

# Genetic association tests: A method for the joint analysis of family and case-control data

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## Abstract

With the trend in molecular epidemiology towards both genome-wide association studies and complex modeling, the need for large sample sizes to detect small effects and to allow for the estimation of many parameters within a model continues to increase. Unfortunately, most methods of association analysis have been restricted to either a family-based or a case-control design, resulting in the lack of synthesis of data from multiple studies. Transmission disequilibrium-type methods for detecting linkage disequilibrium from family data were developed as an effective way of preventing the detection of association due to population stratification. Because these methods condition on parental genotype, however, they have precluded the joint analysis of family and case-control data, although methods for case-control data may not protect against population stratification and do not allow for familial correlations. We present here an extension of a family-based association analysis method for continuous traits that will simultaneously test for, and if necessary control for, population stratification. We further extend this method to analyse binary traits (and therefore family and case-control data together) and accurately to estimate genetic effects in the population, even when using an ascertained family sample. Finally, we present the power of this binary extension for both family-only and joint family and case-control data, and demonstrate the accuracy of the association parameter and variance components in an ascertained family sample.

**Keywords:** ascertainment correction, family-based association, linkage disequilibrium

## Introduction

For much of the past three decades, linkage analysis has been the primary tool for the initial exploration of complex diseases believed to have an underlying genetic aetiology and has resulted in many large cohorts of family data with DNA samples available. Unfortunately, however, the ability of linkage analysis to localise potentially segregating susceptibility or protective genotypes has been limited to, at best, regions of 5–10 centimorgans (cM) in length and, at

worst, 20 cM in length.<sup>1</sup> This limitation has led to a rise in popularity of methods for detecting allelic (or gametic) association in candidate genes, in candidate linkage regions or genome-wide. This allelic association, coupled with linkage, allows for much more precise localisation of regions housing disease genes because, if it is due to linkage disequilibrium (LD), it will span a much shorter distance within the genome than is usually found by linkage analysis. With this rise in association studies, there has been a trend

toward the collection of unrelated case-control samples, often with the abandonment of large family studies. Certainly, these samples are much easier to obtain than are family samples, but they also have certain limitations, even within the context of recent genome-wide association successes.<sup>2,3</sup> Further, allelic association can be due to factors other than LD (which we define as the combination of allelic association and linkage) or pleiotropy (a marker allele itself being involved in the aetiology of the disease).<sup>4</sup>

Population stratification, which exists when multiple strata within a given sample differ with respect to either the underlying trait distribution or the marker genotype distribution (and which leads to spurious association when it occurs with respect to both), is a commonly cited cause of false-positive findings in case-control association studies (eg Knowler *et al.*<sup>5</sup>) and the most likely cause in genetic epidemiological studies. This threat of increased type I error rate has led to the development of many methods that guard against the effects of population stratification. The first two general classes use unlinked loci and can both be subsumed under the term 'genomic control': (1) test for population stratification using unlinked regions of the genome; (2) allow for the population stratification, as estimated from unlinked regions of the genome when performing an analysis of allelic association. The third general class guards against population stratification by using non-transmitted alleles as controls (ie a case-control design perfectly matched for ethnicity by appropriately using family data). While these methods are effective in controlling for population stratification, they each have their limitations with respect to power, efficiency and flexibility.

The limitations of genomic control methods<sup>6–8</sup> are the requirement of having genotypes at many loci unlinked to the disease allele. In the context of a genome-wide association scan, the choice of the best regions to use as a 'control' is difficult, as there is no guarantee that the markers being used are indeed unlinked to a disease gene. Applying this method to a candidate gene study suffers from the same limitation, but also requires significant additional cost and labour to type enough (and how many is enough?) additional loci.

Transmission disequilibrium tests (TDTs) — as they were termed by Spielman *et al.*<sup>9</sup> and are commonly referred to — comprise, in general, a unique study design (rather than a single statistical test) that protects against the effects of population stratification by comparing the frequencies of alleles (haplotypes or genotypes) transmitted from parents to their affected children with the frequencies of non-transmitted alleles to these same children. These tests of allelic association condition on, at least, parental genotypes and offspring disease phenotypes. Many TDT-type designs have been suggested since first proposed by Rubinstein *et al.*,<sup>10</sup> including extensions for multiple siblings, missing parents and extended pedigrees — to name but a few (see Table S1). All of these extensions, however, retain conditioning on part of the data available and therefore share the following limitations: (1) conditional tests are sensitive to sampling strategy, leading to very low power under several conditions;<sup>11</sup> and (2) missing parental data, transmissions from homozygous parents — or from heterozygous parents to heterozygous children — are non-informative, which results in a dramatic reduction of effective sample size and therefore of power, particularly when analysing single nucleotide polymorphism (SNP) data. This may also lead to an increased type I error rate if care is not taken to include the transmissions from two similarly heterozygous parents when the child is heterozygous.<sup>12</sup> Further, as for all tests of allelic association, the power of TDT-type designs rapidly decreases if the marker is not the disease locus and/or if the marker and disease allele frequencies differ.<sup>13–15</sup>

Novel methodological approaches for the analysis of LD in family data include a class of variance component approaches and what are termed family-based association tests (FBATs). Fulker first proposed a test for both between-family association (or, more appropriately, 'among-family', as we typically expect more than two families), which models the phenotypic means given the marker locus genotypes, and within-family association (linkage) by using identity-by-descent status in modelling the sib-pair variance-covariance structure.<sup>16</sup> It was shown that the within-family component provides

an estimate of the additive genetic effect unaffected by population stratification. Sham *et al.*<sup>17</sup> extended this method to incorporate larger sibships, dominance variance and multi-allelic markers. It was further extended to sibships with or without parental genotypes, and to multi-generational pedigree data by Abecasis *et al.*<sup>18</sup> FBAT is a unified approach to family-based tests of association that ‘compares tests for association to their conditional distributions given the minimal sufficient statistics under the null hypothesis for the genetic model, sampling plan and population admixture’,<sup>19</sup> in two steps: (1) building a test statistic that is sensitive to the co-variation of the trait and marker; and (2) finding the distribution of the test statistic under the null hypothesis. Broadly speaking, the test statistic is the ‘covariance between a function of the genotype and a function of the trait’,<sup>20</sup> the dependent variable being the offspring genotype. While the first step gives great flexibility in the choice of test statistic, the second is designed to ensure correct type I error rates (ie validity), regardless of population admixture, genetic model or ascertainment scheme.<sup>21</sup> These approaches are broad, in that they can handle different genetic models, different family structures (including extended pedigrees) and disease phenotypes (qualitative or quantitative, single or multiple). As with the original TDT, however, only heterozygous parents are informative in this framework; non-family data cannot be included and, in the case of FBAT, even if one does have a random sample, the effect size of the allele of interest is not estimated. This can lead to a dramatic loss of effective sample size and therefore potential power and/or precision when compared with an unconditional method such as that presented here and demonstrated in our previous work.<sup>22</sup> Other methods more robust to these particular limitations have been recently proposed for assessing quantitative traits in family-based samples<sup>23</sup> and binary traits in case-control samples, including related individuals.<sup>24,25</sup> Neither of these methods, however, includes an ascertainment correction (central to pooling family and case-control samples), nor do they estimate family or cluster effects. Further, the former does not allow for the

inclusion of case-control data and the latter does not allow for the inclusion of covariates.

Based on the limitations of the existing strategies for testing LD, we present an alternative two-stage family-based association test in which we combine attributes of two existing methods, first to test whether population stratification is present and then appropriately test for and estimate the effect of, LD of a marker to a continuous trait. We further offer extensions of this method that can be applied to binary traits and hence allow an analysis of case-control data together with family data. We illustrate the power of this method for various sample sizes and structures, specifically for joint family and population-based samples that cannot be analysed by existing methods. We also extend this method to allow for the accurate estimation of association parameters and residual variance components from ascertained family data, and demonstrate, via simulation, that this method is effective in controlling ascertainment bias.

## Methods

### Continuous traits

The framework on which our approach is built was first described by George and Elston<sup>26</sup> and Elston *et al.*<sup>27</sup> in the special context of a randomly selected family sample with a measurable, quantitative trait of interest. For any individual  $i$ , with continuous trait (or, as we will later discuss, liability)  $y_i$ ,  $j^{\text{th}}$  covariate values  $c_{ji}$  and a genotype indicator  $z_i$ , we can construct a regression model of the form:

$$h(y_i) = h(\alpha + \gamma_1 c_{1i} + \gamma_2 c_{2i} + \dots + \gamma_n c_{ni} + \delta z_i) + p_i + f_i + f'_i + m_i + s_i + \varepsilon_i, \quad (1)$$

in which the number of  $A_1$  alleles, along with other covariates, is a predictor of phenotype. In this model,  $z_i$  is coded such that the allelic effect of substituting  $A_2$  for  $A_1$  is  $\frac{1}{2} \delta$ . The random components include  $p_i$ , a random polygenic effect,  $f_i$  and  $f'_i$ , random nuclear family effects,  $m_i$ , a random marital effect,  $s_i$ , a random sibship effect and  $\varepsilon_i$ , a random residual individual effect. In addition, the generalised power transformation ( $h$ ),<sup>28</sup> applied to both the

trait and its predictors, when simultaneously estimated under a model that assumes normality of the residuals, helps assure both linearity and normality, thus making the model robust to non-independence (as can be the case in large pedigrees). There are two random nuclear family effects  $f_i$  and  $f'_i$  in model (1) because each individual is potentially a member of two different nuclear families, one in which we include the individual's parents and siblings and one in which we include the individual's spouse and children. All the random effects in the model are assumed to be mutually independent and, after the transformation, normally distributed with zero means and variances  $\sigma_p^2$ ,  $\sigma_f^2 = \sigma_{f'}^2$ ,  $\sigma_m^2$ ,  $\sigma_s^2$  and  $\sigma_\varepsilon^2$  such that:  $V[h(y_i)] = \sigma_p^2 + \sigma_f^2 + \sigma_{f'}^2 + \sigma_m^2 + \sigma_s^2 + \sigma_\varepsilon^2$  for families with more than two generations, and  $V[h(y_i)] = \sigma_p^2 + \sigma_f^2 + \sigma_m^2 + \sigma_s^2 + \sigma_\varepsilon^2$  for families with only two generations. It is important to note that the total variance  $V[h(y_i)]$  is made the same for all individuals by adjusting the residual variance  $\sigma_\varepsilon^2$  separately for each person (see Elston *et al.*<sup>27</sup> for details). This model has recently been further extended to allow for each person to have more than two nuclear family effects, as can occur when there are half-sibships in the data, and other kinds of common environmental cluster effects.

As currently implemented in the S.A.G.E. program ASSOC, the likelihood is maximised numerically over all parameters, and standard errors are determined by numerical double differentiation of the log likelihood. Also,  $p$ -values, based on the likelihood ratio or a Wald test, can be calculated for the transformation parameters, any of the variance components and any regression coefficients. They are two-sided for all transformation parameters and regression coefficients, and one-sided for all variance components.

This method is meant to follow existing evidence of linkage because it does not control for population stratification. With the growing popularity of genome-wide and candidate gene association studies, however, there are likely to be many instances in which linkage is not known *a priori*. For this reason, we suggest — rather than automatically resorting to cumbersome genomic control methods or a less powerful TDT-type

design — using a two-stage procedure to (1) test for a stratification effect and then (2) test for allelic association. If there is no stratification, then the association resulting from model (1) can be interpreted as LD effects. If there is stratification, then one can use the same regression model framework to perform a test like those mentioned above (TDT and FBAT) that conditions on parental genotype.

To test for stratification, we use the same regression model outlined above, but with the addition of transmitted and non-transmitted allele indicators ( $x_{1i}$  and  $x_{2i}$ ) defined as:

$$x_{1i} = \begin{cases} 1 & \text{if A is transmitted from an informative mating} \\ 0 & \text{otherwise} \end{cases}$$

$$x_{2i} = \begin{cases} 1 & \text{if A is not transmitted from an} \\ & \text{informative mating} \\ 0 & \text{otherwise.} \end{cases}$$

Thus, the regression equation (3) for the trait value  $y_i$  is now defined as

$$h(y_i) = h(\alpha + \beta_1 x_{1i} + \beta_2 x_{2i} + \gamma_1 c_{1i} + \gamma_2 c_{2i} + \dots + \gamma_n c_{ni} + \delta z_i) + \eta_i, \quad (2)$$

where  $\eta_i$  is the random effect comprising all of the familial, sibling, marital, polygenic and individual specific errors outlined above. George *et al.*<sup>29</sup> gave details of how the indicator variable  $x_{1i}$  is constructed to form a TDT-type test by substituting it for  $z_i$  in regression model (1). We point out that, because it includes components of a TDT-type test, it requires family data. The variable  $x_{2i}$  is formed analogously for the other allele of an SNP. In the case of a multi-allelic marker, all the other alleles can be pooled into a single allele for this purpose. To test for a stratification effect, we first test the null hypothesis that the genotypic effect is half the difference of the transmitted allele effects; that is,  $\beta_2 - \beta_1 = \frac{1}{2} \delta$ . If we do not reject this null hypothesis at some liberal significance level such as  $p = 0.2$ , we infer that there is no evidence of stratification, set  $\beta_2 = \beta_1 = 0$  and estimate the allele  $A_1$  effect by  $\frac{1}{2} \delta$ . If there is any

evidence of stratification, we set  $\delta = 0$  and estimate the allele  $A_1$  effect by  $\beta_2 - \beta_1$ . Thus, once either  $\beta_2 = \beta_1$  or  $\delta$  is set to 0, as appropriate, we return to a framework in which we simultaneously estimate the effect of allele  $A_1$ , the residual variance components and one or more transformation parameters. We can use asymptotic results to obtain confidence intervals for all parameter estimates in the usual way, and the method can be extended to estimate genotype effects rather than allele effects. While other approaches like the principal component approach proposed by Zhu *et al.*<sup>30</sup> work well within this regression framework and are potentially more informative when many SNPs are available, this new approach is a viable option, even if only one or a few SNPs are typed (ie in the case of a candidate gene study).

### Extension to binary traits

The generalised modulus power transformation mentioned above is fairly effective in inducing approximate normality, but does, of course, assume a continuous trait distribution. In many cases, continuous traits are not available to characterise complex diseases and only the presence or absence of disease is available. Therefore, we propose the following algorithmic extension of Zhu *et al.*<sup>31</sup> Let

$$\mu_{i0} = \begin{cases} 0.9, & \text{if } y_i^* = 1 \\ 0.1, & \text{if } y_i^* = 0 \end{cases}, \quad (3)$$

where  $y_i^*$  is the binary trait of interest, 1 represents affected individuals and 0 represents unaffected, and  $\mu_{i0}$  represents an initial estimate of  $E(y_i^*)$ . Our aim here is to define a new trait  $y_i$  that, if  $\mu_i$  were its expected value, would be approximately normally distributed with mean 0 and variance 1. We use the values of  $y_i$  defined by equations (2) and (3) as the dependent variable in a simple generalised linear regression model of the form

$$\log \text{it}[E(y_i)] = \alpha + \beta_1 x_{1i} + \beta_2 x_{2i} + \gamma_1 c_{1i} + \gamma_2 c_{2i} + \dots + \gamma_n c_{ni} + \delta z_i \quad (4)$$

We have shown that the iterative maximisation procedure currently implemented in our software (ASSOC) is quite robust to these initial estimates,

regardless of family size or misspecified analysis model.<sup>32</sup> We do note, however, that the ease of maximisation and the accuracy of estimates depend on both the sample size and the number of parameters estimated. In general, we recommend at least 20 observations per parameter estimated to ensure accuracy (which can be assessed based on standard errors we provide).

Because the likelihood that is maximised by this process is perhaps not a true likelihood (it is a pseudo-likelihood, in that the estimates of the variance components may be based on incorrect model assumptions), the variance-covariance matrix of the estimators obtained by double differentiation of the likelihood may not equal the true variance matrix, even asymptotically. We may therefore estimate the variance-covariance matrix using the robust sandwich estimator,<sup>33</sup>

$$V_{sand} = \hat{H}_1^{-1} \hat{H}_2 \hat{H}_1^{-1}, \quad (5)$$

where  $\hat{H}_1$  is the estimated Fisher information matrix, which we need not assume is correctly specified, and  $\hat{H}_2$  is the estimated outer product gradient expressed as

$$\hat{H}_2 = \sum_k \hat{D}'_k \sum_k^{-1} [(y_k - \mu_k)(y_k - \mu_k)'] \sum_k^{-1} \hat{D}'_k \quad (6)$$

where, for the the  $k^{th}$  pedigree,  $\sum_k$  is a diagonal matrix with elements  $\mu_{ik}(1 - \mu_{ik})$ ,  $y_k$  is the vector of trait values for the  $k^{th}$  pedigree,  $\mu_k$  is the vector of means specific to the  $k^{th}$  pedigree and  $D_k$ , with transpose  $D'_k$ , is the matrix of first order partial derivatives of  $\mu_k$  with respect to  $\beta$  obtained assuming that the covariates are fixed:

$$D_{Nk \times p}^k = \begin{bmatrix} \frac{\partial \mu_1}{\partial \beta_1} & \frac{\partial \mu_1}{\partial \beta_2} & \dots & \frac{\partial \mu_1}{\partial \beta_p} \\ \frac{\partial \mu_2}{\partial \beta_1} & \frac{\partial \mu_2}{\partial \beta_2} & \dots & \frac{\partial \mu_2}{\partial \beta_p} \\ \vdots & \vdots & \ddots & \vdots \\ \frac{\partial \mu_{Nk1}}{\partial \beta_1} & \frac{\partial \mu_{Nk1}}{\partial \beta_2} & \dots & \frac{\partial \mu_{Nk1}}{\partial \beta_p} \end{bmatrix}. \quad (7)$$

In matrix (7),  $N_k$  is the number of persons in the  $k^{\text{th}}$  pedigree,  $p$  is the total number of regression coefficients in equation (4), including the intercept, and  $\beta_j$  represents any one of them.

### Combining case-control and family data

One of the benefits of the regression framework outlined above is the flexibility to include families of any size or structure. This is vital, given, as mentioned above, that the magnitude of the effects associated with any given gene for a common complex disease is likely to be small. Certainly, provided we are only interested in hypothesis testing (we will discuss parameter estimation later), unmatched case-control data can be easily included as single person pedigrees with only an individual-specific variance. In this framework, however, matched case-control data can also be included by simply specifying the matched pairs as members of the same cluster (a cluster, of course, being a special case of a pedigree). We include in the model a cluster-specific variance component  $\sigma_c^2$ , such that  $V[\text{logit}(y_i)] = \sigma_c^2 + \sigma_e^2$ , and then adjust the residual variance  $\sigma_e^2$  so as to keep the total variance the same for all individuals. This approach does not limit the case-control cluster size or composition, as does conditional logistic regression.

### Correcting for ascertainment

The underlying assumption of the method outlined above is that the sampling units (families, individuals, case-control clusters) represent a random sample from the same population. This is often not the case — particularly when families were sampled for a linkage study — and cannot be the case for case-control samples. The sample association and variance component estimates are thus not representative of the population values. We therefore present an ascertainment correction specifically for family data (and briefly address an extension to case-control data in the discussion).

Let the proband sampling frame (PSF) comprise those individuals who, regardless of phenotype, could have allowed the family to be ascertained by

reason of being in the catchment area (the area from which the sample was collected). Then, let the ascertainment corrected natural log (ln) likelihood be:

$$\ln L(P) = \ln L(P_{All}) - \ln L(P_{PSF}) \quad (8)$$

where  $L(P)$  is the final likelihood,  $L(P_{All})$  is the likelihood for the whole sample on the assumption of random sampling of families and  $L(P_{PSF})$  is the likelihood for the family members in the PSF, similarly on the assumption of random sampling. (For single ascertainment, only the probands are included in the PSF). Maximising this likelihood (8) leads to consistent estimators of all the parameters.<sup>34</sup>

### Power calculations for family data

To assess the power and type I error of our association analysis method as extended to binary traits, as well as to verify the accuracy of both the association parameters and the residual variance components for ascertained data, we simulated 2,000 replicates of samples of 1,000 individuals comprising either 200 nuclear families (two founders, three offspring) or 125 extended pedigrees (three founders, one of whom is a ‘marry-in’: three generations; one sibship of size 3 in generation 2; one sibship of size 2 in generation 3). A continuous liability was created according to the following linear model:

$$y_i = a_i + \gamma p_i + \sum_{j=1}^k \delta_j d_{ji} + \varepsilon e_i, \quad (9)$$

where  $i$  represents the  $i^{\text{th}}$  individual;  $a_i$  is the genotypic effect assigned based on an individual’s major genotype defined as:

$$a = \sqrt{\frac{h_s^2}{2q(1-q)}}, \quad (10)$$

where  $q$  is the allele frequency and  $h_s^2$  is locus-specific heritability, which we varied to have values 0 (the null hypothesis), 0.0025, 0.0125, 0.025, 0.0375, 0.05 and 0.0625;  $\gamma$  is the coefficient (set to 0.25) of the polygenic effect (or ‘polygenotype’)  $p_i$ , generated from a  $N(0,1)$  distribution for founders

and for non-founders derived as  $\frac{1}{2}$  (polygenotype of the mother + polygenotype of the father) + a randomly generated value from a  $N(0, \frac{1}{2})$ ;  $\delta_j$  is the coefficient for the  $j^{\text{th}}$  environmental effect which, in our examples were familial (F), sibling (S) and/or marital (M) (set to 0 when not included in the model and to 0.25 otherwise);  $d_{ji}$  is the environmental factor value assigned to all individuals within the same familial cluster and distributed  $N(0,1)$  across such clusters,  $\varepsilon$  (set to 0.5) is the coefficient of the random effect; and  $e_i$  is generated separately for each individual from a  $N(0,1)$  distribution. The liability  $y_i$  was then transformed to a binary phenotype. First, a standardised liability was created as:

$$z_i = \frac{(y_i - \bar{y})}{\sqrt{1/n - 1 \sum_{i=1}^n (y_i - \bar{y})^2}}, \quad (11)$$

where  $y_i$  is the continuous liability created as defined above and is a mixture of three normal distributions with means equal to the genotypic effects of the three genotypes and a common variance (specific to the variance component model as shown in Table 1),  $\bar{y}$  is the sample mean,  $n$  is the total sample size, and  $1/n - 1 \sum_{i=1}^n (y_i - \bar{y})^2$  is the sample variance. This transformation resulted in three distributions for the  $A_1A_1$ ,  $A_1A_2$ , and  $A_2A_2$  genotypes, with means  $(a - (q^2a - (1 - q)^2a))$ ,  $(0 - (q^2a - (1 - q)^2a))$ , and  $(-a - (q^2a - (1 - q)^2a))$ , respectively, the whole mixture distribution having a

variance of 1. Then, an individual was classified as affected if  $z_i > x$ , unaffected otherwise. For all simulations,  $x$  was fixed at 1.28, corresponding to a disease prevalence of approximately 0.1. Thus,  $A_1$  is the 'risk' allele.

Creation of a random sample was achieved by simply collecting individuals (and thus their entire pedigree) from the simulated population in the order in which they were encountered until the desired sample size (1,000 individuals) was met. All replicates were analysed using both the simulated correlation model and an 'incorrect' correlation model. For example, if data were simulated to have both a familial and polygenic effect, they were analysed under a model (denoted as FP) including both effects and one (denoted P) that included only a polygenic effect. Names for all the models investigated are enumerated in Table 1.

Type I error was calculated as the number of replicates simulated under the null hypothesis meeting a recommended cut-off point for genome-wide association studies by the Wellcome Trust of  $\alpha = 5 \times 10^{-7}$ .<sup>35</sup> Power was calculated as the number of replicates meeting the same criterion but simulated under the alternate hypothesis.

### Sample size estimation for joint family and case-control data

In addition to the simulations outlined above, in order to demonstrate the usefulness of this method for the joint analysis of family and population or

**Table 1.** Total variance of the non-major gene component of the continuous liability underlying the binary trait and the proportion of that variance represented by each variance component for each model

Model name	Simulated proportion of variance for each variance component					
	Total variance	Polygenic	Familial	Sibling	Marital	Random
P	0.3125	0.200	-----	-----	-----	0.800
FP	0.3750	0.167	0.167	-----	-----	0.667
SP	0.3750	0.167	-----	0.167	-----	0.667
MP	0.3750	0.167	-----	-----	0.167	0.667
SMP	0.4375	0.143	-----	0.143	0.143	0.571

F = familial effect; M = marital effect; P = polygenic effect; S = sibling effect.

case-control data, we analytically estimated, for a combination of unrelated individuals (50 per cent cases, 50 per cent controls), nuclear families and/or extended pedigrees, the number of individuals required to detect a given effect size at a fixed type I error rate and power.

For these calculations, we classified families according to the number of founders and non-founders (where unrelated individuals were simply one-founder pedigrees). Suppose there are  $n_i$  families of the  $i^{\text{th}}$  type, each with  $n_{fi}$  founders and  $n_{nfi}$  non-founders. Let  $y_{jm}$  be the trait value underlying liability for the  $m^{\text{th}}$  individual in the  $j^{\text{th}}$  family with polymorphic marker value  $g_{ji}$ , and let  $\alpha_{jm}$  be a row vector whose elements are the intercept and effect of other covariates. Let  $\beta$  be the regression coefficient on the polymorphic marker and define  $Z_{jm} = y_{jm} - \alpha_{jm}1 = \beta(g_{jm} - E(g_{jm}))$ , where  $1$  is a column vector of unities. The three genotypes of the marker are assumed to have the values  $(-2, 1, 1)$ ,  $(-1, -1, 2)$  and  $(-1, 0, 1)$  for dominant, recessive and additive modes of inheritance, respectively. Then, letting  $Z_i' = (z_{f1} \dots z_{fm}, z_{nfi} \dots z_{nfnfi})$ ,  $\Sigma_i^{-1}$  = the inverse of the variance-covariance matrix for the  $i^{\text{th}}$ -type family and assuming multivariate normality, the log likelihood for the  $i^{\text{th}}$ -type family is  $L_i = \text{const} - \frac{1}{2}(Z_i - \beta(G_i - E(G_i)))' \Sigma_i^{-1} (Z_i - \beta(G_i - E(G_i)))$ , giving the maximum likelihood estimator

$$\hat{\beta} = \frac{\sum_i n_i (G_i - E(G_i))' \Sigma_i^{-1} Z_i}{\sum_i n_i (G_i - E(G_i))' \Sigma_i^{-1} (G_i - E(G_i))}, \text{ with}$$

$$\begin{aligned} \text{var}(\hat{\beta}) &= \frac{\sum_i n_i \text{var}((G_i - E(G_i))' \Sigma_i^{-1} Z_i)}{(\sum_i n_i (G_i - E(G_i))' \Sigma_i^{-1} (G_i - E(G_i)))^2} \\ &= \frac{\sum_i n_i (G_i - E(G_i))' \Sigma_i^{-1} (G_i - E(G_i))}{(\sum_i n_i (G_i - E(G_i))' \Sigma_i^{-1} (G_i - E(G_i)))^2} \\ &= \frac{1}{\sum_i n_i (G_i - E(G_i))' \Sigma_i^{-1} (G_i - E(G_i))}. \end{aligned}$$

This is an extension of Nick *et al.*,<sup>36</sup> who gave approximate results for exactly two founders and a dominant mode of inheritance, and assumes the quantitative trait locus and marker variants are in perfect LD. We derived  $\text{var}(\hat{\beta})$  more generally for  $n_{fi}$  founders, for both additive and dominant inheritance, as well as for relative pair specific correlations. We also allowed for incomplete LD by applying a  $1/(0.8)$ -fold factor (equivalent to  $r^2 = 0.8$ ,  $D' \approx 0.9$ ).

For these calculations, we made some simplifying but conservative assumptions. First, we assumed that founder pairs have a correlation of 0 and that parent-offspring correlations ( $\rho_{po}$ ) and sib-sib correlations ( $\rho_{ss}$ ) correspond to a residual heritability of 2  $\rho_{po} = 2 \rho_{ss}$  and that grandparent-grandchild pairs have a residual correlation of  $\rho_{gg}$  corresponding to a residual heritability of 4  $\rho_{gg}$ . We further assumed, for simplicity, that all persons with the same genotype at the disease locus have the same disease risk and that LD between the locus and the closest SNP, assuming the same allele frequencies at the two loci, is given by  $r^2$ . Finally, we imposed the type I error recommended for genome-wide association studies by the Wellcome Trust of  $\alpha = 5 \times 10^{-7}$ ,<sup>35</sup> and assumed a fixed power equivalent to a sample of 500 cases and 500 controls (0.92 for an additive model and 0.86 for a dominant model), and a locus-specific heritability of  $h_{ls}^2$  — see equation (11) — of 0.05. We did this for samples of nuclear families only, extended pedigrees only and mixtures of both, for various sample sizes, and then, demonstrated the approximate linearity of the trend in sample size needed to detect the same effect given a fixed power and type I error.

### Accuracy of association and variance component estimates

In addition to generating random family samples (RAND), we also generated a sample of singly ascertained families (ASC) by assigning each family a probability of entering the sample based on the number of affected members in the family:  $P(\text{family enters sample}) = N_a/N$ , where  $N_a$  is the number of affected members in the family and  $N$  is the number of family members. Each simulation output file was parsed and, if a family had an affected member, the above probability was calculated and, based on the

appropriate Bernoulli distribution, the family was either ascertained or not until the desired sample size was met.

The accuracy of the locus-specific association parameter ( $\beta$ ) and the polygenic and familial variance components were assessed after the appropriate analysis (ie with ascertainment correction if ASC and without if RAND) using the empirically found mean square error (MSE) averaged over 100 replicates, each comprising 1,000 individuals from either 200 nuclear families or 125 extended pedigrees. In all cases, the root mean square error (rMSE) compared with the simulated value (Tables 2–4) is reported. As mentioned, the accuracy of estimates in ascertained case-control samples was not addressed in this study, but is discussed below.

## Results

### Type I error and power in family data

Under both additive and dominant models, the association method we present for detecting diallelic trait loci has stable type I error rates of less than 0.05 (mean = 0.0452) for the RAND sample of both the nuclear families and extended pedigrees.

**Table 2.** Accuracy of the association parameter as In odds of being affected given two copies of the disease allele versus one copy for a sample size of 1,000 individuals

Model*		Nuclear		Extended	
		RAND	ASC	RAND	ASC
FP-FP	Est	2.529	2.479	2.511	2.561
	rMSE	0.1709	0.1210	0.1530	0.2030
FP-P	Est	2.537	2.509	2.517	2.524
	rMSE	0.1789	0.1510	0.1591	0.1661
P-FP	Est	2.780	2.655	2.763	2.722
	rMSE	0.1775	0.0529	0.1603	0.1196
P-P	Est	2.780	2.648	2.768	2.724
	rMSE	0.1775	0.0458	0.1655	0.1212

\*Model indicates the variance components that were simulated followed by those included in the analysis model (F = familial and P = polygenic); Est is the average estimate across all replicates of that model; rMSE is the square root of the mean square error; ASC represents the analysis of an ascertained sample using ascertainment correction and RAND represents the analysis of a random sample without any such correction.

**Table 3.** Accuracy of the association parameter as In odds of being affected given two copies of the disease allele versus no copies for a sample size of 1,000 individuals

Model*		Nuclear		Extended	
		RAND	ASC	RAND	ASC
FP-FP	Est	5.058	4.958	5.022	5.122
	rMSE	0.2936	0.3936	0.3295	0.2296
FP-P	Est	5.074	5.018	5.034	5.048
	rMSE	0.2777	0.3336	0.3176	0.3036
P-FP	Est	5.560	5.310	5.526	5.444
	rMSE	0.1010	0.5696	0.3536	0.4357
P-P	Est	5.560	5.296	5.536	5.448
	rMSE	0.3195	0.5836	0.3437	0.4316

\*Model indicates the variance components that were simulated followed by those included in the analysis model (F = familial and P = polygenic); Est is the average estimate across all replicates of that model; rMSE is the square root of the mean square error; ASC represents the analysis of an ascertained sample using ascertainment correction and RAND represents the analysis of a random sample without any such correction.

The ASC sample had slightly higher type I error rates for the nuclear family sample (0.0523) but not for the extended pedigrees (0.0427). The power reached 100 per cent at a total heritability of 0.25 ( $h_{ts}^2=0.0625$ ) for both the additive and dominant models in both the nuclear family sample and the extended pedigrees, and there was virtually no power to detect a heritability of 0.01. The power curves for the RAND and ASC samples were virtually identical, so for the sake of space only the ASC curves are presented. The nuclear family sample (200 families, 1,000 individuals), outperformed the extended pedigree sample (125 families, 1,000 individuals) under both models. Further, there was a steep decline in power between heritabilities of 0.15 ( $h_{ts}^2 = 0.0375$ ) and 0.10 ( $h_{ts}^2 = 0.025$ ) (Figure 1).

### Sample size estimation in joint family and case-control data

To demonstrate the usefulness of family data in association analysis, as well as the usefulness of combining samples from both linkage (family-based) and association (typically case-control)

**Table 4.** Accuracy of variance components as proportions of the total variance,  $N=1,000$ 

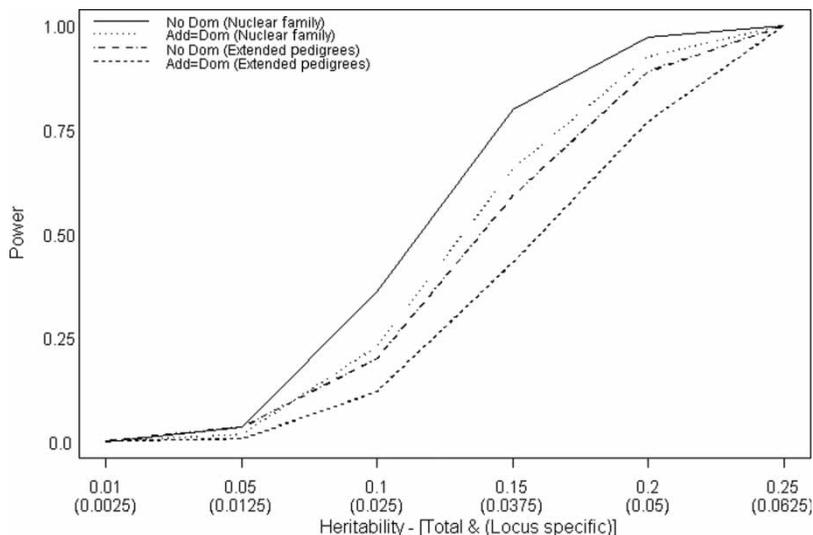
Parameter	Model		Nuclear		Extended			
			RAND	ASC	RAND	ASC		
Marital	MP–MP	Est	0.079	0.068	0.0775	0.0696		
		rMSE	0.0877	0.0990	0.0894	0.0693		
	SMP–SMP	Est	0.1743	0.0636	0.1291	0.0555		
		rMSE	0.0316	0.0781	0.0141	0.0866		
Sibling	SP–SP	Est	0.0574	0.0669	0.0549	0.0713		
		rMSE	0.1095	0.1000	0.1122	0.0959		
	SMP–SMP	Est	0.0549	0.0554	0.057	0.0579		
		rMSE	0.0883	0.1118	0.0860	0.1090		
		Polygenic	FP–FP	Est	0.0896	0.0604	0.1711	0.1388
				rMSE	0.0775	0.1068	0.0000	0.0283
MP–MP	Est	0.0741	0.0655	0.0775	0.0643			
	rMSE	0.0927	0.1015	0.0894	0.1030			
P–P	P–P	Est	0.063	0.0805	0.0602	0.0755		
		rMSE	0.1039	0.1196	0.1068	0.1249		
	SMP–SMP	Est	0.2169	0.0617	0.1759	0.0559		
		rMSE	0.0742	0.0800	0.0332	0.0860		
		SP–SP	Est	0.0962	0.0723	0.0782	0.0603	
			rMSE	0.0707	0.0949	0.0889	0.1068	
Familial	FP–FP	Est	0.1133	0.0122	0.033	0.0139		
		rMSE	0.0539	0.3521	0.1342	0.1530		
	P–FP	Est	0.0198	0.0032	0.048	0.0102		
		rMSE	0.0200	0.0032	0.0480	0.0100		

\*Model indicates the variance components that were simulated followed by those included in the analysis model (F = familial, M = marital, S = sibling and P = polygenic); Est is the average estimate across all replicates of that model; rMSE is the square root of the mean square error; ASC represents the analysis of an ascertained sample using ascertainment correction and RAND represents the analysis of a random sample without any such correction.

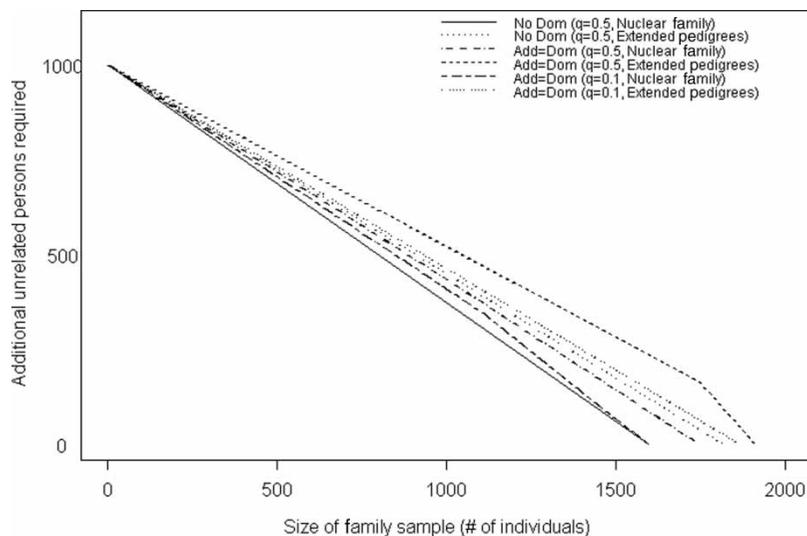
studies, we evaluated the number of unrelated individuals that need to be added to an existing family sample to be able to detect the same effect size as in a population-based sample of 1,000 unrelated cases and 1,000 controls. Beginning with a collection of 125 extended pedigrees or 200 nuclear families (samples that are quite prevalent), one can decrease the number of unrelated samples needed to detect a given effect size ( $h_s^2 = 0.05$ ) by at

minimum close to 40 per cent and at maximum more than 50 per cent (Figure 2).

Note that the equivalence of samples is shown, assuming (1) a common minor allele frequency ( $q = 0.5$ ), for which the family data are not as informative as are the case-control data, and (2) an allele frequency under which the family sample is fairly informative ( $q = 0.1$ ). As expected, the nuclear family sample (assuming an additive model



**Figure 1.** Power to detect association by both total and locus-specific heritability for nuclear families (nuc fam) under an additive model (No Dom) and a model with 50 per cent additive and 50 per cent dominance variance (Add = Dom).



**Figure 2.** Number of unrelated case-control samples needed, in addition to a fixed sample of either nuclear or extended pedigrees, to achieve a power of 92 per cent under an additive model (No Dom) and 86 per cent under a model with 50 per cent additive and 50 per cent dominance variance (Add = Dom). Values were generated for fixed sample sizes of both nuclear families and extended pedigrees, as well as for allele frequencies of both 0.5 and 0.1.

with  $q = 0.5$ ) requires the fewest additional unrelated individuals to detect a given effect, and the extended pedigree sample (assuming a dominant model with  $q = 0.5$ ) requires the most additional unrelated individuals. The extended pedigree sample, under a dominant model with  $q = 0.5$  or

0.1, performed similarly, as did the nuclear family sample under a dominant model with  $q = 0.5$  or 0.1. The extended pedigree and nuclear family samples (assuming an additive model) require approximately the same number of additional unrelated persons to detect the given effect size.

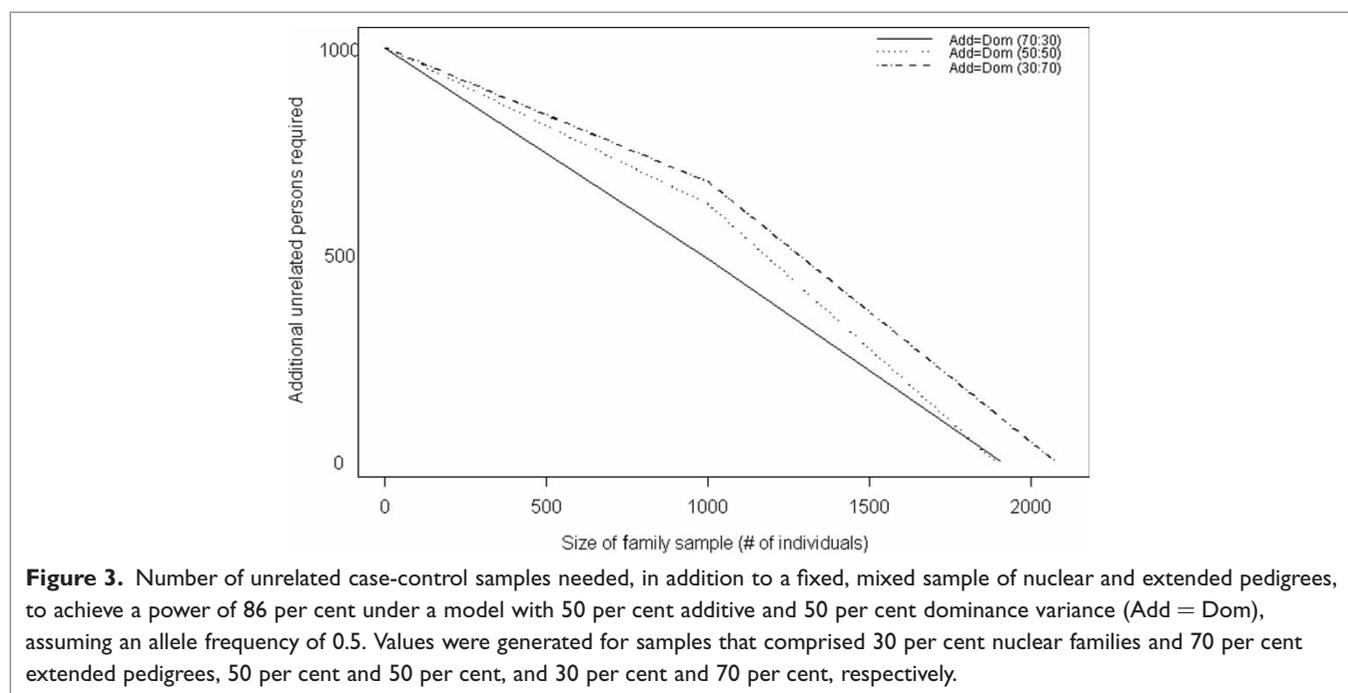
In addition to providing the number of additional individuals necessary to detect a fixed effect size given a sample of nuclear or extended pedigrees, we further provide this information given a sample comprising varying proportions of nuclear and extended pedigrees. We found that, for the additive and dominant models, regardless of the allele frequency, the samples that contained 30 per cent extended pedigrees and 70 per cent nuclear families (30:70) required the fewest additional unrelated individuals (of the three mixtures examined) to attain the same power. For the model in which dominance and additive variance were equal (Add = Dom) with allele frequency 0.5, the sample with equal frequency of nuclear and extended pedigrees (50:50) and the sample that is 70 per cent extended and 30 per cent nuclear (70:30) require similar sample sizes except in the extreme cases where there are very few to no unrelated individuals (Figure 3). The results for the Add = Dom model with  $q = 0.1$  are similar to those for the model with  $q = 0.5$ , except that the divergence of the 50:50 and 70:30 samples is not as large as in the previous case (Figure 4). The additive model indicates the least difference in the three sample types (Figure 5); for example, a sample of 140

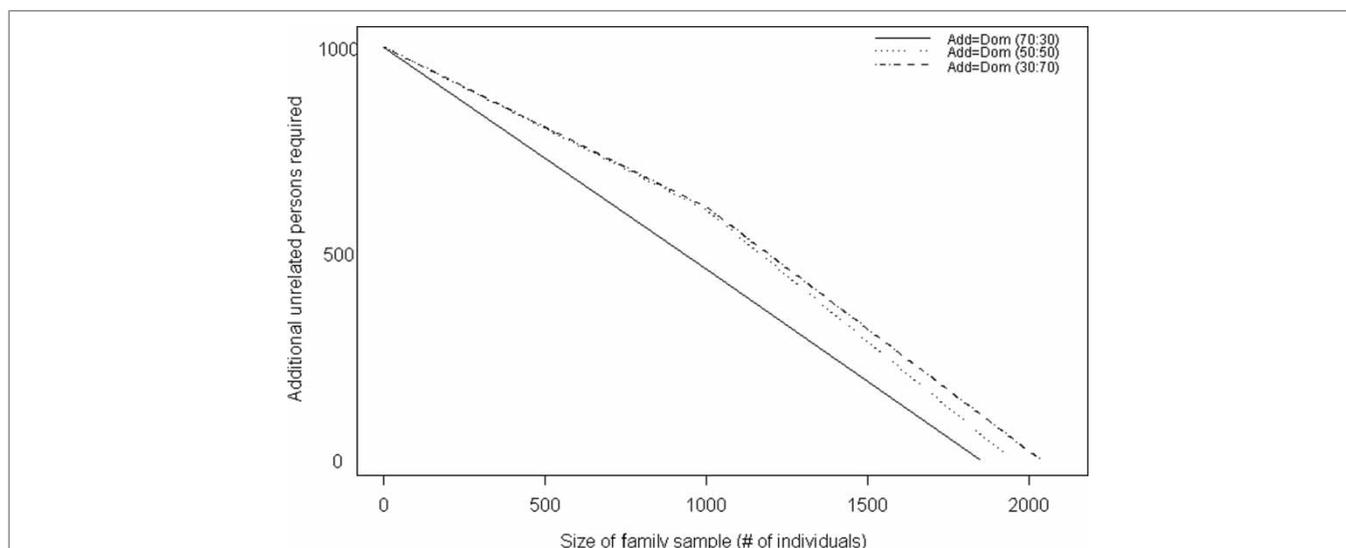
nuclear families and 38 extended pedigrees requires 500 additional individuals to achieve the same power and type I error as 100 nuclear families, 63 extended pedigrees and 625 additional unrelated persons.

### Estimation using family samples

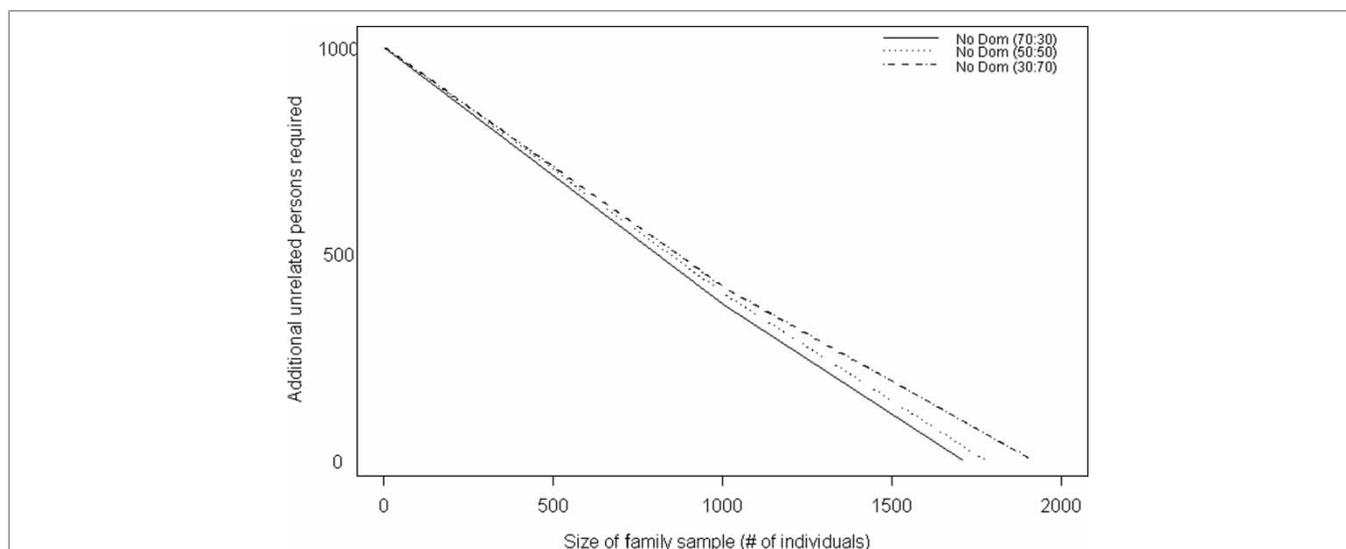
#### *Accuracy of the association parameter*

The estimates of the association parameter (expressed as the ln odds of two copies of the disease allele versus one copy) were, on average, 2.615 for the nuclear family sample and 2.636 for the extended family sample — not too dissimilar to the simulated value of 2.48. The RAND and ASC samples had similar averages of 2.648 and 2.603, respectively. Note that we purposely generated the data under a (probit) model different from the (logit) model used to analyse the data, to illustrate the robustness of the analysis model, and that the accuracy of the ascertainment correction is seen in the small difference in parameter estimates between the RAND and ASC samples. The average estimate for the ascertained extended families (2.633) was overestimated by a factor of 1.06, a slightly larger deviation from the simulated value than seen in the





**Figure 4.** Number of unrelated case-control samples needed, in addition to a fixed, mixed sample of nuclear and extended pedigrees, to achieve a power of 86 per cent under a model with 50 per cent additive and 50 per cent dominance variance (Add = Dom), assuming an allele frequency of 0.1. Values were generated for samples that comprised 30 per cent nuclear families and 70 per cent extended pedigrees, 50 per cent and 50 per cent, and 30 per cent and 70 per cent, respectively.



**Figure 5.** Number of unrelated case-control samples needed, in addition to a fixed, mixed sample of nuclear and extended pedigrees, to achieve a power of 92 per cent under an additive model (No Dom), assuming an allele frequency of 0.1. Values were generated for samples that comprised 30 per cent nuclear families and 70 per cent extended pedigrees, 50 per cent and 50 per cent, and 30 per cent and 70 per cent, respectively.

nuclear family samples, which had an average of 2.573 — only 1.03 times the simulated value (and the closest to it). The rMSE averaged over all models was 0.210 and all estimates were within a factor of 1.15 of the simulated value (Table 2).

Results were similar for estimates comparing the odds of two disease susceptibility alleles to no susceptibility alleles, on average 5.251 — again, not too dissimilar to the simulated value of 5.616. The RAND samples had an average of 5.296 and the

ASC samples almost the same (5.206). Nuclear (NUC) families had the estimate 5.230 and extended families 5.273. In these cases, the random nuclear family samples were the closest to the simulated value. The average rMSE was 0.351 and all estimates were within a factor of 0.88 of the simulated value (Table 3). Notice that for these comparisons the effect was always under- rather than overestimated, whereas in the previous comparisons they were overestimates.

#### *Accuracy of the variance components*

Overall, the rMSEs were, as might be expected, smaller for the RAND samples than for the ASC samples. When comparing the estimated values with the simulated proportions of variance (Table 4), the estimates from the RAND and ASC samples yielded good estimates of the true simulated population values for the polygenic and familial components, but the sibling and marital components were often over- or underestimated in the ASC sample, depending on both model and family structure. Specifically, sibling (S) and marital (M) components were consistently underestimated in the SMP–SMP scenarios and overestimated in all other scenarios.

The accuracy of the variance component estimates were affected by the sampling scheme, as expected. The RAND samples resulted in estimates closest to the simulated population values, but ASC samples yielded estimates reasonably reflective of the population values as well.

## Discussion

The prediction of the future of genetic studies of complex disease is ever changing, but what remains true is that we must have methods of analysis that are both powerful and flexible. Whether searching for common genes with small effect or rare genes with large effect, we shall need large samples that are likely to come only from combining family, population-based and case-control data and we must have methods that analyse these combinations. In fact, the use of family samples was recently highlighted by Visscher *et al.*,<sup>2</sup> showing that including

related individuals results in only a small loss of power but large gains in terms of quality control, flexibility of tests to be performed and ability to control for population stratification. Our results support these assertions and we further recommend that association methods must account for environmental covariates (which are certain to play a role in complex diseases) and must not be restricted by, but rather be effective in controlling for, population stratification. These tools will be powerful in aiding both genome-wide association and candidate gene studies.

We have present here a method to test and estimate the association between an allele or genotype and a continuous or binary trait, as well as approaches to combining family and case-control data that are powerful as well as robust to ascertainment. We also present a two-stage procedure to determine the need for a test that is robust to stratification. A purist would argue that a two-stage approach could affect type I error rate. The important thing to note, however, is that this decision should be made on the basis of the significance, not the magnitude, of the difference in the two estimates of marker effect,  $\beta_2 - \beta_1$  versus  $\frac{1}{2} \delta$ , because a study whose sample size is powered to detect a small effect will automatically be powered to detect the small biases that stratification could induce.

We further present a method for correcting for ascertainment and accurately estimating association parameters, as well as variance components, even in ascertained family data. Two things should be pointed out, however. First, we examined only single ascertainment, when a more complex scheme is used to collect families such that most of the sample is in the PSF and/or the PSF is undefined, the estimates for the association parameter and the variance components will reflect only the effect in the sample. Note, however, that the test for association is still valid and it is only the parameter estimates that are affected. Secondly, when combining data from a case-control sample and an ascertained family sample, for the parameter estimates from this method to be reflective of the population from which the samples were drawn, certain assumptions must be met: (1) the cases in

the population-based data should have been phenotyped in a manner similar to the cases in the family data; (2) there must be appropriate correction for ascertainment; and (3) the non-cases or ‘controls’, although matched, should apart from this also be a random sample — if they are a completely random sample from the same population, it is possible to estimate a relative risk, while if they are a random sample of those showing absence of the phenotype of interest, only an odds ratio can be estimated. If the phenotype is sufficiently rare such that choosing controls based on absence of the trait of interest is essentially the same as random sampling, then the relative risk and odds ratio will be essentially the same. Because this is not the case for common complex diseases, we suggest and will investigate further in future studies, two other ways of combining case-control and family data for accurate estimation: (1) express the likelihood for the case-control data in terms of odds ratios, which are functions of the parameters in the pedigree likelihood, and constrain the maximum likelihood for them such that the marginal probability of disease, given a set of regressors, is finite;<sup>37</sup> and (2) multiply the likelihood by a factor that summarises any information we have about the prevalence of disease independent of the sample data. This factor would be expressed as  $\mu^R(1-\mu)^{N-R}$ , where  $\mu$  is the prevalence of the disease — expressed as a function of the parameters in the full likelihood at particular values of the covariates in the model — and  $R$  reflects our external information about the number of affected persons in a population of size  $N$ . For example, if we have an estimate of  $\mu$ ,  $\hat{\mu}$  and its standard error (s.e.), we can estimate reasonable values for  $N$  and  $R$  by noting  $\text{s.e.} = \sqrt{\hat{\mu}(1-\hat{\mu})/N}$ , and hence  $N = \hat{\mu}(1-\hat{\mu})/(\text{s.e.})^2$  and  $R = N\hat{\mu}$ . It is known that constraining likelihood maximisation so that the estimated disease prevalence is equal to its true prevalence can be equivalent to a correction for single ascertainment.<sup>38</sup> These two options offer simple solutions for ‘non-traditional’ samples and will be examined in future work.

The general method described in this paper, which is currently being implemented in the program package S.A.G.E., is more flexible than

other TDT-type methods and more efficient (in the practical sense) than genomic control methods. Further, we have shown the power of this method for binary traits in various types of family, population-based and combined samples at a constant type I error rate and, while we concede that a population-based sample could sometimes detect a smaller effect size than the respective family-based samples, as mentioned earlier, these scenarios assume the same degree of heterogeneity and sporadic cases in all samples after correction for ascertainment. We know that this is not likely to be the case, as family samples are designed to decrease greatly the number of sporadic cases and, at least to some extent, reduce the amount of heterogeneity in the sample in a manner that makes appropriate ascertainment correction difficult. Further, for most complex phenotypes, family samples of at least the size examined here (and usually much larger) already exist and, as shown in Figures 2 and 3, can drastically reduce the number of population-based samples needed to detect even very small effects. Other benefits of family data, such as increased ability to assess the effects of shared environment and parent-of-origin effects, to detect errors and many others are beyond the scope of this paper, but must also be considered. Finally, while having to correct for ascertainment is one of the reasons often cited for using population-based versus family data, we have demonstrated that, in principle, our method can be used to estimate fairly accurately the effect size of a given allele of interest for a given population, even if using an ascertained sample. For situations where most of the sample is in the PSF (and hence likelihood (8) contains little information), or the PSF is ill-defined, we suggest constraining the likelihood to give an accurate estimate of the disease prevalence. Future investigation will determine the accuracy of estimates obtained in this manner.

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**Table S1.** Summary of TDT-type methods and their respective features

Method/ Reference	Incorporation of:							
	Missing parents	Multiple alleles	Parental phenotypes	Quantitative traits	Extended pedigrees	Different family structures	Multiple markers	Covariates
Curtis (1997) <sup>S1</sup>	*	*						
S-TDT (Spielman and Ewens, 1998) <sup>S2</sup>	*	*				*		
DAT (Boehnke and Langefeld, 1998) <sup>S3</sup>	*	*						
SDT (Horvath and Laird, 1998) <sup>S4</sup>	*	*				*		
NFS (Whittemore and Tu, 2000) <sup>S5</sup>	*	*				*	*	*
TRANSMIT (Clayton, 1999) <sup>S6</sup>	*	*				*	*	
RC-TDT (Knapp, 1999) <sup>S7</sup>	*							
I-TDT (Sun et al., 1999) <sup>S8</sup>	*	*						
Martin et al. (1997) <sup>S9</sup>	*	*				*		
George et al. (1999) <sup>S10</sup>	*			*	*	*		*
P-TDT (Abecasis et al., 2000) <sup>S11</sup>	*			*	*	*		*
Bickeboller and Clerget-Darpoux (1995) <sup>S12</sup>		*						
Spielman and Ewens (1996) <sup>S13</sup>		*						
Purcell et al. (2005) <sup>S14</sup>			*	*				*
TDT(max) (Morris, 1997) <sup>S15</sup>		*						
Lazzeroni and Lange (1998) <sup>S16</sup>		*					*	
Monks and Kaplan (2000) <sup>S17</sup>		*		*		*	*	
Xiong et al. (1998) <sup>S18</sup>		*		*				

Continued

Table S1. Continued

Method/ Reference	Incorporation of:							
	Missing parents	Multiple alleles	Parental phenotypes	Quantitative traits	Extended pedigrees	Different family structures	Multiple markers	Covariates
Fan and Jung (2002) <sup>S19</sup>		*		*		*		*
TDT(Q1) – TDT(Q5) (Allison, 1997) <sup>S20</sup>				*				*
Rabinowitz (1997) <sup>S21</sup>		*		*				*
Allison <i>et al.</i> (1999) <sup>S22</sup>	*	*		*				
Sun <i>et al.</i> (2000) <sup>S23</sup>	*			*		*		*
Schaid and Rowlands (2000) <sup>S24</sup>				*		*		*
Waldman <i>et al.</i> (1999) <sup>S25</sup>				*				*
Sinsheimer <i>et al.</i> (2000) <sup>S26</sup>		*		*	*		*	*
Kistner and Weinberg (2004) <sup>S27</sup>	*	*		*				
QTD (Abecasis <i>et al.</i> , 2000) <sup>S28</sup>	*			*		*		*
Zhu and Elston (2001) <sup>S29</sup>				*	*	*		*
PDT (Martin, 2000) <sup>S30</sup>					*	*		
Goring and Terwilliger (2000) <sup>S31</sup>					*	*		
Clayton and Jones (1999) <sup>S32</sup>				*			*	
ETDT (Sham and Curtis, 1995) <sup>S33</sup>		*						
TDT-EX (Cleves <i>et al.</i> , 1997) <sup>S34</sup>	*	*						
Fulker (1999) <sup>S35</sup>	*			*				*
Fan <i>et al.</i> (2002) <sup>S36</sup>		*		*				*

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