Evaluation of apolipoprotein A-II as a positional candidate gene for familial Type II diabetes, altered lipid concentrations, and insulin resistance

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Abstract

Aims/hypothesis. We hypothesized that apolipoprotein A-II sequence variation was responsible for the observed linkage of Type II (non-insulin-dependent) diabetes mellitus to the apolipoprotein A-II region in Northern European families ascertained for multiple diabetic siblings, and might also influence insulin sensitivity and secretion, non-esterified fatty acids, and lipids.

Methods. We recruited 698 members of 63 families for pedigree studies and additional unrelated people providing 117 diabetic and 130 control subjects. We screened the apolipoprotein A-II gene by single strand conformation polymorphism analysis and fluorescent sequence analysis. Variants were typed by oligonucleotide ligation assay, restriction digest of amplification products, or radioactive fragment analysis for the microsatellite polymorphism. Association of each variant with Type II diabetes was tested in the case-control population by chi-square analysis, or using transmission disequilibrium test in families. Haplotypes were established in families using SIMWALK and

tested for association with diabetes and quantitative traits.

Results. No detected variant altered the coding sequence of the gene. Three single nucleotide polymorphisms showed modest evidence for an association, but no variant or haplotype was associated with diabetes in families. Similarly, we found no association with non-esterified fatty acid concentrations, HDL concentrations, or fasting insulin. In contrast, we found evidence for an association of some haplotypes and individual variants with 2-h post-challenge glucose and measures of insulin secretion.

Conclusion/interpretation. Apolipoprotein A-II is not likely to explain the observed linkage of Type II diabetes, but variation in this gene could alter insulin secretion and post-challenge glucose. [Diabetologia (2002) 45:1026–1033]

Keywords Apolipoprotein A-II, association study, insulin sensitivity, non-esterified fatty acids, insulin secretion, transmission disequilibrium test, Type II diabetes mellitus, single nucleotide polymorphism, single stranded conformational polymorphism, haplotypes.

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Abbreviations: AIR, Δ Acute insulin response to glucose; APOA2, apolipoprotein A2 gene and locus; OGI60, oral compensation index at 60 min glucose; PAP, pedigree analysis package; S, Δ insulin sensitivity index; SNP, single nucleotide polymorphism; SSCP, single strand conformation polymorphism analysis; TDT, transmission disequilibrium test; df, degrees of freedom.

Considerable data support a strong genetic component to Type II (non-insulin-dependent) diabetes mellitus, but the genetic predisposition is probably complex with multiple susceptibility loci interacting with each other and with environmental and life-style factors. Recently genome scans from our laboratory in Caucasian families from Utah [1] and independent scans in Pima Indians [2], French Caucasian families [3], British Caucasian sib pairs [4], and Amish families [5] (A. Shuldiner, personal communication) have mapped one locus to chromosome 1q21-q23. Utah, Pima, and French studies all show peak linkage to the apolipo-

protein A2 locus (*APOA2*). Although multiple positional candidate genes exist within the confidence interval for linkage, considerable data support the *APOA2* gene as a functional candidate in addition to its location within a well replicated region of linkage. *APOA2* was linked to non-esterified fatty acid concentrations (NEFA) in mice and humans [6]. Increased NEFA concentrations have been shown to decrease insulin action in muscle [7], increase hepatic glucose output [8], and possibly alter insulin secretion [9, 10, 11]. A common polymorphism in the 3' flanking region of the *APOA2* gene has been associated with increased *APOA2* serum levels, coronary risk, and altered lipid profiles [12, 13, 14, 15, 16, 17].

Mouse models provide additional evidence that the *APOA2* locus might alter insulin sensitivity. Heterozygous and homozygous mice made deficient in apolipoprotein A-II by homologous recombination showed profound decreases in HDL cholesterol, reduced NEFA concentrations, reduced glucose, and low insulin concentrations, thus suggesting improved insulin sensitivity [18]. In contrast, transgenic mice overexpressing *APOA2* have increased atherogenesis [19, 20], evidence for insulin resistance with higher fasting insulin concentrations and delayed clearance of a glucose bolus, increased obesity, increased triglycerides, and increased NEFA concentrations [21]. Thus, mice overexpressing and underexpressing *APOA2* show altered insulin sensitivity and glucose homeostasis, consistent with a direct or indirect role for *APOA2* in diabetes risk.

Because the *APOA2* locus can be implicated in Type II diabetes susceptibility by linkage studies with Type II diabetes mellitus, linkage to NEFA concentrations, and a role in glucose homeostasis in experimental animal models, we evaluated the role of common variants in this gene in members of Caucasian families ascertained for at least two diabetic siblings. We screened the exons and flanking regions for novel variants in family members and control subjects, and evaluated the common variants for association with Type II diabetes in both case-control and family-based studies. We also evaluated the role of these common variants in glucose homeostasis, control of NEFA concentrations, and HDL and triglyceride metabolism.

Subjects and methods

Study population. All subjects studied were of Northern European ancestry and were ascertained in Utah under a protocol approved by the University of Utah Institutional Review Board. All subjects provided informed consent. Family based studies included members of 63 families whose characteristics have been described [1, 22, 23]. Genotypic data were available on up to 698 people, including 283 affected, 388 unaffected, and 27 subjects who were considered to have an unknown diagnosis. Non-diabetic family members were characterized by glucose and insulin concentrations during a standard 75 gm

OGTT. Non-esterified fatty acids concentrations were available on a subset of 475 family members, mostly from families ascertained later in the study. Direct measures of insulin sensitivity and secretion derived from the tolbutamide-modified frequently sampled intravenous glucose tolerance tests were available for 126 members of 26 kindreds [23, 24]. Case-control studies were conducted on 117 unrelated, non-diabetic control subjects and 130 unrelated diabetic patients, of whom 69 were unrelated members of families that met the criteria of two diabetic siblings. Not all diabetic family members were included in earlier linkage studies [1].

Mutation detection and typing. We selected 25 unrelated diabetic family members from families with the greatest evidence for linkage to chromosome 1q21-q23 and 10 unrelated control subjects from the same population. Primers were designed to amplify six fragments covering the region from 430 bp upstream of exon 1 to 527 bp of the 3' flanking region, along with intron sequences flanking the exons. Mutations were detected by single strand conformation polymorphism analysis (SSCP) under five nondenaturing conditions on fragments of under 350 bp: 5% polyacrylamide with and without 10% glycerol and at room temperature or at 4°C, and mutation detection enhancement gels (MDE; FMC Bioproducts, Rockland, Me., USA) at room temperature. We used bidirectional fluorescent sequencing [25] on a LI-COR GR4200 (LI-COR, Lincoln, Neb., USA) to confirm all variants and to check the sensitivity of our SSCP screening in 16 diabetic patients. Finally, single nucleotide polymorphisms (SNPs) were searched for using the published databases [26, 27] and literature, and confirmed using SSCP and sequence analysis.

The microsatellite polymorphism in intron 2 was typed radioactively [1]. SNPs at locations -262, 904, and 1099 were typed using an oligonucleotide ligation assay, with radioactive detection on a Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, Calif., USA) after polyacrylamide gel separation. Primer sequences for SNPs 1, 2 and 3 are shown (Table 1). For semi-automated scoring we used GeneProfiler software (Scanalytics, Fairfax, Va., USA) on digitized images. The SNP at position 1860 (SNP 4) was typed using restriction enzyme Msp I and the fragments detected on agarose gels [17].

Statistical analysis. Association of each SNP with Type II diabetes was tested in the unrelated population by chi-square analysis of allelic association or by Fisher's Exact test [28]. Additionally, based on the prior evidence for a recessive locus [1], we tested homozygotes for each allele against homozygotes and heterozygotes for the other allele. Family based associations with Type II diabetes were tested using a modified transmission disequilibrium test (TDT) [29] which used likelihood analysis and full pedigree data to probabilistically infer missing parental genotypes and haplotypes. The analysis compared the model with a maximised likelihood to the likelihood of no increased transmission ($\tau=0.5$). The likelihoods of the genetic models [30] were computed using the Pedigree Analysis Package [31] and the maxima were obtained using GEMINI [32]. Hypothesis testing compared the likelihood of a submodel ($\tau=0.50$; null hypothesis) to the likelihood of a more general model (transmission maximised). Twice the natural logarithm of the ratio of the likelihoods has an asymptotic chi-square distribution. The allele frequency of each SNP was estimated from unrelated pedigree members, assuming Hardy-Weinberg equilibrium, but using genotypes on all pedigree members to identify when individuals had not been genotyped.

Haplotypes were derived from all family data using the program SIMWALK2 version 2.60 [33]. For haplotype analysis, we included the nine observed alleles of the microsatellite polymorphism. Haplotype frequencies were estimated similar-

Table 1. Genotyping primers for APOA2 single nucleotide polymorphisms by oligonucleotide ligation assay

SNP	Allele specific primers	Common primer
SNP1 (-262)	CTTGGAATCCTGCTTCCTGTTGCGT TATTCTTGGAATCCTGCTTCCTGTTGCGC	TGAGAGGCAGGGGGTACAGCTCTTA
SNP2 (904)	AACAGCAAGAACTGGGCCTGG TCTTAACAGCAAGAACTGGGCCTGC	GTGGGAGGTCAGAGCAGACTG
SNP3 (1099)	AAACTCTAATCCCCTCACCCCTATC CTAGAAACTCTAATCCCCTCACCTATT	GTTCCGTTCCAGCCTTCTTGATC

Primer sequences for oligonucleotide ligation assays. Conditions are available from the authors. Numbers in parentheses refer to the location relative to the start of exon 1 (see Table 2)

ly by using PAP and assuming tight linkage between the SNPs, and haplotype transmission was tested using a transmission disequilibrium test as described above. We combined all rare haplotypes into a single haplotype 9. We used likelihood analysis to compute the probability of the correct genotype assignment for each subject.

For quantitative trait analyses, we tested only non-diabetic subjects for insulin, glucose, and NEFA, and we tested both non-diabetic subjects and all subjects for triglycerides and HDL cholesterol. All skewed measures, including insulin, glucose, BMI, and lipid measures were log-transformed to normality and adjusted for age and sex prior to analysis. An effect of each SNP on the quantitative traits was tested using a measured genotype analysis in PAP. A mean for each genotype was estimated and tested against a single mean in a two degrees of freedom (df) chi-square test. An effect of the microsatellite or haplotype on the quantitative traits was tested using measured allele analysis in PAP. Effects of each allele were estimated and tested against no effect of any allele (9 df) or no effect of a single allele (1 df). Polygenic inheritance was included for both measured genotype and measured allele analysis. Analysis of subjects who had undergone frequently sampled intravenous glucose tolerance tests was done using mixed effects linear regression analysis in SPSS v 10.1 (SPSS, Chicago, Ill., USA) [34]. Family membership was a random factor, genotype and diagnosis (normal and IGT) were fixed factors, and age and ln-transformed BMI were covariates, as we have described previously [35]. BMI, insulin sensitivity index (S_I), acute insulin response to glucose (AIR_g), and disposition index ($S_I \cdot AIR_g$) [36] were all ln-transformed prior to analysis. This analysis provides results comparable to that conducted in PAP to account for exact pedigree relationships [35]. Because many of the tests were correlated, and because of the presence of a gene in this region, we present p values without correction for multiple testing.

To examine a parameter comparable to disposition index in a larger group of subjects, we derived an index which we call

the “oral compensation index” (OCI). We used the 60 min insulin and glucose to measure insulin secretion, as these are most widely available in our population, and derived the 60 min oral compensation index (OCI₆₀) as $OCI_{60} = \ln[(I_{60} - I_0)/G_{60}] + \ln[1/I_0 \cdot G_0]$, where I_0 and I_{60} are baseline and 60 min insulin concentrations, and G_0 and G_{60} are baseline and 60 min glucose concentrations. The correlation with disposition index is 0.42.

Linkage disequilibrium was calculated after establishing haplotypes using gene-counting and the expectation maximisation (E-M) algorithm [37] using the EH and 2LD computer programs [38].

Results

The *APOA2* gene comprises four exons and three introns (Fig. 1). In contrast to the published reports, which predict at least 10 SNPs, we identified only three SNPs by a combination of detection methods: a T/C at position -262 in the 5' flanking region (SNP1); a G/C substitution at position 904 in intron 3 (SNP2); and a C/T substitution at position 1099 in intron 3 (SNP3) (all locations are given relative to exon 1 start

Fig. 1. Map of Apolipoprotein A2 Gene. The figure shows the approximate location of the four single nucleotide polymorphisms identified for this analysis, the GT repeat microsatellite, and the four exons. All numbers are relative to the transcription start site; 5' flanking polymorphisms are denoted as negative numbers. Distances were taken from GenBank accession XO4898. The four SNPs are labelled as in Tables 1 and 2

Table 2. Allelic association of APOA2 single nucleotide polymorphisms and diabetes in unrelated subjects

Polymorph	Location	Freq, FT2DM	Freq, T2DM	Freq, Control	<i>p</i> , FT2DM	<i>p</i> , T2DM	Relative risk
SNP1	-262 T/C	0.500	0.602	0.593	0.051	0.434	0.69 (0.45,1.05)
SNP2	904 G/C	0.883	0.883	0.794	0.139	0.239	1.43 (0.81, 2.53)
SNP3	1099 C/T	0.938	0.924	0.872	0.036	0.157	2.23 (0.97,5.14)
SNP4	1860 C/T	0.098	0.150	0.189	0.030	0.291	0.47 (0.23, 0.97)

Freq, FT2DM: allele frequency in members of familial kindreds with Type II (non-insulin-dependent) diabetes mellitus (T2DM); Freq, T2DM, allele frequency in all diabetic subjects; Freq, Control: frequency in unrelated non-diabetic subjects; *p*, FT2DM: significance on 2X2 χ^2 analysis, diabetic family mem-

bers vs control subjects; *p*, T2DM: significance for all diabetic patients vs control subjects. Relative risk is shown for allele 1 for diabetic members of familial T2DM kindreds vs control subjects, with 95% CI in parentheses. Location is relative to the start of exon 1

Table 3. Linkage disequilibrium between APOA2 single nucleotide polymorphisms

	SNP1	SNP2	SNP3	SNP4
SNP1	–	–1.000 ^a	–0.758 ^b	1.000 ^a
SNP2	–	–	–0.711 ^d	–1.000 ^c
SNP3	–	1.000 ^a	–	–
SNP4	–	–	–	–

All pairwise disequilibrium statistics are significant, as noted:

^a $p < 0.000001$, ^b $p < 0.0001$, ^c $p < 0.01$, ^d $p < 0.05$

Linkage disequilibrium statistic (D') is shown for each biallelic SNP from 5' (SNP1) to 3' (SNP4), as shown in Figure 1

site). The T/C substitution that altered an MspI site at position 1860 in the 3' flanking region was beyond the region we selected for screening but has been described well before. All SNPs detected have been reported previously [27, 39], and none alter the coding sequence. In addition, we identified the known polymorphic microsatellite (GT)_n repeat seven nucleotides upstream of exon 3 in intron 2. The location of each SNP and the GT repeat are shown (Fig. 1).

Although we found nominal associations ($p \leq 0.05$) for SNPs 1, 3 and 4 when unrelated diabetic family members were compared with control subjects, this significance was lost when all available unrelated diabetic patients were examined (Table 2). The estimated relative risks were approximately two for the risk allele for each variant (Table 2) but the confidence intervals were wide and not significant, consistent with the marginal *p* values. The four SNPs were in substantial but incomplete linkage disequilibrium (Table 3), consistent with the short distance between the most 5' and most 3' SNP. The lack of significant linkage disequilibrium between SNPs 2 and 3 might represent a recombination hotspot introduced by the microsatellite repeat [40].

Based on the strength of the APOA2 gene as a positional candidate and the suggestion of an association for SNPs 1, 3 and 4 among family members, we typed the four SNPs and the microsatellite polymorphism in all family members from our initial linkage study, regardless of original evidence for linkage to this re-

gion. We tested each variant independently and as haplotypes for excess transmission to diabetic family members using the transmission disequilibrium test. We observed 34 of the 160 possible haplotypes, of which only 8 haplotypes were present in 10 or more diabetic patients. Haplotypes 1 and 2 accounted for 55% of all subjects (Table 4). This relative lack of haplotype diversity is consistent with the strong linkage disequilibrium observed in pairwise analysis (Table 3). No single SNP was over-transmitted to diabetic patients. We noted trends to over-transmission of microsatellite alleles 1 ($p = 0.021$) and 4 ($p = 0.051$). Only haplotype 9 (the combined rare haplotypes) showed evidence of over-transmission at $p = 0.039$. Given that we tested nine haplotypes, nine microsatellite alleles, and three SNPs, these values clearly would not be significant after Bonferroni correction.

Given the prior evidence for a role of APOA2 in insulin sensitivity, HDL concentrations, and NEFA concentrations [6, 21, 41, 42, 43], we examined the effect of APOA2 variation and haplotypes on ten related traits: NEFA, HDL cholesterol, triglycerides, fasting insulin, fasting glucose, 60 and 120 min glucose during an OGTT and a measure of insulin secretion at 60 min that is normalized for the insulin sensitivity (OCI60; see Methods). Surprisingly, we found little evidence for an effect of either individual variants or haplotypes on triglycerides, HDL cholesterol, NEFA, or fasting insulin. Microsatellite allele 4 and haplotype 2 showed marginal effects on triglyceride concentrations ($p = 0.029$ and $p = 0.046$, respectively) and alleles 7 and 8 showed a marginal effect on NEFA ($p = 0.035$ and $p = 0.046$, respectively), but none would be significant after Bonferroni correction. We found a trend to effects on fasting glucose for microsatellite allele 1 and haplotype 5 (Table 4) ($p = 0.049$ and $p = 0.003$, respectively), and evidence for an effect of APOA2 on 120 min glucose during the OGTT, with SNP1 (promoter region), microsatellite allele 1, SNP 2, SNP4, and haplotypes 1 and 3 all showing evidence for an association (Table 5).

Among 113 subjects from 26 families who were both successfully genotyped and who had undergone intravenous glucose tolerance tests with Minimal

Table 4. Haplotypes observed in at least ten diabetic patients

Assignment	1	2	3	4	5	6	7	8
Haplotype	26112	13112	11211	12122	16122	12211	27112	15112
Frequency	0.325	0.225	0.093	0.076	0.035	0.030	0.032	0.024

The table shows the eight haplotypes that were observed in ten or more diabetic family members with the population frequency estimated from the family data. The major allele for each single nucleotide polymorphism is denoted as 1 and the minor allele

as 2. The microsatellite alleles were numbered 1–10, but allele 9 was not observed in this study. The order of the individual markers in the haplotype was SNP1, APOA2 microsatellite, SNP2, SNP3, and SNP4 (see Fig. 1)

Table 5. Significant measured genotype and allele effects for SNPs, microsatellite alleles, and haplotypes for quantitative traits

Variant	Trait	Number	<i>p</i> value
Microsatellite allele 1	fasting glucose	429	0.049
Haplotype 5	fasting glucose	331	0.003
SNP3 (1099)	60 min glucose	360	0.012
SNP1 (-292)	120 min glucose	331	0.008
SNP2 (904)	120 min glucose	349	0.003
SNP4 (1860)	120 min glucose	335	0.029
Microsatellite allele 1	120 min glucose	418	0.015
Haplotype 1	120 min glucose	323	0.017
Haplotype 3	120 min glucose	323	0.008
Microsatellite allele 1	OCI60	395	0.003
Microsatellite allele 5	OCI60	395	0.010
Haplotype 3	OCI60	302	0.039
Haplotype 4	OCI60	302	0.038

Traits showing significant ($p < 0.05$) association with single nucleotide polymorphisms (SNPs), individual microsatellite alleles, or haplotypes. Number, number of non-diabetic subjects tested; OCI60, oral compensation index at 60 min, as defined in the Methods. *p* values are shown without Bonferroni correction for multiple tests, since the tests are not fully independent

Model analysis [23, 24], no variant altered insulin sensitivity (S_I). We found marginal effects ($p = 0.030$) of SNP3 on first phase insulin response (acute insulin response to glucose, or $AI R_G$), and some evidence for an effect of SNP2 and SNP4 on disposition index ($S_I * AI R_G$), at $p = 0.032$ and $p = 0.034$, respectively, with interactions with family membership showing more significance ($p < 0.001$). To further explore these suggestive effects of APOA2 on insulin secretion in response to insulin sensitivity in a larger sample, we used a derived index of insulin secretion that is analogous to the disposition index from the OGTT data (OCI60, see Methods). In support of the smaller intravenous glucose tolerance test study, two microsatellite alleles and two haplotypes showed evidence for an association with OCI60 (Table 5).

Discussion

Linkage data in mice and humans [6], in experimental mouse models [43], transgenic mouse models [21, 41] and knockout [43] mouse models all support a role for

APOA2 in HDL and NEFA metabolism and in insulin sensitivity. Such a pleiotropic effect is consistent with the hypothesis that increased NEFA alter insulin sensitivity, increase hepatic glucose production, and can alter insulin secretion [44, 45]. Human studies also support an association of APOA2 variation with atherosclerosis or hypertriglyceridaemia [12, 17, 46]. Finally, linkage of both Type II diabetes [1, 2, 3, 4] and familial combined hyperlipidaemia [47, 48] to the region of chromosome 1q21-q23 that includes the APOA2 locus would potentially support APOA2 as a candidate for both disorders. Studies from our laboratory found linkage in 42 multigenerational Caucasian families under a recessive model that reached genome-wide significance ($p < 0.05$ for the genome wide scan, and logarithm of odds score of 4.29) with a peak at this locus [1].

Based on these data, we carefully screened the APOA2 gene for new variants in a population showing linkage of this region to Type II diabetes and examined the association with diabetes and with a number of diabetes related traits. No variation was identified within the coding sequence and no variant would be expected to alter the protein. We tested the four SNPs identified in addition to the microsatellite (simple tandem repeat) variant that sits within intron 2, very close to the upstream splice acceptor site of exon 3. Although we find marginal evidence for an association when we compare diabetic members of families used for the linkage study with control subjects from the same population, this significance is lost when we include a larger sample of diabetic patients from this same population. This finding might be expected if the families selected for multiple diabetic siblings were different from other diabetic patients in the population and if the true variant were in a region that we did not test. However, using haplotypes constructed from other family members and the transmission disequilibrium test (TDT) implemented in families [29], we found little evidence that either individual variants or haplotypes were transmitted from parents to their diabetic offspring more often than expected by chance alone (equal transmission of both alleles from heterozygous parents). We have over 70% power (depending on the allele frequency) to detect a 15% increase in transmission of an allele to affected family members under an additive model. This increased transmission

is equivalent to a relative risk of diabetes of 1.3 for those carrying one copy of the susceptibility allele. Nonetheless, this test could have limitations in a recessive disease, particularly if a combination of two haplotypes contributes to the risk, as has been proposed for the *NIDDM 1* or *calpain 10* gene on chromosome 2q [49]. Nonetheless, *APOA2* variation does not seem likely to account for the linkage to this region.

Based on the previous human and animal studies, we also tested for an association of both individual variants and haplotypes with diabetes and lipid associated traits. We were only able to show modest evidence for an association of microsatellite alleles 7 and 8 with NEFA concentrations and microsatellite allele 4 and haplotype 2 with triglyceride concentrations and we found no evidence for an association of any variant with HDL cholesterol. These findings seem to contrast the earlier linkage studies of NEFA [6]. The association findings are consistent with our inability to show linkage of these traits to this region using variance component quantitative trait linkage analysis. Our inability to replicate the earlier linkage is not surprising. Replication of linkage studies has been difficult to do and might require a much larger study than ours [28]. Furthermore, these families were ascertained for two or more diabetic siblings and thus represent a different population than the earlier study. However, none of the common variants seem likely to affect triglyceride, NEFA or HDL cholesterol concentrations in this population. Furthermore, we were not able to show an effect on insulin sensitivity, either using fasting insulin among non-diabetic subjects, homeostatic model-derived insulin sensitivity [50], or the minimal model-derived insulin sensitivity index in a subset of subjects who underwent intravenous glucose tolerance testing.

Several findings in this study were surprising and suggest a role for this gene in the contribution to diabetes susceptibility apart from our failure to reject the null hypothesis convincingly regarding the association with diabetes. Firstly, the common microsatellite allele 1 and the haplotype 5 were associated with fasting glucose, albeit with marginal significance ($p=0.049$) in the first case. Secondly, this same microsatellite allele, SNP2, and SNP4 (the previously described Exon 4 variant) were all associated with the glucose concentration 2 h after a 75 g oral glucose load, with significance of $p=0.003$ to $p=0.029$, and haplotypes 1 and 3 were similarly associated ($p=0.017$ and $p=0.008$, respectively). We have analysed ten traits for five variants (four SNPs and the microsatellite), but neither the traits (insulin and HOMA, 60 min glucose and OCI60) nor the variants can be considered independent. Thus, a Bonferroni correction for 50 tests, which would require a p value of 0.001 for a nominal significance of 0.05, is overly conservative and might result in the rejection of a real effect. This is particularly true for 2-h

glucose and 60-min glucose, where several tests show a trend and microsatellite allele 1 and SNPs 1 and 2 are very close to the corrected p value.

An additional finding was the association of SNP2 and SNP4 with disposition index. We have shown previously that disposition index is heritable in these families [23] and *APOA2* variation could contribute to this polygenic heritability. Further support for this finding comes from our exploration of the OCI60 index, which is analogous to the ln-transformed disposition index but derived from the OGTT (see Methods). Using this index, we again find modest associations of all microsatellite alleles, the specific microsatellite alleles 1 and 5, and haplotypes 3, 4, and 8. Taken together, the findings of an association of *APOA2* variation with insulin secretion and post-challenge glucose might suggest that the beta-cell response to insulin resistance is impaired, leading to increased 2-h glucose after a glucose load. However, the overlap between these traits and the associated variants is incomplete, and no single variant accounts for these findings.

The variant most likely to explain our findings could be the microsatellite (tandem repeat). This dinucleotide repeat ends only seven nucleotides upstream from the intron 2 splice acceptor site and the beginning of exon 3. We have observed previously ten alleles, of which only nine were observed among the family members included in this study. Microsatellite allele 1 is associated with 120 min glucose, OCI60, and is present on haplotype 3 that also shows an association with these traits. However, no consistent association of a size range of alleles is apparent in our study, and the limited haplotype diversity suggests that the microsatellite allele might be in disequilibrium with another variant at some distance. A large population study that can focus on the microsatellite population and these traits could be required to determine whether susceptibility and protective alleles exist in this region.

In conclusion, we have evaluated one of the strongest candidate genes that might explain linkage to both Type II diabetes and to familial combined hyperlipidaemia. Our data suggest possible effects of *APOA2* on post-challenge glucose and on insulin secretion related traits but we find little convincing evidence that *APOA2* variation results in altered NEFA concentrations, altered insulin sensitivity, or measurable alterations in the risk for Type II diabetes. If this gene contributes to the observed linkage to Type II diabetes, it must do so together with other factors in this region.

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