A High-Density Whole-Genome Association Study Reveals That *APOE* Is the Major Susceptibility Gene for Sporadic Late-Onset Alzheimer's Disease

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Objective: While the apolipoprotein E (*APOE*) ε4 allele is a well-established risk factor for late-onset Alzheimer's disease (AD), initial genome scans using microsatellite markers in late-onset AD failed to identify this locus on chromosome 19. Recently developed methods for the simultaneous assessment of hundreds of thousands of single nucleotide polymorphisms (SNPs) promise to help more precisely identify loci that contribute to the risk of AD and other common multigenic conditions. We sought here to demonstrate that more precise identification of loci that are associated with complex, multigenic genetic disorders can be achieved using ultra-high-density whole-genome associations by demonstrating their ability to identify the *APOE* locus as a major susceptibility gene for late-onset AD, despite the absence of SNPs within the *APOE* locus itself, as well as to refine odds ratios (ORs) based on gold-standard phenotyping of the study population.

Method: An individualized genome-wide association study using 502,627 SNPs was performed in 1086 histopathologically verified AD cases and controls to determine the OR associated with genes predisposing to Alzheimer's disease.

Results: As predicted, ultra-high-density SNP genotyping, in contrast to traditional microsatellite-based genome screening approaches, precisely identified the *APOE* locus as having a significant association with late-onset AD. SNP rs4420638 on chromosome 19, located 14 kilobase pairs distal to the *APOE* ε4 variant, significantly distinguished between AD cases and controls (Bonferroni corrected p value = 5.30×10^{-34}, OR = 4.01) and was far more strongly associated with the risk of AD than any other SNP of the 502,627 tested.

Conclusion: This study provides empirical support for the suggestion that the *APOE* locus is the major susceptibility gene for late-onset AD in the human genome, with an OR significantly greater than any other locus in the human genome. It also supports the feasibility of the ultra-high-density whole-genome association approach to the study of AD and other heritable phenotypes. These whole-genome association studies show great promise to identify additional genes that contribute to the risk of AD.

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder typically characterized by progressively disabling impairments in memory and other cognitive domains but also by noncognitive behavioral symptoms. AD preferentially affects individuals over 60 years, with prevalence rates as high as 40% in nonagenarians.[1] Sporadic AD is multifactorial and genetically complex. Twin studies suggest that genetic factors may account for as much as 80% of the disease risk.[2] While several genes and genetic polymorphisms therein have been suggested as AD susceptibility factors, the only well-verified susceptibility gene for AD is the apolipoprotein E (*APOE*) ε4 allele.[3,4]

Located on chromosome 19, the *APOE* gene encodes the apolipoprotein E protein, which is known to play a central role in the regulation of the cholesterol and triglyceride metabolism.[5] The 3 common *APOE* alleles (ε2, ε3, and ε4) are distinguished from each other on the basis of 2 single nucleotide polymorphisms (SNPs), resulting in 2 amino acid changes at positions 112 and 158. The *APOE* ε3 allele is characterized by cysteine at positions 112 and

158, the *APOE* ε4 allele by cysteine at position 112 and arginine at position 158, and the *APOE* ε4 allele by arginine at both positions. A significant association of the *APOE* ε4 allele with AD was initially demonstrated in 1993.[3] This finding was replicated in numerous studies and is now considered the best-established genetic association with AD.[6] The magnitude of the effect of the *APOE* ε4 allele as a risk factor for AD is influenced by a person's age and ethnicity. The highest odds ratios (ORs) are detected in the Japanese population (OR = 5.6 for *APOE* ε4 allele heterozygous, OR = 33.1 for *APOE* ε4 allele homozygous). In Caucasian populations through meta-analysis of multiple studies (with primarily antemortem study populations, which have, at best, 90% diagnostic accuracy), *APOE* ε4-heterozygous individuals have been calculated to have an approximately 3-fold increased risk and homozygous persons to have an approximately 15-fold increased risk for developing AD by age 75 years compared with *APOE* ε3-homozygous individuals.[4] Although the association between the *APOE* ε4 allele and AD is consistent and strong, initial genome scans using microsatellite markers in late-onset AD failed to identify this locus on chromosome 19.[7]

The completion of the human genome sequencing effort, the cataloging of the majority of interindividual DNA variants (SNPs) in public databases, and the evolution of massively parallelized SNP genotyping technologies has permitted the undertaking of non–hypothesis-driven whole-genome association studies. Alleles of every gene in the genome can now be queried for imbalances between cases and controls. It has been proposed that at least 300,000 SNPs across the genome are required to perform a genome-wide association study in an outbred population,[8] and technologies have only recently become available to permit this kind of study. One such technology, the 500K GeneChip from Affymetrix (Affymetrix, Santa Clara, Calif.), was used in the present study. According to the manufacturer, the 500K array allows ~80% of the genome in the Caucasian population to be queried for association signals. Interestingly, the *APOE* functional variants are not represented on the GeneChip. However, if our whole-genome approach has adequately high resolution (i.e., SNPs close enough to the actual SNP involved in the susceptibility to the phenotype; high linkage disequilibrium [LD]) and adequate statistical power, the *APOE* locus should be ascertained with SNPs on the GeneChip other than, but close to, the *APOE* ε2/3/4 variant.

We have investigated the hypothesis that ultra-high-density SNP genotyping approaches in histopathologically confirmed and clinically assessed samples will identify loci that have been largely undetectable using traditional microsatellite-based protocols. To test the feasibility of this approach, we sought to demonstrate the ability of our high-density whole-genomic association method to distinguish AD cases from controls as well as permit us to confirm the widespread, but not yet empirically confirmed, belief that the *APOE* locus is more strongly associated with the risk of AD than any other locus in the human genome. Finally, we have been able to refine the OR of the *APOE* ε2/3/4 carriers relative to AD.

METHOD

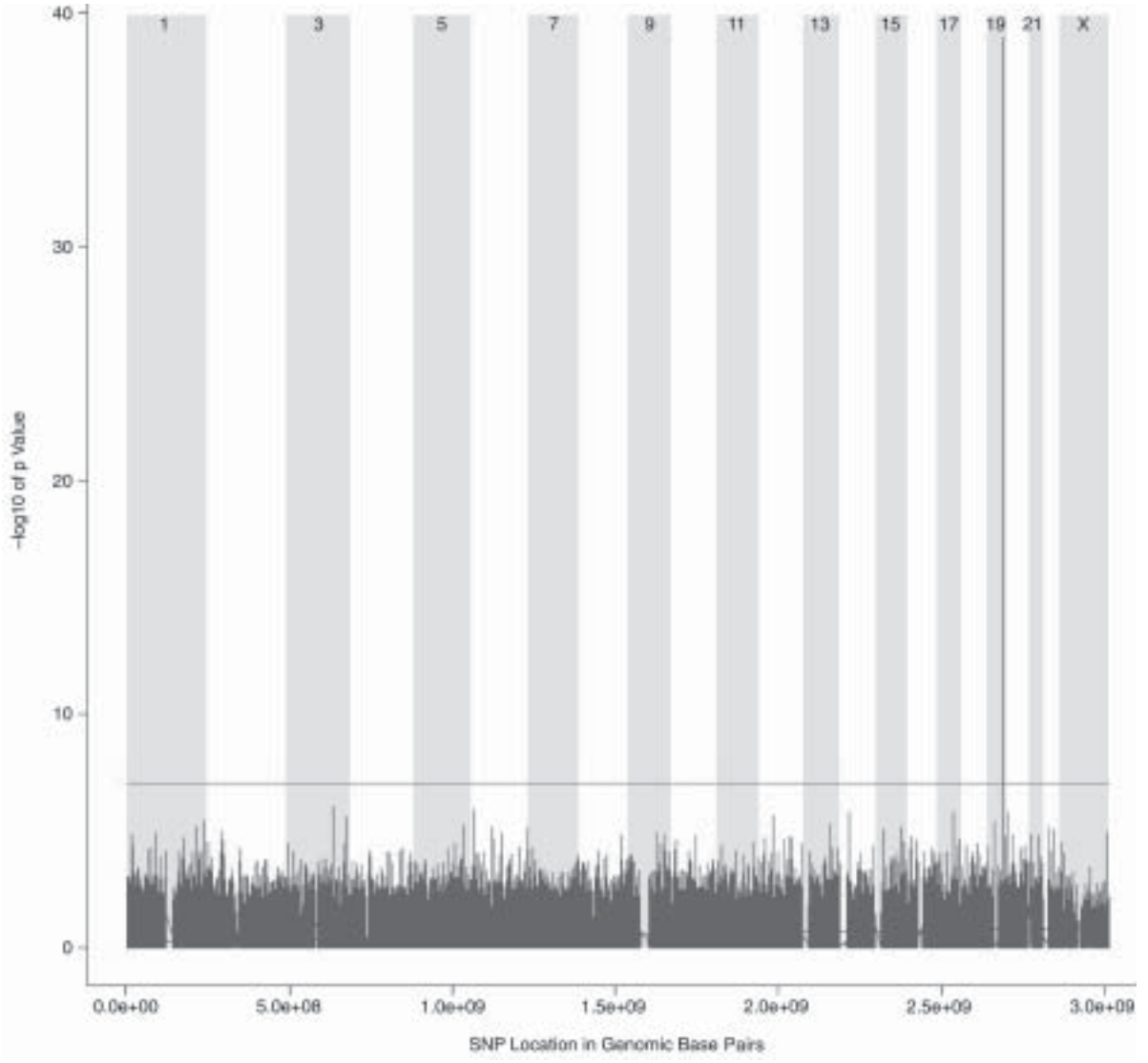
Samples

DNA samples were extracted from brain tissue of 1086 brain donors who were at least 65 years of age at the time of their death. The donors included 664 patients who satisfied clinical and neuropathologic criteria for the diagnosis of AD and 422 persons with no known AD symptomatology or pathology (controls). Their brain tissue and neuropathologic diagnoses were supplied by investigators from 12 National Institute on Aging Alzheimer's Disease Centers in accordance with an agreement with these Centers, the National Institute on Aging, and the National Alzheimer's Coordinating Center. Additional postmortem samples were received from Sun Health Research Institute and the Netherlands Brain Bank. Eighteen percent of the patients and 16% of controls were assessed using Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria,[9] which provide information about neuritic plaque density; 59% of the patients and 75% of the controls were also assessed using Braak and Braak staging,[10] which assesses the distribution of neurofibrillary tangles. *APOE* genotypes were obtained by either pyrosequencing[11] or restriction fragment length polymorphism analysis.[12] The patients included 362 females and 302 males with a mean ± SD age of 82 ± 7.7 years at the time of death. The controls included 255 females and 167 males with a mean ± SD age of 79 ± 11.0 years at the time of death. No population stratification was present between cases vs. controls using the genomic control method with 50 randomly chosen SNPs.[13]

Affymetrix 500K GeneChip SNP Genotyping and Analysis

Array-based SNP genotyping. Each of the 1086 brain tissue samples was processed using the Gentra DNA isolation protocol (QIAGEN Inc., Valencia, Calif.) to obtain high molecular weight DNA, which was then diluted to 50 ng/mL with reduced Tris-ethylenediaminetetraacetate (Tris/EDTA; TE) buffer. Samples were processed as described in the Mapping 500K Protocol (Affymetrix). Briefly, quality and relative concentration of each pool or sample was assessed on 2% Tris-acetate-EDTA (TAE) agarose gel; 250 ng (5 mL) of DNA from each sample was digested in parallel with 10 units of Nsp I and Sty I restriction enzymes (New England Biolabs, Beverly, Mass.) for 2 hours at 37°C. Enzyme specific adaptor oligonucleotides were then ligated onto the digested ends with T4

Figure 1. Ultra-High-Density SNP Whole-Genome Association Scans Identify *APOE* as the Locus Most Strongly Associated With AD in 1086 Individuals (664 with AD, 422 controls)[a]



[a]The 500K GeneChip unambiguously identified the *APOE* locus through a non–hypothesis-driven scan. The identified SNP rs4420638 is ~14 kb distal to the *APOE* locus and was found to have, by far, the most significant association p value via Fisher exact test.
Abbreviations: AD = Alzheimer's disease, SNP = single nucleotide polymorphism.

DNA Ligase (Invitrogen, Carlsbad, Calif.) for 3 hours at 16°C. After dilution with water, 10 mL of the diluted ligation reactions were subjected to polymerase chain reaction (PCR). PCR was performed using Titanium Taq DNA Polymerase (BD Biosciences, San Jose, Calif.) in the presence of 100 mM PCR primer 002 (Affymetrix), 350 mM each dNTP, 1M G-C melt (Clontech, Mountain View, Calif.), and 1X Titanium Taq PCR Buffer (BD Biosciences). Cycling parameters were as follows: initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 15 seconds repeated a total of 30 times, with a final extension at 68°C for 7 minutes. PCR products from 3 reactions were combined and purified using the Clontech Clean-Up Kit 96-well PCR purification plates (Clontech) according to the manufacturer's directions. PCR products were then verified to migrate at an average size between 200–800 base pairs (bps) using 2% TAE gel electrophoresis. Ninety micrograms of purified PCR products were then fragmented using 0.25 units of DNAase I (Invitrogen) at 37°C for 35 minutes. Complete fragmentation of the products to an average size less than 180 bps was verified using 2% TAE gel electrophoresis. Following fragmentation, the DNA was end

labeled using 105 units of terminal deoxynucleotidyl transferase (Invitrogen) at 37°C for 4 hours. The labeled DNA was then hybridized onto the respective Mapping 500K array at 49°C for 18 hours at 60 rpm. The hybridized array was washed, stained, and scanned according to the manufacturer's (Affymetrix) instructions. Genotypes were extracted using the SNiPer-HD[14] software. All data are stored in a GeneChip Operating Software (Affymetrix) enterprise server at Translational Genomics Research Institute, Phoenix, Ariz. Raw data for the 300 kilobase pairs (kb) encompassing the *APOE* locus are available at http://www.tgen.org/neurogenomics/data.

Statistical analysis. Perl scripts were constructed to intercalate, corresponding to chromosomal position, the 502,627 SNPs that were collectively genotyped on the Sty I and Nsp I arrays for each sample (http://bioinformatics.tgen.org/software/tgen-array/). Calculation of an SNP's allelic frequency distribution between cases and controls was based on the Fisher exact test using each SNP sequentially. Bonferroni correction was performed on the resultant p values, and all SNPs were plotted across the genome using R. The *APOE* locus had the highest corrected significance level and thus the strongest single allelic association with AD.

Haplotyping and Linkage Disequilibrium Mapping

LD mapping of the 300 kb surrounding the *APOE* locus was performed importing genotypes into the Haploview program v. 3.3 (Broad Institute, Cambridge, Mass.). When variants of 2 genetic loci are in strong LD (measured by D′ statistic), the variant seen at one locus is predictive of the variant found at the other on an individual chromosome. LD thus represents the likelihood that 2 genetic markers will be inherited together. Pairwise LD values (as measured by D′) for each pair of SNPs across the 300 kb interval were calculated using the Haploview software and plotted (see Figure 2).[15]

RESULTS

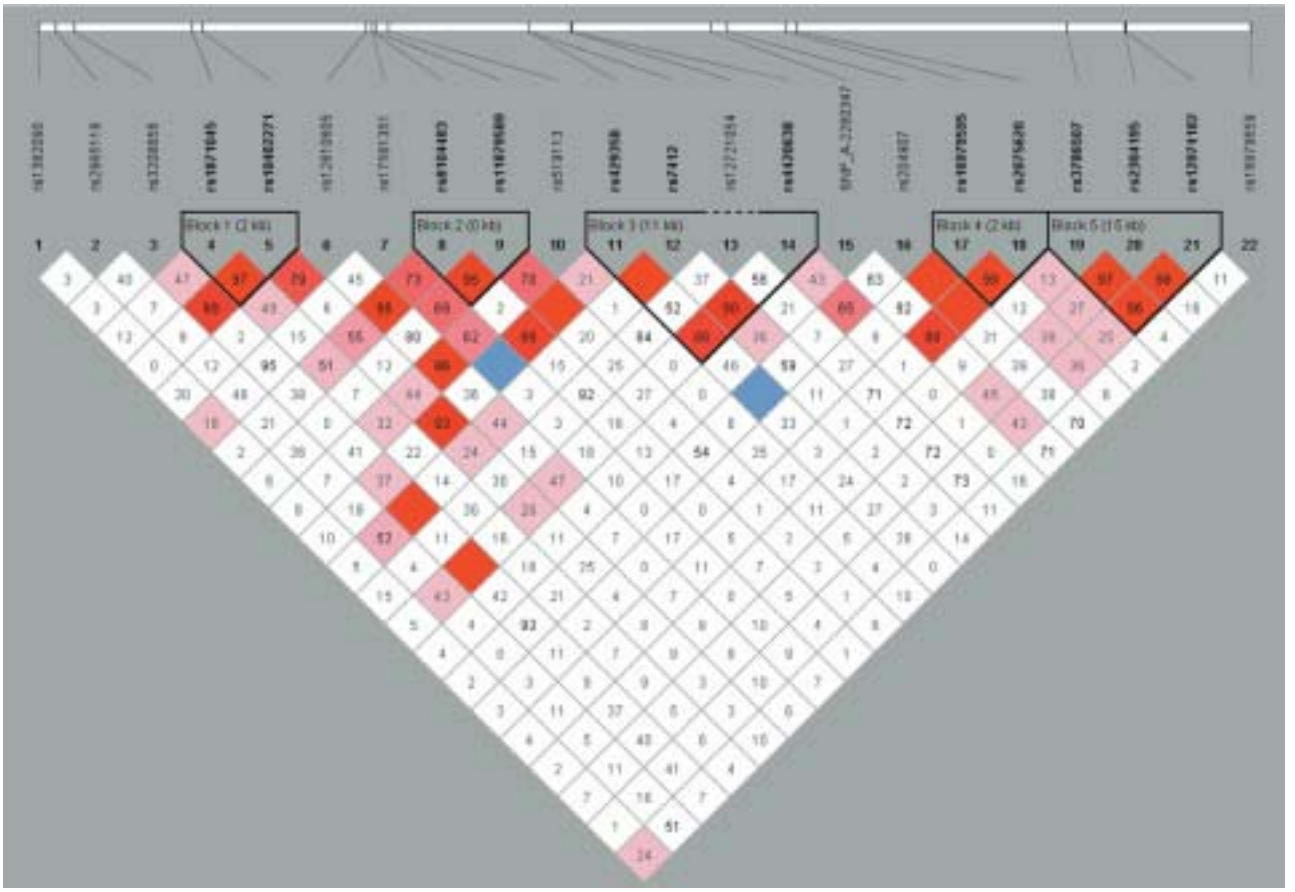
Unlike traditional microsatellite-based genome screening approaches, the ultra-high-density SNP genotyping performed herein was able to precisely identify the *APOE* locus as having a significant association with late-onset AD. Whole-genome scans of 502,627 SNPs were performed for each of the 1086 samples. The most significant SNP identified, rs4420638 (uncorrected p value = 1.06×10^{-39}, Bonferonni corrected p value for multiple hypothesis testing = 5.30×10^{-34} based on 500,000 comparisons), was located on chromosome 19, 14 kb from the *APOE* ε4 variant.[16] Figure 1 displays the magnitude of this association peak in comparison to other SNP p values across the entire human genome. The specific *APOE* ε4 functional variant is not included on the 500K GeneChip but has been repeatedly identified as a prominent genetic risk factor in AD.[17] Figure 2 illustrates the LD (as measured by D′) between rs4420638 and *APOE* locus as defined by the 2 functional variants that define ε2/3/4 status. Haplotype block 3 (Figure 2) is composed of rs4420638 and the 2 *APOE* SNPs: rs429358 and rs7412 at a D′ = 0.86 and 0.90, respectively (rs12721054 being uninformative with a minor allele frequency of 0.0060). Pairwise D′ values for all SNPs in the surrounding ~300 kb are illustrated. The *APOE* locus would have been missed with the 500K array if not for a single SNP in LD with the functional variant, and speaks to the need for increased SNP density for complete coverage. This locus was 27 orders of magnitude more significant than the next most significant SNP by p value. Table 1 depicts refined odds ratios in a large sampling of very carefully phenotyped study samples, which illustrate the increase in effect of *APOE* genotype in AD predisposition. As an example, previous meta-analyses have indicated an OR for *APOE* ε4 homozygotes of 14.9[4] and 15.6,[18] whereas here we calculate the OR as 25.3 through only including both histopathologically verified cases and controls who were also assessed for dementia prior to death.

DISCUSSION

The *APOE* gene, known to mediate the regulation of cholesterol and triglyceride metabolism,[5] has been repeatedly implicated in the pathogenesis of AD.[3,4,6,16] The *APOE* gene confers differential susceptibility to AD etiology depending on the combination of the 3 alleles (ε2, ε3, and ε4) as well as the age and ethnicity of the person. The ε4 allele is most highly associated with AD at a large range of ages and in all ethnic groups.[4] Thus, despite the inability of microsatellite-based genome screens to identify a locus on chromosome 19,[7] the *APOE* gene is consistently and strongly associated with late-onset AD and is considered the single most important genetic factor in AD.[6]

Three major conclusions can be reached based on the results of this study. First, whole-genome association studies using carefully phenotyped cases and controls, when rigorously controlled, adequately powered, and performed using a sufficiently reliable and dense set of markers, can indeed identify the allelic variants that contribute risk toward complex disease. Second, the rare allele of rs4420638, the only informative SNP on the 500K array in LD with the *APOE* locus, is strongly associated with sporadic late-onset AD, having a p value of 1.06×10^{-39} and an OR of 4.01, the highest (in both cases) across the entire human genome in this sampling. Using rs4420638 as an example, however, caution is warranted about making claims regarding holistic extraction of association findings, as this is the only SNP that detected the major known *APOE* association and speaks to the need for increased density on SNP arrays even for less-ancient outbred populations. Third, we have refined the ORs associ-

Figure 2. Disequilibrium Values[a] for Each Pair of Single Nucleotide Polymorphisms Across the ~300 Kilobase Pairs Interval Surrounding the *APOE* Locus[b]



[a]As measured by D′.
[b]The SNP identified in this investigation (rs4420638) was found be in significant linkage disequilibrium with SNPs that characterize the *APOE* ε2/ε3/ε4 locus, rs429358 and rs7412, with D′ values of 0.86 and 0.90, respectively. This figure illustrates this significant linkage disequilibrium between rs4420638 and *APOE* ε2/ε3/ε4 as well as the density and distribution of assayed SNPs within the 300 kb region surrounding the *APOE* locus.
Abbreviation and figure legend: LOD = log of the odds. The color code on the plot follows the standard color scheme for Haploview: white boxes, |D′| < 1, LOD < 2; shades of pink/red, |D′| < 1, LOD ≥ 2; blue, |D′| = 1, LOD < 2; bright red, |D′| = 1, LOD ≥ 2.

ated with *APOE* genotype status based on a postmortem sampling, which will ultimately provide more accurate risk assessment when prospectively implemented.

CONCLUSION

The findings from this whole-genome association study, utilizing > 500,000 SNPs in a sample of 1086 histopathologically well-characterized AD cases and age-matched unaffected controls, immediately implicate *APOE* as the single major pathogenic locus (at this density of genome coverage and using Caucasian-specific population LD and SNP informativeness characteristics) and validate the strategy of whole-genome association studies in complex heritable traits. Ultra-high-density SNP genotyping can be successfully utilized to identify risk loci in complex diseases when rigorously controlled, adequately powered, and performed using a sufficiently reliable and dense set of markers. In the case of AD, the most prominent risk locus was determined to be *APOE*, which has been repeatedly identified as a major risk factor in AD pathogenesis. This study is the first phase in a larger effort to determine other alleles that are significantly associated with AD.

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Table 1. The Effect of *APOE* Genotype on Predisposition to Alzheimer's Disease

APOE Genotype	Controls, N	Cases, N	Odds Ratio (vs. ε3/ε3)	Previously Reported	
				Farrer et al.[4]	Bertram et al.[18]
ε2/ε2	15	4	0.42	0.6	…
ε2/ε3	35	13	0.41	0.6	0.6
ε2/ε4	5	17	3.49	2.6	…
ε3/ε3	259	214	1.00	1.0	1.0
ε3/ε4	76	287	4.32	3.2	4.3
ε4/ε4	7	113	25.31	14.9	15.6

Symbol: … = not reported.

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