well-known power figures [6-8] and confirmed that bivariate association analysis outperforms univariate analysis when the QTL exerts pleiotropic effects and the relative increase in power is greatest when correlation of the QTL is opposite in sign to the residual correlation. For all the investigated genetic models, the power of bivariate as well as that of univariate analysis was greatest in the selected than in the unselected samples. The most powerful sampling selection design varied with the genetic model, specifically with the size and the direction of the induced-QTL correlation. Applying truncate selection on one trait was found the most efficient sampling design when the genetic and the residual correlations are opposite in signs. Conversely, when the QTL exerts pleiotropic effects and both sources (QTL and residual) of co-variation are of same sign, applying selection criteria on both traits was found the optimal sampling selection design. When the QTL does not exert pleiotropic effects, extreme selection based on one trait was found more powerful than that based on the two traits. Overall, in the selected samples, SUR-based bivariate analysis remains more powerful than univariate analysis when the genetic and residual correlations are opposite in sign. Interestingly, and in contrast to the results observed in the unselected samples, the power to detect association in the selected samples may be greater when the signs of the genetic and the residual correlations are the same than when they differ. Finally, when the QTL affects one trait only, the power of the SUR-based bivariate association test was found as good as or better than that of univariate association test, depending on the size of the residual correlation.

So far, two studies have investigated the power of bivariate association in unselected population-based data, and they both applied bivariate association test based on

Generalized Estimating Equations [16,18]. The former applied a general GEE-based model that allows, as the SUR model, for different QTL effects on the two traits. The second study applied the traditional GEE-based bivariate model that assumes same QTL effects on the phenotypes. Our results are congruent with those reported by the first study. The traditional (restricted) bivariate test estimates, as the univariate test, a single parameter (i.e., the SNP regression coefficients on each trait are all set equal). Thereby, when the QTL affects similarly each trait, the advantage of the restricted over the general bivariate model is expected to be twofold: at a given nominal P-value, the bivariate restricted model shows greater power than the general model and the gain in power of bivariate analysis over univariate analysis is, thus, enhanced. Conversely, when the QTL does not affect similarly each trait, especially when the QTL affects one trait only, the power of the bivariate restricted model is expected to be lower than that of separate univariate analysis. Clearly, the traditional (restricted) bivariate model is appropriate when the QTL is pleiotropic and affects all traits similarly. But rarely, knowledge of this magnitude about a complex trait is known *a priori*. Thus, we do not recommend using restricted bivariate models even in unselected data.

Our bivariate genome-wide association analysis of Lumbar Spine and Femoral Neck BMD values, conducted in a sample of unrelated males with low BMD (LS Zscores ≤-2) and high BMD (LS and FN Zscores >0.5), consistently demonstrated the advantage of the SUR-based bivariate test over separate univariate analysis. All of the top hits in univariate analysis also showed strong evidence of association in bivariate analysis. Conversely, additional SNP associations were detected with the bivariate method that did not reach nominal significance in single-trait analyses: this was achieved without adjusting significance of univariate analyses for multiple testing.

In conclusion, our results showed that SUR-based models are useful to detect association for correlated phenotypes: they show greater or same power than univariate analysis even when the QTL does not exerts pleiotropic effects, in both unselected and selected samples. However, our results also showed that similar power levels can be achieved whether the QTL exerts or not pleiotropic effects. Thus, disentangling pure pleiotropic from residual covariation remains a challenge even in bivariate association analysis.

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**Conflict of interest**: none

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**Titles and legends to figures**

**Figure 1:** Power rates at $\alpha=10^{-5}$ of SUR-based bivariate analysis and univariate analysis adjusted for multiple testing by Bonferroni correction (U_b), in samples of N=1 000 subjects and under various parameters settings: QTL heritability ($h^2_1/h^2_2$), sign of the induced genetic correlation (rG), residual correlation (rE).

(A) Power estimates against QTL heritability for moderately (rE=0.2) or strongly (rE=0.6) correlated traits, in randomly selected samples (Su)

(B) Power estimates under extreme selection (S1 or S2) for moderately (rE=0.2) or strongly (rE=0.6) correlated traits and QTL heritability ($h^2_1=h^2_2=0.005$)

**Figure 2:** Overlap in significance of results from bivariate and univariate (Best_U) association analysis.

(A) Top 100 hits in SUR-based bivariate association test: $-\log_{10}$ P-values of univariate analysis against $-\log_{10}$ P-values of SUR-based bivariate analysis

(B) Top 100 hits in univariate association test: $-\log_{10}$ P-values of SUR-based bivariate analysis against $-\log_{10}$ P-values of univariate analysis

**Table 1**: Mean (and sd) of the SUR-based bivariate association statistic (F test) in samples of N=1 000 subjects for various parameter settings: QTL heritability ($h^2_1/h^2_2$), sign of the induced genetic correlation (rG), residual correlation (rE), and sampling selection design.

| | | | [1]Sampling | | |
|---|---|---|---|---|---|
| | | | Su | S1 | S2 |
| $r_E$ | $r_G$ | $h^2_1/h^2_2$ | μF (sd) | μF (sd) | μF (sd) |
| 0.2 | 0 | 0.005/0 | 3.69 (2.61) | 10.10 (4.34) | 9.23 (4.18) |
| | | 0.01/0 | 6.17 (3.32) | 18.94 (5.86) | 17.97 (5.74) |
| | | 0.03/0 | 17.02 (6.08) | 59.02 (10.56) | 55.3 (10.32) |
| | +1 | 0.005/0.005 | 5.08 (2.99) | 11.42 (4.86) | 15.17 (5.41) |
| | | 0.005/0.01 | 7.45 (3.66) | 14.07 (5.04) | 19.08 (6.58) |
| | | 0.01/0.01 | 9.50 (4.42) | 22.89 (6.86) | 29.84 (7.89) |
| | | 0.03/0.03 | 26.90 (7.34) | 72.04 (12.89) | 92.88 (14.41) |
| | -1 | 0.005/0.005 | 7.26 (3.92) | 13.91 (5.30) | 9.57 (4.48) |
| | | 0.005/0.01 | 10.57 (4.36) | 17.22 (5.65) | 11.05 (4.88) |
| | | 0.01/0.01 | 13.69 (5.42) | 27.72 (7.40) | 19.29 (6.56) |
| | | 0.03/0.03 | 39.95 (9.40) | 89.83 (13.86) | 68.63 (13.23) |
| 0.6 | 0 | 0.005/0 | 4.88 (3.04) | 11.26 (4.51) | 9.96 (4.47) |
| | | 0.01/0 | 8.79 (4.04) | 22.54 (6.75) | 19.78 (6.16) |
| | | 0.03/0 | 25.04 (7.34) | 69.67 (11.85) | 62.92 (11.30) |
| | +1 | 0.005/0.005 | 4.09 (2.69) | 10.41 (4.49) | 12.22 (4.78) |
| | | 0.005/0.01 | 6.36 (3.60) | 12.67 (5.06) | 15.60 (5.52) |
| | | 0.01/0.01 | 7.33 (3.85) | 20.35 (6.34) | 23.81 (6.76) |
| | | 0.03/0.03 | 20.42 (6.53) | 63.56 (11.19) | 73.11 (11.61) |
| | -1 | 0.005/0.005 | 13.70 (5.32) | 20.94 (6.59) | 16.02 (5.84) |
| | | 0.005/0.01 | 19.71 (6.52) | 27.35 (7.55) | 21.20 (6.92) |
| | | 0.01/0.01 | 26.06 (7.40) | 42.83 (9.58) | 34.26 (8.87) |
| | | 0.03/0.03 | 78.65 (14.17) | 143.61 (19.15) | 124.18 (17.77) |

[1] Su: unselected sample; S1: sample selected on T1 distribution; S2: sample selected on T1 and T2 distributions.

**Table 2:** Mean (and sd) of the traditional univariate association statistic (t test) in samples of N=1 000 subjects for various parameter settings: QTL heritability ($h^2_1/h^2_2$), sign of the induced genetic correlation (rG), residual correlation (rE), and sampling selection design.

| | | | [1]Sampling | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Su | | S1 | | S2 | |
| $r_E$ | $r_G$ | $h^2_1/h^2_2$ | T1 μt (sd) | T2 μt (sd) | T1 μt (sd) | T2 μt (sd) | T1 μt (sd) | T2 μt (sd) |
| 0.2 | 0 | 0.005/0 | 2.26 (1.01) | -0.03 (1.00) | 4.23 (0.99) | 0.98 (1.02) | 4.02 (1.00) | 2.59 (1.05) |
| | | 0.01/0 | 3.15 (1.01) | -0.02 (0.97) | 5.95 (0.98) | 1.41 (1.00) | 5.78 (0.96) | 3.60 (1.00) |
| | | 0.03/0 | 5.53 (1.06) | 0.00 (1.00) | 10.69 (0.96) | 2.33 (0.96) | 10.34 (0.98) | 6.34 (1.01) |
| | +1 | 0.005/0.005 | 2.23 (1.00) | 2.20 (0.98) | 4.15 (0.97) | 3.21 (1.07) | 4.99 (0.97) | 4.83 (1.03) |
| | | 0.005/0.01 | 2.19 (0.97) | 3.23 (1.02) | 4.19 (0.97) | 4.21 (0.99) | 5.36 (1.02) | 5.69 (1.07) |
| | | 0.01/0.01 | 3.18 (1.00) | 3.19 (1.04) | 5.96 (1.01) | 4.72 (1.04) | 7.06 (0.96) | 6.93 (1.09) |
| | | 0.03/0.03 | 5.57 (1.01) | 5.57 (0.98) | 10.64 (1.01) | 8.60 (1.07) | 12.28 (0.96) | 12.57 (1.13) |
| | -1 | 0.005/0.005 | 2.20 (1.01) | -2.26 (1.01) | 4.18 (1.00) | -1.26 (0.99) | 3.15 (0.99) | 0.36 (1.02) |
| | | 0.005/0.01 | 2.26 (0.99) | -3.22 (0.97) | 4.21 (0.92) | -2.16 (0.99) | 2.69 (1.03) | -0.60 (0.99) |
| | | 0.01/0.01 | 3.18 (1.04) | -3.17 (1.04) | 5.95 (0.98) | -1.95 (1.01) | 4.56 (0.96) | 0.44 (0.97) |
| | | 0.03/0.03 | 5.57 (1.01) | -5.58 (1.04) | 10.68 (0.97) | -3.91 (1.01) | 8.51 (0.96) | 0.29 (0.95) |
| 0.6 | 0 | 0.005/0 | 2.23 (1.00) | 0.00 (0.96) | 4.19 (0.97) | 2.35 (0.98) | 3.89 (0.99) | 2.69 (0.97) |
| | | 0.01/0 | 3.13 (0.99) | -0.05 (0.98) | 6.01 (1.00) | 3.26 (0.97) | 5.64 (0.97) | 3.87 (0.98) |
| | | 0.03/0 | 5.55 (1.02) | 0.01 (0.98) | 10.62 (0.98) | 5.44 (0.96) | 10.08 (0.96) | 6.63 (0.96) |
| | +1 | 0.005/0.005 | 2.22 (1.00) | 2.24 (0.99) | 4.17 (1.00) | 4.06 (1.02) | 4.58 (0.99) | 4.59 (1.01) |
| | | 0.005/0.01 | 2.25 (1.01) | 3.24 (1.03) | 4.17 (1.00) | 4.79 (1.03) | 4.90 (0.94) | 5.40 (0.99) |
| | | 0.01/0.01 | 3.17 (1.03) | 3.18 (1.00) | 5.97 (1.01) | 5.83 (1.01) | 6.52 (0.96) | 6.58 (1.00) |
| | | 0.03/0.03 | 5.55 (1.01) | 5.60 (1.03) | 10.70 (0.98) | 10.47 (0.99) | 11.48 (0.93) | 11.75 (0.99) |
| | -1 | 0.005/0.005 | 2.20 (1.02) | -2.30 (1.02) | 4.13 (0.97) | 0.48 (0.95) | 3.23 (0.97) | 0.81 (0.92) |
| | | 0.005/0.01 | 2.23 (1.00) | -3.21 (1.00) | 4.15 (1.00) | -0.20 (1.00) | 2.97 (0.99) | 0.03 (0.97) |
| | | 0.01/0.01 | 3.10 (1.02) | -3.22 (1.01) | 5.99 (0.96) | 0.70 (0.97) | 4.75 (0.97) | 1.11 (0.95) |
| | | 0.03/0.03 | 5.52 (1.04) | -5.60 (1.05) | 10.68 (0.95) | 0.57 (0.93) | 8.89 (0.92) | 1.69 (0.88) |

[1] Su: unselected sample; S1: sample selected on T1 distribution; S2: sample selected on T1 and T2 distributions.

**Table 3:** Power rates at $\alpha = 10^{-5}$ of SUR-based analysis and univariate analysis adjusted by a Bonferroni correction (U_b) under varying parameter settings: QTL heritability ($h^2_1/h^2_2$), sign of the induced genetic correlation (rG), residual correlation (rE) and sampling selection design.

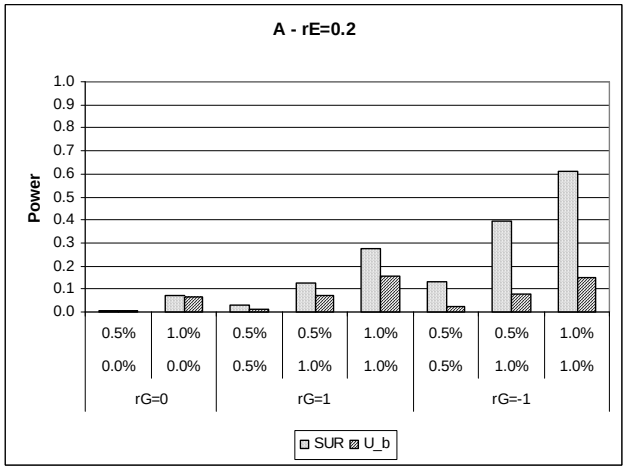
| | | | | [1]Sampling | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Su | | S1 | | S2 | |
| $N^2$ | rE | rG | $h^2_1/h^2_2$ | SUR | U_b | SUR | U_b | SUR | U_b |
| 1 000 | 0.2 | 0 | 0.005/0 | 0.008 | 0.008 | 0.329 | 0.349 | 0.273 | 0.277 |
| | | | 0.01/0 | 0.072 | 0.068 | 0.901 | 0.913 | 0.872 | 0.888 |
| | | +1 | 0.005/0.005 | 0.032 | 0.013 | 0.437 | 0.326 | 0.725 | 0.729 |
| | | | 0.005/0.01 | 0.127 | 0.072 | 0.675 | 0.510 | 0.889 | 0.891 |
| | | | 0.01/0.01 | 0.277 | 0.153 | 0.966 | 0.932 | 0.999 | 0.998 |
| | | -1 | 0.005/0.005 | 0.132 | 0.023 | 0.646 | 0.317 | 0.280 | 0.068 |
| | | | 0.005/0.01 | 0.393 | 0.077 | 0.844 | 0.316 | 0.406 | 0.033 |
| | | | 0.01/0.01 | 0.610 | 0.150 | 0.994 | 0.918 | 0.893 | 0.468 |
| | 0.6 | 0 | 0.005/0 | 0.034 | 0.012 | 0.440 | 0.323 | 0.325 | 0.217 |
| | | | 0.01/0 | 0.234 | 0.060 | 0.967 | 0.925 | 0.926 | 0.845 |
| | | +1 | 0.005/0.005 | 0.016 | 0.015 | 0.368 | 0.399 | 0.514 | 0.568 |
| | | | 0.005/0.01 | 0.094 | 0.096 | 0.564 | 0.602 | 0.760 | 0.797 |
| | | | 0.01/0.01 | 0.140 | 0.130 | 0.926 | 0.930 | 0.982 | 0.988 |
| | | -1 | 0.005/0.005 | 0.613 | 0.022 | 0.945 | 0.293 | 0.763 | 0.079 |
| | | | 0.005/0.01 | 0.899 | 0.079 | 0.990 | 0.310 | 0.934 | 0.042 |
| | | | 0.01/0.01 | 0.989 | 0.148 | 1.000 | 0.909 | 1.000 | 0.550 |
| 300 | 0.2 | 0 | 0.01/0 | 0.002 | 0.003 | 0.074 | 0.074 | 0.075 | 0.080 |
| | | | 0.03/0 | 0.051 | 0.048 | 0.880 | 0.895 | 0.850 | 0.864 |
| | | +1 | 0.005/0.01 | 0.005 | 0.002 | 0.024 | 0.014 | 0.079 | 0.089 |
| | | | 0.01/0.01 | 0.010 | 0.006 | 0.124 | 0.089 | 0.286 | 0.306 |
| | | | 0.03/0.03 | 0.211 | 0.117 | 0.963 | 0.920 | 0.995 | 0.994 |
| | | -1 | 0.005/0.01 | 0.015 | 0.001 | 0.062 | 0.006 | 0.017 | 0.001 |
| | | | 0.01/0.01 | 0.024 | 0.001 | 0.222 | 0.086 | 0.088 | 0.011 |
| | | | 0.03/0.03 | 0.551 | 0.106 | 0.991 | 0.908 | 0.932 | 0.530 |
| | 0.6 | 0 | 0.01/0 | 0.009 | 0.004 | 0.120 | 0.086 | 0.085 | 0.056 |
| | | | 0.03/0 | 0.204 | 0.069 | 0.955 | 0.900 | 0.913 | 0.832 |
| | | +1 | 0.005/0.01 | 0.002 | 0.002 | 0.017 | 0.027 | 0.051 | 0.061 |
| | | | 0.01/0.01 | 0.005 | 0.002 | 0.102 | 0.124 | 0.150 | 0.187 |
| | | | 0.03/0.03 | 0.112 | 0.104 | 0.925 | 0.943 | 0.968 | 0.973 |
| | | -1 | 0.005/0.01 | 0.103 | 0.004 | 0.229 | 0.012 | 0.119 | 0.000 |
| | | | 0.01/0.01 | 0.185 | 0.005 | 0.583 | 0.077 | 0.395 | 0.018 |
| | | | 0.03/0.03 | 0.960 | 0.120 | 1.000 | 0.880 | 1.000 | 0.575 |

[1] Su: unselected sample; S1: sample selected on T1 distribution; S2: sample selected on T1 and T2 distributions; [2] sample size**;**

**Table 4:** Association results for the top 10 most associated SNPs from SUR-based bivariate analysis of LS and FN BMD

| | | | | | | Bivariate analysis | | Univariate analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | LS | | | FN | | |
| **Chr. (Locus)** | **Closest Gene** | **Pos (bp)** | **SNP** | **[1]Min.** | **[2]MAF** | **P** | **[3]$R^2$ (%)** | **P** | **[4]Rank** | **[5]$R^2$ (%)** | **P** | **[4]Rank** | **[5]$R^2$ (%)** |
| 2q37.1 | SP100 | 231 037 761 | rs1649866 | A | 0.33 | 2.03E-05 | 3.41 | 0.54 | (-) | 0.12 | 0.09 | (-) | 0.95 |
| | | 231 042 007 | rs1678160 | G | 0.33 | 1.45E-05 | 3.52 | 0.57 | (-) | 0.10 | 0.07 | (21 500) | 1.04 |
| 3q25 | LEKR1 | 158 200 574 | rs6799034 | C | 0.43 | 1.81E-05 | 3.45 | 0.97 | (-) | 0.00 | 2.41E-02 | (7 166) | 1.63 |
| 6q22.1 | LOC643884 -LOC728590 | 113 858 994 | rs2049924 | A | 0.29 | **8.42E-06** | 3.69 | 1.80E-03 | (607) | 3.09 | 0.36 | (-) | 0.27 |
| 6q25 | TIAM2 | 155 533 083 | rs998318 | G | 0.31 | 2.94E-05 | 3.30 | 1.30E-05 | (2) | 5.94 | 1.22E-05 | (1) | 5.98 |
| 12p13-p12 | PZP - A2MP | 9 254 198 | rs1017301 | C | 0.34 | 2.24E-05 | 3.38 | 0.22 | (-) | 0.48 | 1.13E-03 | (348) | 3.36 |
| 15q14-15 | RYR3 | 31 680 776 | rs2437143 | C | 0.38 | **6.97E-06** | 3.75 | 8.41E-03 | (2 635) | 2.21 | 1.65E-05 | (3) | 5.80 |
| 19p13.11 | FAM125A | 17 392 450 | rs2303680 | G | 0.41 | 1.92E-05 | 3.43 | 0.74 | (-) | 0.03 | 4.66E-02 | (13 886) | 1.27 |
| 22q11.2 | SLC2A11 | 22 534 158 | rs2275979 | A | 0.18 | **5.44E-06** | 3.85 | 0.52 | (-) | 0.13 | 0.07 | (20 101) | 1.08 |
| 22q13 | LL22NC03-75B3.6 | 43 026 421 | rs3935378 | T | 0.49 | 1.64E-05 | 3.48 | 3.54E-06 | (1) | 6.69 | 2.95E-05 | (8) | 5.47 |

[1]: Minor allele; [2]: Minor allele frequency; [3]: r-square of the whole system taking into account the residual (co)variance matrix; [4]: rank of the identified SNP; [5]: r-square from linear regression.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Power
1.0
0.9
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rG=0
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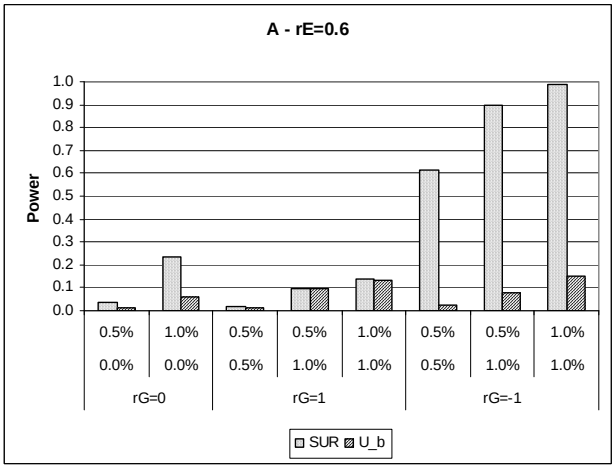
rG=-1
SUR
U_b


A - rE=0.6
Power
1.0
0.9
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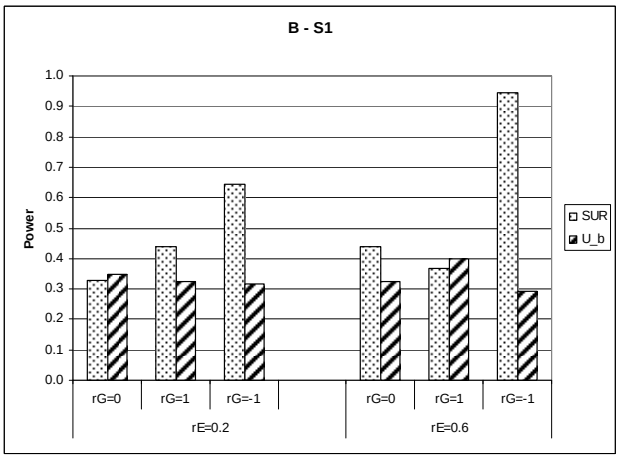
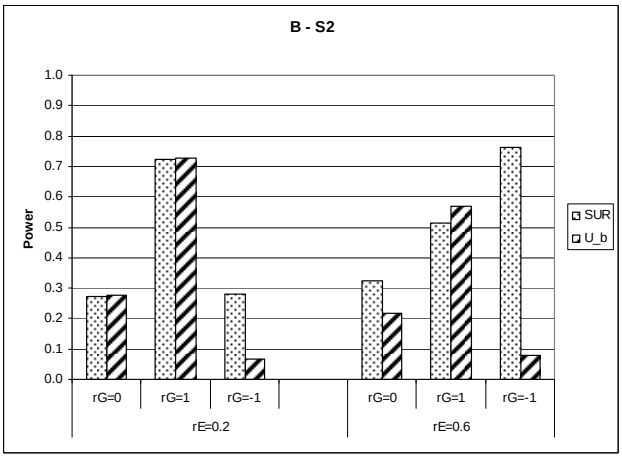
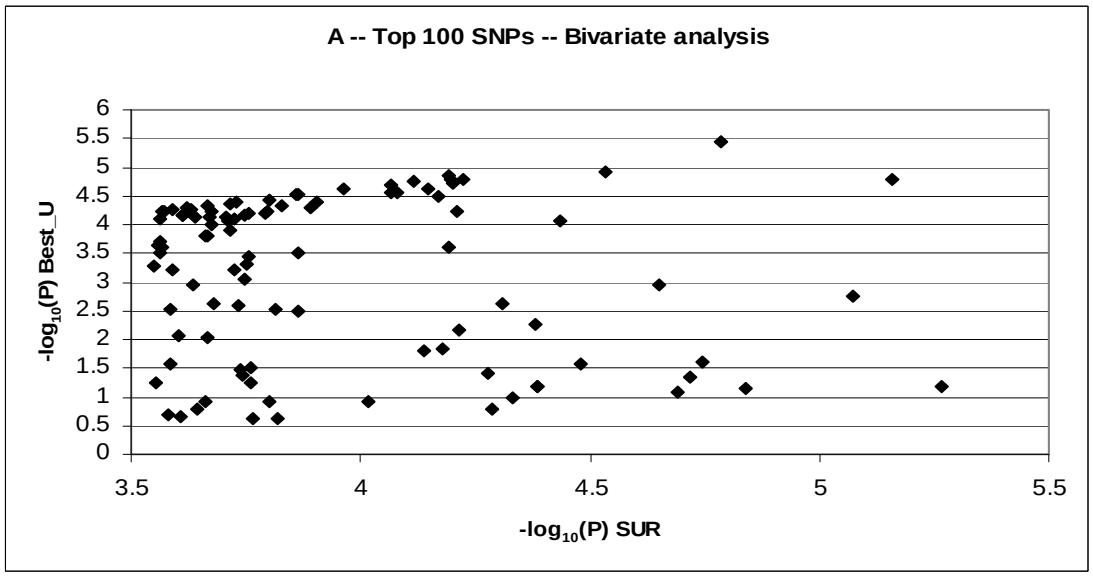
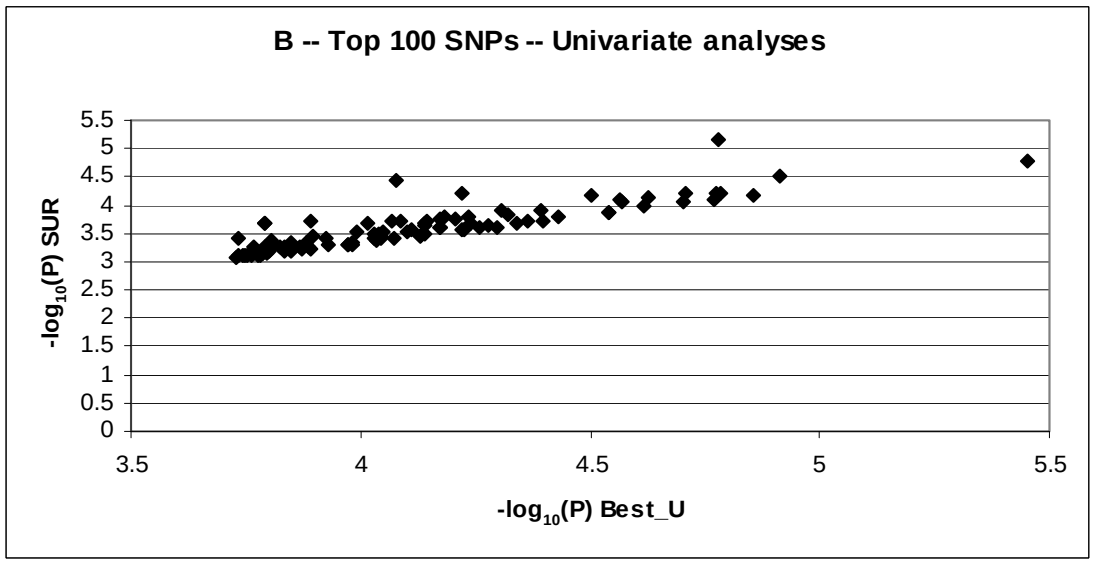
rG=0
rG=1
rG=-1
SUR
U_b


B - S1
Power
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rG=0
rG=1
rG=-1
rG=0
rG=1
rG=-1
rE=0.2
rE=0.6
SUR
U_b


B - S2
Power
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rG=0
rG=1
rG=-1
rG=0
rG=1
rG=-1
rE=0.2
rE=0.6
SUR
U_b


A -- Top 100 SNPs -- Bivariate analysis
-log10(P) Best_U
-log10(P) SUR
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3.5
4
4.5
5
5.5


B -- Top 100 SNPs -- Univariate analyses
-log10(P) SUR
-log10(P) Best_U
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3.5
4
4.5
5
5.5