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Genetic association studies in Alzheimer's disease research: challenges and opportunities

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SUMMARY

Genetic association studies have identified important risk factors for Alzheimer's disease and other diseases. However, the ease with which these methods can be applied and the shear number of polymorphisms in the human genome has led to a well-characterized multiple comparison problem—given the number of genetic variants being tested, it is likely that many of the positive findings reported in the literature to date will prove to be false positive findings explained simply by random fluctuation in data and type I error. The disparity of findings in initial positive reports versus subsequent negative replication studies observed in the Alzheimer's disease literature underscores this problem. The problem of a high false positive rate can be addressed in part by using statistical correction for multiple comparisons in larger and statistically more powerful samples and in meta-analyses of smaller samples. National initiatives are now being considered to address this problem by encouraging sharing of genetic material. Of equal concern in planning future initiatives are methodological issues that are the domain of the epidemiologist. In fact, it is possible that disparate findings across case–control studies reported to date may be explained in part by problems in the design, analysis and interpretation of these studies. The involvement of epidemiologists may improve the situation in this regard. For example, population stratification bias, control selection bias and prevalent case bias can be minimized by careful study design and by appropriate statistical analysis. Regarding interpretation of case–control studies, a more careful consideration of the strength of evidence for a given genetic variant may help to temper enthusiasm for, or appropriately qualify, positive findings. Epidemiologists have well-developed causal criteria for this purpose. This paper reviews the current state of case–control studies of genetic variants in Alzheimer's disease from the epidemiological perspective. The problem of multiple comparisons and a high false positive rate is reviewed. The potential for bias in case–control studies of Alzheimer's disease is reviewed by way of example. Future initiatives to promote case–control studies of genetic variants in Alzheimer's disease can only benefit from increased awareness the tools of epidemiology.

KEY WORDS: Alzheimer's disease; case–control study; type I error; multiple comparisons; stratification bias; selection bias

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1. INTRODUCTION

Genetic association studies play an integral role in mapping genes of complex disorders, as they are often used to follow-up on regions of interest identified through linkage analyses or to test candidate genes identified from metabolic pathways. The basic premise of these studies is to compare the genotype or allele frequency distribution of a candidate-gene between cases and controls. A significant increase (or decrease) of genotype frequencies in the cases indicates a positive (or negative) association between the gene and the trait. This approach was used to describe the association between apolipoprotein E (APOE) genotype and Alzheimer's disease. The association between APOE and Alzheimer's disease was initially reported in a case–control sample of 30 Alzheimer cases with a family history of disease and a convenience sample of 91 unrelated controls [1], and has been consistently replicated [2]. In a meta-analysis of clinic- and autopsy-based association studies, the APOE $\varepsilon 3/\varepsilon 4$ genotype was associated with a 3.2 fold increased risk and the $\varepsilon 4/\varepsilon 4$ genotype a 14.9 fold increased risk relative to subjects homozygous for the common $\varepsilon 3$ allele among Caucasians [2]. Approximately 50 per cent of the Alzheimer's disease cases had an $\varepsilon 4$ allele [2], and the $\varepsilon 4$ allele is presumed to explain the disease in a substantial fraction of these cases. Rare mutations that cause early onset Alzheimer's disease have been described on the genes APP, PS1 and PS2. These mutations were identified by linkage analysis. The APOE $\varepsilon 4$ allele is the only accepted genetic risk factor for Alzheimer's disease identified by the case–control analysis.

The APOE success story is reason for optimism regarding the genetic association study approach. However, we have now seen a proliferation of genetic association studies, and their contribution to our understanding of disease is being questioned [3]. While many statistically significant genetic associations have been reported, most have not been replicated by subsequent validation studies. This high frequency of apparent false positive findings may be explained in part by publication bias and the shear number of hypotheses being tested. Even ignoring publication bias, we can expect that 100α per cent of null hypotheses tested will result in a false positive finding, where α is the *a priori* type I, or accepted false positive, error rate used in hypothesis testing. The shear volume of positive findings that have resulted from genetic association studies reported to date may be more of a distraction than a contribution to our understanding of disease, especially when you consider that most positive findings will prove to be false. This problem has now been well characterized for complex diseases in general [4], and for Alzheimer's disease in particular [3, 5–9]. By one account, 80 positive genetic associations with Alzheimer's disease have been reported in the literature, with only one of them, the APOE $\varepsilon 4$ variant, being consistently replicated [5].

The large number of studies being performed and the large number of candidate genes being tested contribute to the high number of false positive findings. This problem is compounded by the number of hypothesis tests often performed in the course of assessing a single candidate polymorphism. For example, testing of a given candidate variant is often performed overall and within subsets of the data determined by demographic variables and genetic variables. Testing for association within all possible subgroups determined by sex, dichotomized age, APOE $\varepsilon 4$ carrier status and subgroups determined by two-way interactions of these variables leads to 19 hypothesis tests performed in the course of assessing a single candidate polymorphism (Table I). Multiple hypothesis testing under different assumptions about mode of inheritance (recessive, multiplicative, dominant) further contributes to this problem.

Table I. Many case–control studies report odds ratios for candidate variants for subsets of the data defined by age, gender and APOE genotype. Hence, a large number of potential hypotheses may be performed in the course of analysing a single variant site.

Subgroups tested	No. of tests
Overall	1
By age	2
By gender	2
By APOE	2
By age and APOE	4
By age and gender	4
By gender and APOE	4
Total	19

Considering publication bias and the number of possible unreported statistical tests performed in the course of a given analysis, false positive findings are not unexpected.

Concerns about false positive findings may be somewhat overstated, however. There are a number of plausible explanations for the lack of replication of initial positive findings. Some validation studies are under powered, meaning the sample size is too small to reasonably expect a statistically significant finding even if the predicted signal were present in the data. Random chance can also lead to such *false negative* findings even in appropriately powered follow-up studies. Further, unknown genetic or environmental factors that interact with a candidate variant to increase risk for a phenotype may be present in some study populations and not in others. Considering the positive findings that have been the subject of multiple validation studies (reviewed, e.g. in Reference [3, Table I]), a number of observations can be made. First, none of the findings excepting the APOE finding have been consistently validated. Some have been consistently invalidated. On the other hand, a number of positive findings have been validated by half or more of the follow-up studies performed, suggesting a factor beyond random chance explains the initial and followup findings. Factors beyond chance to consider include the possibility that study design bias is consistently present in initial reports and validation studies, or, optimistically, that a true risk factor has been identified. If even one or a few of the positive findings reported to date prove to be valid risk factors for Alzheimer's disease, our collective scientific endeavor will be deemed a success. Deciphering the accumulating data on genetic associations with Alzheimer's disease without inadvertently discarding true risk factor variants is a major challenge confronting the Alzheimer's disease research community.

The multiple comparison problem can be addressed in a number of ways. Larger and statistically more powerful samples will allow smaller p-values and a tighter control of the type I error rate. The number of hypotheses tested in the course of analysing a single variant can also be reduced. For example, subgroup testing (Table I) could be replaced with testing for effect modification testing via logistic regression (although this will reduce the power to detect subgroup specific effects). Multiple testing under different genetic models (recessive, multiplicative, dominant) could be replaced with the so-called *efficiency robust* testing that does not require specification of a model but still maintains relatively high power to detect effects regardless of the underlying genetic model [10]. Larger validation samples and

meta-analyses of smaller samples will also help in sorting through positive findings to date. National initiatives are now being considered to address this by encouraging sharing of genetic material (John Hardy, personal communication). Large samples with small α, and appropriate control for multiple comparisons, will go a long way toward addressing the problem of a high false positive rate in genetic association studies. In particular, large and statistically powerful case–control samples available for validation studies will speed the process of weeding out important from unimportant initial positive findings.

We suggest also that concern for study design and study bias should play a prominent role in future efforts to understand the potential importance of reported genetic associations with Alzheimer's disease. It is likely that a meaningful proportion of the disparate findings between initial reports versus followup studies in the literature today can be explained by spurious findings in one or both of the reports. Hence, an understanding of study design bias is critical to meta-analytic reviews of currently reported putative genetic associations. Study design considerations are equally important as we plan future initiatives to fund large-scale case–control studies. For example, large but biased studies may do more harm than good, because even subtle biases can lead to statistically significant but entirely spurious findings if the sample size is large enough. This manuscript attempts to review by way of example some of the potential sources of bias in case–control studies of Alzheimer's disease. Stratification bias, convenience sample bias, case selection bias and control selection bias are addressed. It is hoped that this review will underscore the importance of study design and study performance in case–control studies of Alzheimer's disease.

2. STRATIFICATION BIAS

Stratification bias occurs when genetic risk factors are investigated in a sample that includes an admixture of genetically distinct populations. If disease frequency varies across the populations, then *any* genetic variant which varies across the populations will be associated with the disease. A classic example used to describe population stratification is that of a population of Native American Pima-Papago Indians admixed for a number of generations with European decendents [11]. The frequency of diabetes mellitus approaches 50 per cent among Pima-Papago Indians, substantially higher than the frequency of about 10 per cent among persons of European decent. Under this circumstance, any polymorphism more common among Pima-Papago Indians will be positively associated with diabetes in the admixed population, and, conversely, any polymorphism more common in Europeans will be negatively associated with (and apparently protective against) diabetes in the admixed population. The description by Knowler *et al.* of a polymorphism which was strongly associated with diabetes in this admixed population, but not significantly associated with diabetes after controlling for degree of admixture in the analysis, highlighted the potential for stratification bias to genetics researchers [11].

Stratification bias can be avoided in a number of ways, for example, by restricting the sample to a homogenous subgroup (e.g. just Pima-Papago Indians) or by controlling for the genetic subgroups in the analysis (i.e. by performing a stratified analysis) [11]. However, these approaches can be difficult in practice, as subpopulation membership is not always clear. For example, ethnicity is typically used as a surrogate to subpopulation membership, but ethnic classifications may not always correspond to meaningful genetic subgroups [12].

Alternatives for addressing population stratification bias that avoid the need to establish ethnicity include the family-based association study design. Family-based association studies sample the controls from unaffected relatives of the cases (see Reference [12] for a review). If siblings or parents are used as the controls, then this design eliminates any concern for stratification bias [12]. There is a cost, however, in the sense that family-based studies are less efficient. That is, they require a larger sample to detect a given genetic effect [13]. Population stratification can also be controlled for analytically using genomic controls [14] or structured analysis [15, 16]. These methods use the more efficient population-based case–control design, although they require the genotyping of additional markers beyond the genetic variants of research interest.

In many situations, stratification bias may be of little practical concern relative to other sources of bias discussed in this review. For example, Wacholder *et al*. demonstrated empirically and mathematically that stratification bias is of little practical concern in analyses restricted to U.S. Caucasians of non-Hispanic European descent—within this population there exist substrata defined by country of European origin, but most disease frequencies do not differ across these substrata in a way that would induce substantial bias for most imaginable disease and admixture scenarios [17]. Similar investigations will have to be performed for other study populations. However, the implication is that stratification bias is not substantial in studies performed on U.S. Caucasian populations of non-Hispanic descent [17]. This statement depends on the assumption that disease rates do not vary markedly across ethnic subgroups represented in the admixed population [17]. For the case of Alzheimer's disease, there is preliminary evidence of a modest north–south gradient in incidence across the European continent, but no evidence of large differences in disease frequency across European populations [18].

This is not to say that information on ethnic origin should be ignored if it is available. Relatively recent immigration to some geographic regions of the U.S. makes it feasible to measure ethnic history in some populations. For example, the elderly residents of southeastern Minnesota include second and third generation Swedish, Norwegian, German and other immigrants, so that measuring population substructure is possible in this population [19]. Information on population substructure allows investigators to test for and control potential confounding by population stratification. Importantly, it also allows investigators to test for effect modification by ethnic background (e.g. Reference [20]). The observed effect size for genetic risk factors that have an effect in only certain genetic backgrounds is diluted in pooled analyses, but may become apparent in analyses stratified by ethnic background. The mathematical formulas of Wacholder [17] imply that false positive findings are unlikely to be induced by population stratification; however, effects that are unique to a single genetic or ethnic background are likely to be missed in pooled analyses that ignore population stratification.

3. BIAS IN CONVENIENCE SAMPLES

Much of the discussion in Section 2 is predicated on the analyses being performed on population-based samples of cases and controls. Often, far less rigorously obtained case–control series are used for genetic association studies. For example, tertiary care cases may be compared to convenience sample controls, or cases and controls from different geographic regions may be analysed without consideration of sample sources. In the worst-case

Table II. Association between diabetes and Alzheimer's disease in early prevalent case-based studies versus later incident case-based studies. Case selection criteria used in the prevalent case-based studies may have systematically introduced bias to these studies.

Prevalent case–control studies	Relative risk estimate
Heyman *et al.* (1984) [21]	0.8
Wolf-Klein *et al.* (1988) [22]	0.3
Broe *et al.* (1990) [23]	0.6
Ferini-Strambi *et al.* (1990) [24]	0.2
Incident case-based studies	
Leibson *et al.* (1997) [25]	1.6
Ott *et al.* (2000) [26]	1.9
Luchsinger *et al.* (2001) [27]	1.3
Edland *et al.* (2002) [28]	1.8

scenario, all of the cases in a convenience sample are obtained from one subpopulation and all of the controls are obtained from another subpopulations. Since by design the disease rate is 100 per cent in one subpopulation and 0 per cent in the other subpopulation in this constructed sample, the range of potential bias is unbounded [17]. For example, in a highly socioeconomically stratified community, tertiary care referral cases may come entirely from an advantaged ethnic subpopulation, introducing substantial spurious associations between any polymorphism unique to this ethnic subpopulation and Alzheimer's disease. These are extreme examples of selection bias. Extreme examples aside, the important point is that unknown and unbounded potential bias is introduced when convenience samples are employed for case–control studies. For this reason, epidemiologists attempt to avoid case–control analysis of convenience samples. Rather, more subtle sources of case selection bias and control selection bias in appropriately designed studies are the primary concern of epidemiology. Some examples of case selection bias and control selection bias are outlined in the sections below.

4. CASE SELECTION BIAS/PREVALENT CASE BIAS

One source of case selection bias is the use of prevalent cases in a case–control study. For chronic diseases such as Alzheimer's disease, genetic and environmental factors associated with survival with disease are over-represented in prevalent cases. These factors will in turn be (spuriously) associated with disease in case–control studies using prevalent cases. More generally, case selection bias refers to the problem that any factor related to case selection may be spuriously associated with disease in a case–control study.

Example: An example of case selection bias is illustrated in Table II. Early case–control studies of Alzheimer's disease relied by necessity on selected samples of prevalent cases. These early studies fairly consistently found that diabetes mellitus was less common in Alzheimer's disease cases than in controls [21–24]. Subsequent incident case-based studies, on the other hand, have consistently found the opposite trend, with history of diabetes mellitus being associated with increased risk of Alzheimer's disease [25–28]. The signal in the prevalent case–control studies may reflect some degree of prevalent case bias, but probably

reflects mostly the tendency to restrict to 'clean' Alzheimer's cases free of vascular dementia risk factors in early case–control studies of Alzheimer's disease [24].

This example illustrates the potential magnitude of selection bias in prevalent case–control studies of Alzheimer's disease looking at environmental and medical history exposures. Findings related to environmental exposures based on prevalent case–control studies should be interpreted with caution. Likewise, we believe that prevalent case–control series should not be used for testing gene–environment interactions. This example also illustrates the potential magnitude of bias in studies of genetic exposures. For example, a hypothetical pleiotropic genetic risk factor for Alzheimer's disease and diabetes would be missed by the prevalent case series studies, not an unrealistic scenario, as several candidate genes for diabetes are also candidate genes for Alzheimer's disease, most recently the gene encoding insulin degrading enzyme [29]. Hence, even with regard to gene–disease associations, findings from highly selected prevalent case-based series should be interpreted with caution.

Potential magnitude of case selection bias: Unlike stratification bias, it is difficult to bound the potential magnitude of case selection bias mathematically, and empirical descriptions may be the best we can do. Lehmann *et al.* [5] present an example of possible prevalent case bias. In a meta-analysis of investigations of the BCHE K variant, they found that the expected association between the BCHE K variant and Alzheimer's disease among elderly (age >75) persons with an APOE $\varepsilon 4$ allele was present in studies where the age of cases was determined as age of onset (allelic odds ratio $=2.8$, $p<0.05$), while the association was not present in studies where the age was determined as current age or age at death (allelic odds ratio $=1.0$). The implication is that the latter studies did not have access to true age at onset, and therefore the cases used in these studies include a mix of late-onset cases and early-onset cases who have survived with disease beyond the age of 75 years [5]. We caution that the values reported in the meta-analysis are allelic odds ratios rather than genotypic odds ratios, and therefore are difficult to interpret [30]. Moreover, it remains possible that the positive findings in this meta-analysis can be explained as false positive findings, since a large number of hypotheses (by age, gender and APOE genotype) were performed to arrive at the proposed relevant subset within which the BCHE K variant is associated with Alzheimer's disease [5]. Nonetheless, this example illustrates the potential magnitude of bias present in studies using prevalent disease cases.

5. CONTROL SELECTION BIAS

Bias can also be introduced by selecting controls whose exposure history is different from the population that is the source of the cases [31]. Potential problems in control selection cannot be underestimated, and understanding the subtle issues of control selection bias is a major concern of epidemiology [31, 32]. For example, controls are not matched on age in some case–control studies of Alzheimer's disease. This may introduce bias due to cohort effects and bias due to genetic variants associated with longevity occurring with different frequency in the cases and controls. Since age is an accepted risk factor for Alzheimer's disease, most epidemiologic studies of Alzheimer's disease attempt to frequency match on age when acquiring controls and to control for age in the analysis. Some of the problems that can be introduced when age is not considered in the design and analysis are outlined in the following paragraphs.

Cohort effects: In the context of genetic studies, cohort effects refer to the possibility that different birth cohorts may have different ethnic backgrounds or different histories of environmental exposures. Differences in ethnic background across birth cohorts in a geographically defined population are related to historic migration patterns in the geographic region. If cases are born many years before controls in a case–control study performed in North America, it is hypothetically possible that some of the difference in gene frequencies between the two groups follows from demographic shifts in the base population during the time period in question. A related concern is that cohorts may have different histories of exposure to environmental risk factors. For example, a person 70 years old in the year 2000 was born during the depression and therefore was more likely to have experienced malnutrition during childhood as compared to a person 60 years old in the year 2000. Factors other than diet, such as environmental exposures and changes in work environment, also may vary dramatically across birth cohorts within North America populations. Cohort effects are a concern in epidemiologic studies of environmental risk factors. The extent to which cohort effects bias estimates of genetic association or gene–environment interaction is unknown, however.

Longevity effects: The single most important known risk factor for Alzheimer's disease is age. The median age at onset of Alzheimer's disease in the United States is approximately 80 years in most population-based, incident-case samples [33–35], significantly older than the median age at death in the American population. Hence, ironically, longevity genes may be over represented in Alzheimer's disease samples [36].

Little is known about the genetics of longevity [37], and it is difficult to quantify the potential magnitude of bias that could result from comparing gene frequencies in older cases with that in younger controls. One of the few established genetic risk factors for early death is the APOE $\varepsilon 4$ allele. In this example, the association between Alzheimer's disease and APOE $\varepsilon 4$ would actually be attenuated by using a younger control group. Longevity genes unrelated to risk of Alzheimer's disease, on the other hand, would be over-represented in cases and positively associated with disease in a case–control study with younger controls. Hence, positive and negative biases can be imagined. Confounding of genetic associations with Alzheimer's disease by longevity is probably negligible in most case–control studies. However, if the age difference between cases and controls spans a range where mortality is high (e.g. cases in their 80s compared to controls in their 70s) it is conceivable that the frequency of longevity genes will be different between the two groups.

Other design-induced control selection biases: Cohort effects and longevity effects are just two examples of potential sources of control selection bias. Another example is choosing controls from a geographically distinct population, which may include a different mix of ethnic subgroups. The example of bias introduced when case and control membership is related to socioeconomic status was reviewed in the section on convenience sample bias above (Section 3). Many more examples, each dependent on the case selection mechanism used in a given study, can be imagined. General principles of control selection have been reviewed [31, 32, 38–40], and are beyond the scope of this review.

6. DISCUSSION

This paper has focused on potential problems with the case–control design. Our intention is not to disparage the case–control design, but rather to emphasize the importance of appropri-

ately designed and performed case–control studies. We feel strongly that case–control studies can contribute to our understanding of the genetics of Alzheimer's disease if epidemiologic considerations are brought to bear in interpreting the results of existing studies and in designing future studies. In fact, the well advertised failures of the case–control design, attributed mainly to the multiple comparison problem, may be traced in some part to an under emphasis on the epidemiological considerations of design, analysis and interpretation in existing studies.

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