Case-control studies in the genomic era: a clinician's guide

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The goal of case-control association studies is to find genetic variants in the human genome that influence common traits. The Human Genome and HapMap projects have added fresh impetus to this goal by cataloguing the raw genetic data behind human DNA variation. Studies that associate these genetic variants with phenotype improve both molecular diagnostics and drug discovery and offer clinicians important opportunities to improve care of patients. In this review I focus on case-control studies, which are the most widely used design and expected to be the most powerful. I also address the problem of case-control non-replication, which is widespread despite enormous effort and use of resources. Important causes of non-replication include inadequate statistical power to detect small and moderate effects, phenotype heterogeneity, population stratification, publication bias, and multiple comparison testing.

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Introduction

Although there are several designs for genetic association studies (case-control, cohort, family-based), the case-control study is expected to be the most powerful for detecting modest risk variants in common disease. Case-control studies search for differences in **allele** frequency between disease carriers (cases) and non-carriers (controls) with the assumption that differences in frequencies are associated with the disease outcome. Despite enormous potential, few days go by without one case-control study linking a genetic variant to a disease, such as epilepsy or parkinsonism, and another refuting a previous association. This problem has led to justified scepticism among clinicians; for example, a large study that examined all previous claims of association in temporal-lobe epilepsy did not replicate the results of a single study.[1] Recently, well-designed, genome-wide, case-control studies have emerged after decades of small underpowered studies and unfulfilled academic and commercial promises.[2–4] Many of these studies are public–private collaborations, such as The Wellcome Trust case control consortium, which aims to test 10 billion genotypes (675 000 polymorphisms in 15 000 individuals) in ten common diseases.

In this review, I update clinicians with current strategies in population-based genetic approaches to complex disease. The emphasis is on the case-control study because this design dominates research. I hope that the reader will learn to critically appraise published associations and identify previous sources of false-positive and false-negative data, such as inadequate power, population stratification, and multiple comparison testing.[5]

The human genetic code

Polymorphism is variation in the DNA sequence of a gene that differs among people. There are several types of polymorphism, such as tandem repeats and insertion or deletion, but single nucleotide polymorphisms (SNPs) are by far the most common and widely studied. Each person has about 10 million SNPs that are common in a given population and an inestimable number of rarer variants.[6] This extensive human variability accounts for the tremendous genetic diversity in populations and the genetic uniqueness of individuals. Extant human genetic variation is non-random and arises through chance, natural selection, and the development of societies and migrations of our ancestors.[7]

As well as finding disease-causing variants, **population genetics** asks how many genetic variants contribute to each trait and at what allele frequency? There are two theoretical hypotheses but little empirical data. The "common disease–common variant hypothesis" proposes that a few common polymorphisms cause the same disease in most affected individuals. Conversely, the "multiple rare-variant hypothesis" proposes that disease susceptibility arises from different rare genetic variants in different individuals.[8–11] Equally unclear is the proportion of variance, or effect, that each risk-variant has on the overall phenotype and the associated importance of "silent" polymorphisms versus polymorphisms that directly change proteins by changing an amino acid.[12–15]

Allele
One of the alternative forms of a DNA sequence that may occur at a given position, or locus, on the chromosome.

Population genetics
The study of factors that influence the frequency of genetic traits in a population; differs from mendelian genetics, which involves the study of single families with highly penetrant traits.

The Human Genome Project

The Human Genome Project is the primary source of raw genetic data for geneticists. This project is a high-quality reference map for nearly all 3 billion nucleotides in the genome and is freely accessible via various websites (panel 1).[16–18] These websites enable researchers to integrate DNA and protein sequences, gene and chromosome maps, and haplotype structure without having to laboriously produce reagents, such as clones and sequences. One problem is that some websites are not clinically orientated and are difficult to navigate; however, helpful online interactive tutorials are available.

For **The Wellcome Trust Case Control Consortium** see http://www.wtccc.org.uk

The International HapMap project is a by-product of the Human Genome Project. This project catalogues variation such as polymorphisms, their frequency in different populations, and physical relation on the chromosome, termed **haplotype** structure. Phase 1 of the HapMap project contains 1 million SNP genotypes for 269 individuals from four world populations (Han Chinese, Japanese, European, and Yoruba [Nigeria]). Phase 2 will add an additional 4·6 million SNPs[19–21]

The Human Genome Project and HapMap have given geneticists the raw genetic data to understand common diseases. These projects do not interpret it, nor can they associate genetic and phenotypic variation as can be done in a case-control study.

Haplotype
A series of alleles found at linked loci on a single chromosome; polymorphisms on this section of the chromosome will be very similar in large portions of a given population.

Panel 1: **Freely downloadable teaching websites dedicated to population genetics**

Access to the Human Genome Project and associated initiatives
- National Centre for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov
- The International HapMap Project: http://www.hapmap.org
- Ensembl browser: http://www.ensembl.org
- The genome browser at the University of California (UCSC): http://genome.cse.ucsc.edu
- NCBI SNP site: http://www.ncbi.nlm.nih.gov/projects/SNP/
- ENCODE (Encyclopedia Of DNA Elements): http://www-shgc.stanford.edu/genetics/encode.html

Interactive and clinically based websites explaining population genetic methods and terminology
- The National Human Genome Research Institute: http://www.genome.gov/glossary.cfm
- DNA from the Beginning, maintained by the Cold Spring Harbor Research Laboratory for basic genetics information: http://www.dnaftb.org/dnaftb/
- US Department of Energy Office of Science—educational link: http://doegenomes.org/
- Glossary of genetic terms: http://www.weihenstephan.de/~schlind/genglos.html

Linkage disequilibrium
The non-random association between alleles of different loci that arises because these alleles are physically close on the chromosome and are rarely separated by recombination. When two loci (A and B) have high levels of linkage disequilibrium between them, the genotype at position A can predict the genotype of position B; this forms the basis of haplotype tagging.

Recombination
The crossing over of genetic material between parental chromosomes during meiosis to form new haplotypes.

Haplotype tagging
Polymorphisms in a particular part of the chromosome can be efficiently represented by genotyping of a subset of genes.

Candidate-gene and genome-wide approaches

The first step in a well-designed case-control study is to find a plausible candidate gene to test for trait influencing variants. Genes become candidates when genetic or biological data establish a link to the trait of interest. For example, the genes encoding ion channels may influence sporadic epilepsy because ion-channel mutations cause familial epilepsy and many effective antiepileptic drugs target ion channels.[22,23] Until recently, research in neuroscience was dominated by studies testing single polymorphisms in candidate genes. Gradually, this approach has been supplanted by whole gene studies, where all of the polymorphisms in a candidate gene are tested.[24–26] There have unfortunately been few successes by either approach, albeit that inadequate study design may be an important cause. There is a need for non-hypothesis or non-candidate driven genome-wide studies. One advantage is the absence of a-priori assumptions and the potential for and the identification of new targets and disease pathways. For example, the association between the cholesterol transporter APOE and Alzheimer's disease was an unlikely initial hypothesis; however, this association has been replicated over 100 times.[27]

Genome-wide studies have major financial, technical, and statistical drawbacks for hospital-based clinicians or scientists. In the short to medium term, only large institutions will have the resources to take these drawbacks on, even with improved efficiency of SNP genotyping and lower costs.[28,29] The current focus is therefore on shortcut strategies that minimise the number of SNPs genotyped but capture the maximum amount of genetic variability. For a clinician to understand these strategies he or she must first understand **linkage disequilibrium**—the method used by geneticists to make these shortcuts.

What is linkage disequilibrium?

A haplotype is a stretch of DNA sequence that is similar in most of any studied population. Polymorphisms in a haplotype that are in linkage disequilibrium with each other have been inherited together on the same haplotype more commonly than would be expected by chance. This is usually because they are physically close and are rarely unlinked by **recombination**.

The Human Genome Project has revealed an unexpected block structure to human linkage disequilibrium, where long segments of chromosome comprise SNPs that remain ancestrally associated with each other (high levels of linkage disequilibrium), punctuated intermittently by hot-spots of recombination (low levels of linkage disequilibrium).[30,31] As a result, there is commonly only few haplotypes within a linkage disequilibrium block compared with the mathematical possibilities; this forms the basis of a shortcut strategy called map-based association. In **haplotype tagging**, a reduced number of marker SNPs can be used to indirectly track disease-causing variants.[32–35] This process relies on linkage disequilibrium; for example, if the levels of linkage disequilibrium between position A and position B are high enough for position A to predict the genotype of B, then B is redundant from a genotyping point of view. This approach minimises genotyping work whereas maximising (or at least quantifying) the information content.

Map-based approach

Tagging SNPs are selected by their ability to make indirect and efficient statistical assessment on the genotype of those polymorphisms with which they have ancestrally high levels of linkage disequilibrium (figure).[32,35] Ahmadi and colleagues[36] showed the power of the tagging SNP approach in a study where only 200 such tags efficiently captured the common genetic variation for the 55 most important genes regulating human drug metabolism.

For the candidate gene, the tagging approach is straightforward. First, identify tagging SNPs using HapMap and an appropriate tag-selection program.[33,37] Second, test these tags and the resultant tag-defined haplotypes in a case-control study. For large institutions that are prepared to take on genome-wide tagging studies, it is estimated that 260 000 SNPs in Chinese and Japanese people and 474 000 in west Africians (because of lower levels of linkage disequilibrium) would efficiently capture the common SNPs in the phase 1 HapMap data-set.[21] More importantly, it can be inferred that the number needed to tag all common variation in the human genome will probably not be much bigger. However, these map-based approaches have disadvantages, particularly in the detection of low-frequency variants. Some researchers have proposed a sequence-based approach as an alternative.[38]

Sequence-based approach

The critical assumption made by this strategy is that SNPs influencing complex neurological traits are located in

exons or other regions regulating gene function. For genome-wide studies there may be as few as 50 000–100 000 such variants.[38] Refining the criteria to indicate factors such as the severity of amino-acid change and evolutionary conservation reduces this estimate further.[39]

Sequence-based approaches are better than map-based approaches for detecting rare variants (ie, the multiple rare-variant hypothesis). Sequence-based approaches also represent a numerically manageable genotyping proposition and are compatible with DNA pooling technologies. However, variation in non-regulatory parts of the genome is overlooked, and we know very little about the importance of these regions.

How to judge a case-control study

Case-control studies encourage clinicians and geneticists to associate these genetic approaches with phenotypic variation. However, case-control studies have a reputation for producing results that do not withstand independent replication—a major review of 166 positively reported associations found only six associations that were reproduced consistently.[40] For non-replicated gene–disease associations, the critical question is whether these refute the original positive report—ie, the initial result was a false positive—or whether they are false negatives because they were underpowered to re-detect the original association. Lohmueller and colleagues[41] partly addressed this question with a pooled meta-analysis of 25 non-replicated associations and found eight that probably supported the original association. These data suggest that false-positive associations are very common but equally that there are many real associations that are not confirmed because follow-up studies were underpowered. These results are further confounded by the fact that the effect size found in an initial study is commonly much bigger than that ultimately found in pooled analyses, a trend known as the winner's curse.[41,42]

To preserve future confidence, clinicians and geneticists should ensure rigorous standards before publishing association studies. These standards must emphasise the aim of minimising false positives and false negatives and in the overall assessment of significance, initial reports should be presented and interpreted cautiously with inference being based primarily on subsequent replications (panel 2). Here, I discuss six factors that I consider important when considering the quality of genetic association.

Phenotype

The importance of phenotype is generally underdiscussed. Studying phenotypes where heritability is a known epidemiological risk seem more attractive than those without; however, this method may be impractical in the study of genetic variability to drug response. The onus is on clinicians to find ways to ensure that phenotypic variation is captured with the same precision as genetic variation and there have been calls for a Human Phenome Project.[43] Two major problems with phenotypes are misdiagnosis and aetiological heterogeneity.

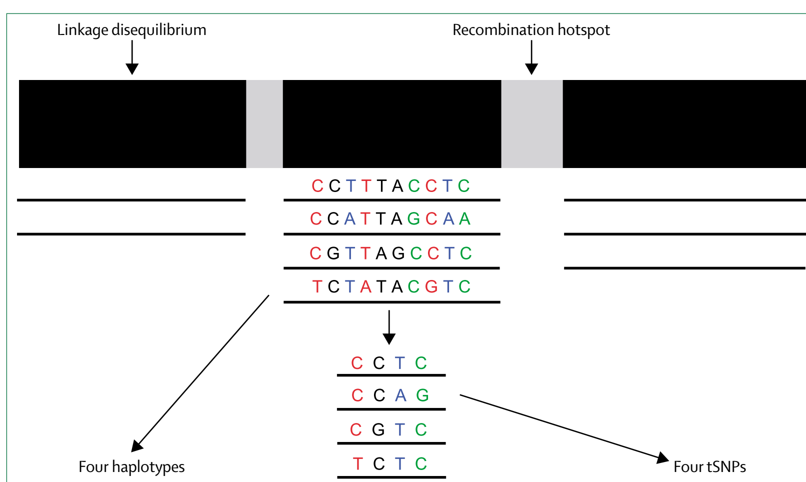

Figure: **Linkage disequilibrium blocks, hotspots, haplotypes, and tagging SNPs**
The black bars represent linkage disequilibrium blocks and the grey bars recombination hotspots. In the middle of the diagram are four haplotypes in a linkage disequilibrium block. Ten SNPs are localised in order along the chromosome. Because of linkage disequilibrium each red SNP can predict the allelic state of the other red SNPs. The same can be said of other SNPs of a designated colour. As a result, the selection of just one SNP from each coloured group represents all in that group. Hence, haplotype diversity of this block can be represented by just four tagging SNPs (tSNPs), rather than the original ten. Typically, linkage disequilibrium patterns will not be so clear cut and statistical strategies are required to select tagging SNPs. Modified with kind permission from Prof D B Goldstein, Duke Institute for Genome Sciences and Policy, USA.

Misdiagnosis can be improved by universally adopted diagnostic criteria, which add standardisation across different institutions.[44–46] In this regard, additional levels of proof, such as radiological or pathological data, improve diagnostic accuracy and add objectivity to clinical acumen.[47,48] However, the importance of experienced clinicians cannot be overstated.

Aetiological heterogeneity is an even less tractable problem. Take the example of stroke. First, not all stroke types are equally heritable; thrombotic and embolic stroke probably have different genetic risk factors, but they are commonly indistinguishable at the bedside.[49–51] Second, the heritability of stroke also indicates the heritability of stroke risk factors and intermediate phenotypes. Finally, preventive treatments, such as antihypertensive drugs and carotid surgery, change stroke penetrance and many strokes are probably clinically silent.

Intermediate phenotypes (eg, blood pressure, carotid-artery stenosis) have distinct advantages over clinical endpoints (disease state) because phenotype more directly relates to the gene product and therefore is probably influenced by fewer individual genetic or environmental factors.[52,53] In other words, genetic variants will probably have a greater effect on the proportion of variance with intermediate phenotypes than with clinical endpoints.

The prospective cohort study is the gold standard of investigation; this longitudinal analysis of healthy individuals recruited on the basis of exposure characteristics and followed up over time to compare outcomes in subsets of the cohort with different levels of

Panel 2: **Important considerations in the design and assessment of case-control association studies**

Phenotypic analysis

Rigorous phenotype characterisation to minimise misdiagnosis and aetiological heterogeneity
Phenotypes with epidemiological evidence of heritability will probably be more successful than those without
Larger sample sizes provide greater statistical power
Consider unified diagnostic criteria
Consider objective outcome measures (eg, pathology or radiology)
Consider intermediate phenotypes
Case-control matching for ethnicity and factors affect the frequency of trait; therefore consider using unlinked markers as genomic control; in general prospective studies provide better controls than case-control studies

Genetic analysis

Candidate-gene analysis has greater sensitivity than candidate-polymorphism studies
A plausible biological role for the candidate gene in the trait of interest increases the possibility that the association is true
Find the causal variant, rather than an associated marker variant (eg, associated region, comparative genetics, functional experiments)
Quality control measures (eg, genotype validation steps, blinded genotype calls)

Statistical analysis

Indicate if the study is hypothesis testing or hypothesis generating
To minimise false-positive reports, initial studies should be interpreted cautiously with inference placed on replication in independent studies
Prospective power calculations demonstrate adequacy of power or sample size; important variables such as effect size, linkage disequilibrium patterns, heterogeneity (allelic and genetic), and sample size should be stated
Significance thresholds should be corrected for multiple comparisons; authors must declare all evaluated polymorphisms, rather than submitting a single significant result
Hardy-Weinberg equilibrium tests in cases and controls; if violated, the first consideration should be genotyping error
Consider meta-analysis of new data with existing data
Consider multivariable analysis (eg, gene–gene interaction, clinical heterogeneity)
Negative results: to minimise publication bias, journals should publish high-quality negative association studies; a web-based repository of all associations (positive and negative)

This outline is meant as a guideline and is not exhaustive.

exposure.[54] An example is the UK Biobank, which aims to recruit 500 000 individuals.[55] Prospective studies are attractive because phenotype data are available before disease onset—eg, diet, blood pressure, and cholesterol concentrations; control data have minimum risk of stratification; and the genetic and environmental data are available irrespective of disease status and not limited to survivors. However, prospective studies are expensive and beyond the resources of most institutions.

Study power

The power of a study is the statistical probability that study will detect a true association if one is present. Unfortunately, chance still plays a major part; for example, a study with only 20% power may yield a true positive, whereas a study with 90% power may yield a false negative.

For **Genetic Power Calculator, SGDP Statistical Genetics Group, Kings College London** see http://statgen.iop.kcl.ac.uk/gpc/

Power calculations are based on variables such as sample size, the prevalence and effect of the risk factor, and the strength of linkage disequilibrium between the marker and causal variant. There are various genetic-power calculation programs, including the Genetic Power Calculator, although most ignore genetic heterogeneity (different causal genes in different individuals) and aetiological heterogeneity (non-genetic causal factors) and therefore probably overestimate statistical power.[56] Most studies in neuroscience are too small and powered only to detect the strongest genetic associations. Therefore, in the judgment of a negative association shrewd clinicians look for prospective power calculations. If researchers do not have enough power to detect a moderate risk variant the result could be a false negative.

500 cases and 500 controls will provide a study of 100 SNPs with 80% power to detect one susceptibility variant, which accounts for 5% of the relative risk (assuming an experiment-wide false-positive rate of 5%).[57] One easy way to improve the power of a study is to increase the sample size, for example by sharing clinical material across institutions. The European Multiple System Atrophy Study Group's collaboration gathered a cohort of over 600 patients—a task beyond any individual institution, given that the prevalence of multiple system atrophy is 2–5 per 100 000.[58,59]

Significance thresholds and multiple comparison testing

A statistical test can only produce a quantitative p value that is above or below an arbitrary significance threshold; it is not an infallible true or false answer to the test made. When the significance threshold is set at 0·05 then 5% of results will be false positives because of random statistical fluctuations. To minimise false positives, there is widespread acceptance that significance thresholds should be lowered, although no limit has been universally adopted;[60–63] this will necessitate bigger case-control studies in order to maintain adequate power. For example, for implementation of the relatively conservative significance threshold of 0·005, as proposed by Ott,[61] about three times more cases and controls (based on 90% power) are needed. However, this rigorous alpha level and larger sample sizes may not be needed for replication studies and multiple replication in large samples is still the most straightforward way to produce robust associations.

A further consideration is the problem of multiple comparison testing. In assessing any association study, clinicians should check how many chances the investigators gave themselves before finding a positive result. In simple terms, the more often the investigators rolled the dice, the greater likelihood of a positive result; the basic rule is that significance thresholds should be experiment wide rather than restricted to selective tests. There are various ways to statistically correct the significance threshold for multiple comparisons, although none are entirely satisfactory.[64,65] However, replication is probably most important in the end, and

researchers should be encouraged to validate positive findings in a second independent sample.

Multiple comparison testing is an even greater concern in genome-wide studies because thousands of tests are made. For example, a significance threshold of 0·005 in a study of 100 000 SNPs, where only two SNPs are causal, will yield 500 false-positive results (as well as the two true positives, if adequately powered). Hence, genome-wide searches should be viewed as hypothesis generating and not hypothesis testing, and significance thresholds should be corrected for the number of tests, thereby offsetting the small prior probability of true association.

Finally, probably the most problematic concern, for which there is no clear statistical solution, is that of the rogue investigator who does multiple tests but only reports the positive ones. One suggestion might be to insist that all authors declare all the hypotheses that they have tested prior to publication.

Population stratification

Population stratification bias arises when a case-control sample contains subgroups with allele-frequency differences. In practice this is most commonly due to mixing of different racial ethnicities in a single sample. False-positive claims can arise if one subgroup (or ethnic group) has high disease prevalence because any polymorphism that is more common in that subgroup will tend to be associated with the disease even if it does not influence it.[66–68] There is substantial reliance in studies in neuroscience on the use of homogeneous populations or ethnically matched controls to protect against population stratification. However, the evidence that this method is reliable is conflicting.[69–72] There are formal methods to measure covert stratification. In family-based studies the transmission-disequilibrium test is the most common.[73] In case-control studies, where parental genotypes are generally unavailable, multiple unlinked marker polymorphisms can be genotyped across the genome under the presumption that these are independent of the disease state and therefore can detect or correct for covert differences in the genetic makeup between cases and controls.[74–77]

Publication bias

Small, positive genetic-association studies are not difficult to do and can get published at the expense of larger, more definitive, negative studies, which commonly remain lost in editorial offices or PhD theses. In assessing a gene–disease association, the clinician should beware of this “file-drawer problem” and view small positive studies as hypothesis generating until such time as they have withstood replication in larger studies.[78,79] To ensure that the neuroscience community has access to all the data, especially unpublished negative studies, one strategy is to have an unbiased web-based repository for all studies, regardless of outcome. When there are enough studies, meta-analysis will have a valuable place, not least as this can make a coarse statistical assessment of publication bias.[80,81]

Causal inference and finding the causal variant

Being satisfied that a study has established a robust gene–disease association, the clinician should look to see if the researchers have found the causal variant rather than simply a marker variant that is in linkage disequilibrium with this. To infer a direct pathogenic effect from an associated marker variant is a serious pitfall because association depends on the strength of linkage disequilibrium between the associated and causal variant and theoretically these two variants can reside in different genes. Therefore, finding associated markers rather than causal variants could undermine the positive and negative predictive value of future clinic-based tests.

So far, few studies have followed initial positive associations with the identification of the causal variant. One strategy to do this is to establish the patterns of linkage disequilibrium that surround the associated marker variant and define the associated region.[82,83] Potential causal variants in this region can then be ranked for closer assessment with strategies such as phylogenetic footprinting and functional experiments.[84]

New methods are being developed that assess gene function and expression; however, these are beyond the scope of this review.[85] For example, the Encyclopedia of DNA Elements (ENCODE) project was established in 2003 to understand the sequence elements that confer biological function.[86]

Finally, comparative genetics compares gene products across different species with the underlying assumption that aspects of function can be inferred from the evolutionary conservation of DNA sequence across species. The development of resources, such as the chimpanzee genome and human-mouse homology maps, has added real impetus to this approach.[87–89]

And the future

Population genetics promises much to clinicians—personalised medicine based on genetic makeup and new drug targets to treat or modify individual disease risk. The identification of real gene–disease associations in autoimmune disorders, Alzheimer’s disease, age-related macular degeneration, and others provides optimism for current approaches.[2–4,27]

As discussed, vast quantities of raw genetic data generated by the Human Genome and HapMap projects await interpretation. Success, or otherwise, will depend upon our ability to correctly identify and replicate true associations between markers and disease. This endeavour requires cooperation between clinicians and scientists and efforts from both sides to recognise and understand each other’s frequently impenetrable jargon.

Many important questions still need answering. Do single or multiple genes influence common diseases? Are risk alleles rare or common? Does disease result

Search strategy and selection criteria

References for this review were identified by searches of PubMed from 1966 to April 2006. Articles were also identified through searches of public genome websites. Search terms included “population genetics”, “complex trait analysis”, “HapMap”, and “the Human Genome Project”. Only papers published in English were reviewed. The final reference list was generated on the basis of originality and relevance to the topics covered in the review and, where possible, definitive references were used.

from too many susceptibility alleles or too few protective alleles? How important are environmental exposures and epigenetic factors? Some of these answers may be found in parallel biological endeavours such as transcriptional profiling and comparative genetics as well as future discoveries on the functional anatomy of the brain.[90,91]

There must be a qualitative shift in the mindsets of clinicians, away from the concept of genetic diseases to the concept that probably all diseases (and drug responses) have some genetic component. In the future, genetic counselling on the basis of multiple low-penetrance factors will be possible, in addition to the current decisive single-gene-disorder genetic testing. Technical developments will probably outpace the collection of epidemiological data needed to interpret genetic tests and recommend sensible treatment or prevention options, and some tests will probably be offered prematurely. The expected increasing uncertainty of the implications of genetic information will necessitate fuller integration between the basic genetic sciences and clinical neurology.[92–95]

Conflicts of interest
I have no conflicts of interest.

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