

Subsets of Finns with High HDL to Total Cholesterol Ratio Show Evidence for Linkage to Type 2 Diabetes on Chromosome 6q

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Key Words

Linkage analysis · Heterogeneity · Type 2 diabetes · HDL cholesterol · Ordered subsets analysis · Chromosome 6q

Abstract

Objectives: The purpose of this study was to examine carefully heterogeneity underlying evidence for linkage to type 2 diabetes (T2DM) on chromosome 6q from two sets of FUSION families. **Methods:** Ordered subsets analysis (OSA) was performed on two sets of FUSION families. For OSA results showing significant improvement in evidence for linkage, T2DM-related phenotypes were compared between individuals with T2DM within the subset versus the complement. **Results:** OSA analysis revealed 105 families with the highest average HDL to total cholesterol ratio (HDL ratio) that had strongly increased evidence for linkage (MLS = 7.91 at 78.0 cM; uncorrected p = 0.00002). Subjects with T2DM within this subset were significantly leaner, had lower fast-

ing glucose, insulin, and C-peptide, and more favorable cardiovascular risk profile compared to the complement set of subjects with T2DM. OSA also revealed 33 families with the lowest average fasting insulin that had increased evidence for linkage at a second locus (MLS = 3.45 at 128 cM; uncorrected p = 0.017) coincident with quantitative trait locus linkage analysis results for fasting and 2-hour insulin in subjects without T2DM. **Conclusions:** These results suggest two diabetes susceptibility loci on chromosome 6q that may affect subsets of individuals with a milder form of T2DM.

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Introduction

Type 2 diabetes mellitus (T2DM) is a complex disease with both genetic and environmental determinants [1–3]. We [4] and others [5–16] have completed genome-wide scans in a variety of populations with varying degrees of

evidence for linkage to T2DM across the genome. Given the complex nature of T2DM, it is likely etiologic heterogeneity contributes to the variability in these results and may attenuate linkage signals within individual studies. Statistical approaches to account for this complexity have been introduced and have the potential to increase power to detect linkage [17–20]. However, many of these approaches require a priori stratification of the data or do not take advantage of disease-related phenotype information.

We introduced ordered subset analysis (OSA) [19, 21] which uses disease-related phenotype information in an attempt to reduce heterogeneity in a given set of families by identifying relatively homogeneous subsets of the cohort showing evidence for linkage. As exemplified in our Finland-United States Investigation of Non-insulin Dependent Diabetes Genetics (FUSION) study [22], we observed evidence for linkage to T2DM on chromosome 6q in our original FUSION families (F1) [4] and in a second, independent set of similarly recruited families (F2) [23] (F1 MLS = 0.89, F2 MLS = 2.31, F1 and F2 MLS = 2.55). OSA further revealed interesting subsets of these families showing greater evidence for linkage in the same regions of chromosome 6q [23]. The most interesting result was a subset of 105 F1 and F2 families with highest HDL to total cholesterol ratio (HDL ratio).

In our original report, we reported the LOD scores associated with our OSA analyses within the context of our genome-wide linkage analyses [23]. The purpose of this study was to examine further and characterize the HDL ratio OSA result on chromosome 6q. If the OSA results truly represent subsets identified through disease-related phenotypes, then we might expect those affected individuals in the subset to appear phenotypically different from the complement set of affected subjects. Furthermore, the characteristics of the individuals within the subset could provide clues to the physiologic mechanisms underlying genetic predisposition for T2DM. Our analyses revealed that families within the OSA subset show not just strong evidence for linkage to T2DM, but also display a distinct phenotypic pattern suggesting a milder form of T2DM. In addition, we also report a series of overlapping OSA and quantitative trait locus (QTL) linkage analysis results further distal on chromosome 6q [4, 24]. These linkage results appeared to be consistent with observations originally reported in Mexican Americans [25, 26] and subsequently in a Chinese sample [27].

Methods

Subject Recruitment, Phenotyping, and Genotyping

Subject recruitment, phenotyping, and genotyping have been described in previous publications [4, 22–24]. Briefly, the F1 collection consisted of 1,129 affected individuals in 495 families ascertained on affected sibling pairs (ASP) with T2DM, while the similarly ascertained F2 sample consisted of 580 affected individuals in 242 families. Subjects were classified as having T2DM if they were currently taking medication for diabetes or had a medical record diagnosis conforming to WHO criteria [28]. All affected individuals were screened for potential type 1 diabetes as previously described [22] and families were excluded from analyses if so identified. Fasting blood for glucose, insulin, C-peptide, and lipids was collected on all subjects, as was DNA. Additionally, oral glucose tolerance tests (OGTT) conforming to WHO criteria [28] and tolbutamide-modified intravenous glucose tolerance tests were performed in unaffected spouses and offspring of index cases and siblings affected with T2DM from F1.

The linkage genome scan for F1 was performed in-house and initially consisted of 408 microsatellite markers, yielding an average density of 8 cM [4]. The genome scan for F2 was performed at the Center for Inherited Disease Research and consisted of 392 microsatellite markers with an average density of 9 cM [23]. There were 34 markers in common between the two genome scans. An additional 227 microsatellite markers were genotyped to close large gaps and increase resolution in regions of interest (cf. [23] for details). For this report, a total of 61 microsatellite markers (20 unique to F1, 14 unique to F2, and 27 common to both) were genotyped on chromosome 6 yielding an average density of 3.9 cM for F1 and 4.4 cM for F2.

Linkage Analysis

Genome-wide affection status-based linkage analysis was performed using Genehunter Plus as previously described [29]. The family-specific z-score was weighted by the square root of $s-1$, where s represents the number of affected individuals in the family. In addition, OSA was performed [4, 19, 21] using the program OSA V2.1 (<http://www.chg.duke.edu/software/osa.html>). Briefly, a family-based measure of a quantitative trait of interest, e.g., mean BMI, for all affected members is used to rank order all families. Families are sequentially entered into the analysis in rank order and with each addition of a family, likelihood maximization is performed for the given subset as a function of the excess allele sharing parameter δ [29]. Once all families have entered into the analysis, the subset with the overall maximum LOD is identified and statistical significance determined by permutation test. In the permutation test procedure, we assess the significance of the increase in the LOD score in the identified subset compared to the baseline LOD score comprising all families for chromosome 6. We randomly permute the rankings for the families and repeat OSA for each permutation. The chromosomal p value for the OSA increase in LOD score is estimated as the proportion of permutations giving maximum OSA LOD greater than or equal to that observed in the original data. For OSA, the following thirteen traits were examined in F1 and F2: age of diagnosis, body mass index (BMI), waist-to-hip ratio (WHR), fasting glucose, fasting insulin, fasting C-peptide, total cholesterol, LDL cholesterol, HDL cholesterol, HDL to total cholesterol ration (HDL ratio), tri-

Table 1. Phenotype correlations with HDL ratio and comparison of phenotypes for high HDL ratio OSA subset in F1 and F2

	Correlation with HDL ratio*	Subset	Complement	p value**
Subjects	720	220	1,352	
Age, years	0.02 (0.56)	65.6 (10.5)	65.2 (11.3)	1.0
Age of diagnosis, years	-0.06 (0.16)	52 (12)	52 (13)	1.0
Duration of disease, years	0.06 (0.14)	11.4 (11.7)	10.9 (12.0)	1.0
BMI, kg/m ²	-0.18 (<0.001)	28.5 (5.6)	29.5 (6.2)	0.015
WHR	-0.14 (<0.001)	0.93 (0.10)	0.94 (0.10)	0.006
Glucose, mM	-0.17 (<0.001)	8.2 (3.5)	9.6 (4.7)	<0.002
Insulin, pM	-0.25 (<0.001)	72 (66)	102 (78)	<0.002
C-peptide, nM	-0.25 (<0.001)	1.3 (1.3)	1.6 (1.5)	0.03
Triglycerides, mM	-0.69 (<0.001)	1.3 (0.6)	2.0 (1.4)	<0.002
Cholesterol, mM	-0.56 (<0.001)	5.1 (1.1)	5.7 (1.4)	<0.002
HDL cholesterol, mM	0.77 (<0.001)	1.4 (0.4)	1.0 (0.3)	<0.002
HDL ratio, HDL/total	-	0.28 (0.08)	0.19 (0.07)	<0.002
LDL cholesterol, mM	-0.52 (<0.001)	3.0 (1.0)	3.6 (1.2)	<0.002
Diastolic BP, mm Hg	-0.02 (0.52)	84 (13)	85 (13)	1.0
Systolic BP, mm Hg	0.004 (0.92)	150 (26)	150 (30)	1.0

Data are shown as Pearson correlation coefficient (p-value) or median (interquartile range).

* Correlations are based on age and gender-adjusted phenotype value, except for age, age of diagnosis, and duration of disease, which are unadjusted.

** Bonferroni-corrected for the number of traits tested (n = 15).

glycerides, systolic blood pressure, and diastolic blood pressure. For F1, 24-hour urine creatinine and albumin were also analyzed in T2DM patients. Family-specific mean values were computed after adjusting for age and gender.

We adjusted our chromosome-based OSA p values for multiple comparisons, given that multiple traits and two different rankings, low-to-high and high-to-low, were examined for each chromosome. For F1 analyses, permutation-based OSA p values were Bonferroni-adjusted by multiplying by 30 to account for the 15 traits and 2 sorting directions. F2 and joint F1 and F2 OSA p values were multiplied by 26 due to the lesser number of traits [13] examined. The underlying correlation among phenotypes examined makes these corrections conservative, as each trait is considered to be independent.

Phenotype Comparisons

Pearson correlation coefficients between age and gender-adjusted HDL ratio and the constellation of diabetes-related phenotypes, also age and gender-adjusted, were computed by randomly selecting a single affected individual from each family. For each subset identified by OSA, we compared phenotypes of the affected individuals within the subset with the identical phenotypes in the complement set. Phenotype data were compared using generalized estimating equations (GEE) to account for the correlation among related individuals. All phenotype data were statistically transformed to approximate univariate normality and adjusted for age and gender, and where appropriate, BMI. We report Bonferroni-corrected p values to account for the multiple traits examined (n = 15, cf. table 1). As noted above, the underlying correla-

tion among phenotypes increases the conservative nature of the Bonferroni correction.

Medication use was compared by χ^2 analysis. Medications were classified into broad categories using Finnish medication codes and their presumed effect on specific quantitative traits. The p values for the test of association were Bonferroni-corrected for the six drug categories examined: insulins, sulphonylureas, metformin, glucocorticoids, lipid lowering, and anti-hypertensive.

Phenotype values are reported as median with interquartile range in parenthesis, unless otherwise specified.

Results

As previously reported [23], linkage analysis based on F1 families revealed a modest signal (fig. 1A, MLS = 0.89) at 96 cM on our FUSION genetic map (between D6S1546 and D6S268). In F2, linkage to chromosome 6q was the strongest genome-wide signal [23] and was nearly coincident with the signal observed in F1 (fig. 1B, MLS = 2.31 at 95 cM, 1-LOD support: 90–109 cM). Joint analysis of F1 and F2 (fig. 1C) yields an MLS of 2.55 (1-LOD support: 82–109 cM) at 100 cM between D6S268 and D6S474.

In F1, OSA identified 93 families with the highest HDL ratio showing strongly increased evidence for linkage

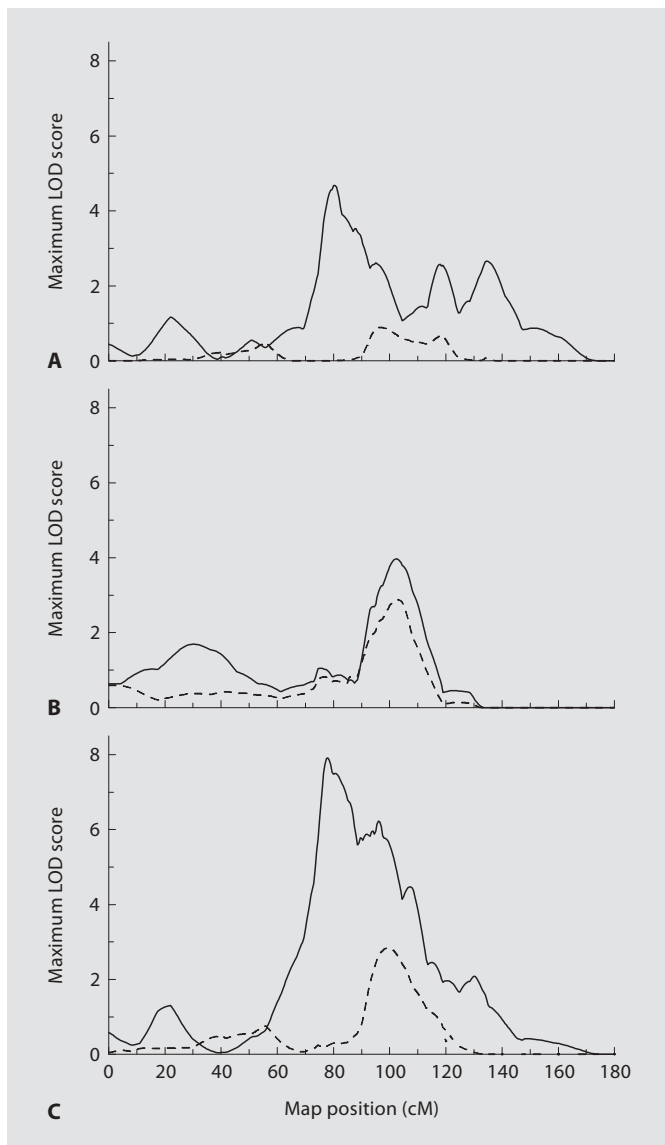


Fig. 1. High HDL Ratio OSA Results. Linkage analysis results using all families are shown as dashed lines and OSA results are shown in solid lines. Panel **A**: F1. Panel **B**: F2. Panel **C**: Joint F1 and F2.

(fig. 1A, MLS = 4.68 at 81 cM near D6S1045; $p = 0.003$). This subset represented 19% of F1 families. A subset of 141 F2 families (70%) with the highest HDL ratio also showed an increased linkage signal in the same region (fig. 1B, MLS = 3.97 at 103 cM between D6S268 and D6S474), but this improvement in linkage was not statistically significant ($p = 1.0$). In the joint analysis (F1 and F2), a strongly increased MLS of 7.91 ($p = 0.0006$) was observed at 78 cM (between D6S445 and D6S1601) with

Table 2. Use of anti-diabetic and selected other drugs by the high HDL ratio OSA subset and the complement in F1 and F2

	Subset	Complement	p value*
Insulins	82 (36.3)	590 (40.5)	1.0
Sulphonylureas	131 (58.0)	879 (60.3)	1.0
Metformin	64 (28.3)	437 (30.0)	1.0
Glucocorticoids	77 (34.1)	554 (38.0)	1.0
Lipid lowering	61 (27.0)	459 (31.5)	1.0
Anti-hypertensives	148 (65.5)	1,034 (70.9)	1.0

Data are shown as counts (%).

* Bonferroni-corrected for the number of drug categories tested ($n = 6$).

a subset of 105 families (15%, fig. 1C). This increase in LOD score would remain significant ($p = 0.014$) even with an additional genome-wide correction for the number of chromosomes. Although not statistically significant after correcting for multiple comparisons, OSA also identified a signal for a low total cholesterol subset (291 families, MLS = 5.71 at 102 cM; uncorrected $p = 0.003$) and a marginal signal for a high HDL cholesterol subset (56 families, MLS = 4.32 at 78 cM; uncorrected $p = 0.053$), consistent with the high HDL ratio result. Complete OSA results are presented in the supplemental tables.

HDL ratio was predictably highly correlated with other lipid measurements with correlation coefficients ranging from -0.52 for LDL cholesterol ($p < 0.015$) to 0.77 for HDL cholesterol ($p < 0.015$). The magnitude of the correlations between HDL ratio and other phenotypes were more modest, ranging from a low of 0.02 (study age; $p = 0.56$) to a high of -0.25 (C-peptide; $p < 0.015$). While the 220 affected individuals in the high HDL ratio subset of 105 F1 and F2 families and the complement set of 1,352 affected subjects in 591 families were similar in age at ascertainment and age-at-diagnosis for T2DM, the two subsets differed in several important ways (table 1). By selection, the high HDL ratio subset had higher HDL cholesterol and lower LDL cholesterol. They also had lower BMI, WHR, fasting glucose and insulin, and total triglycerides (table 1). These phenotypic differences persisted when analyses were performed separately for F1 and F2 (data not shown).

The differences in phenotypes between the high HDL ratio subset and the complement could not be accounted for by differences in medication use at the time of study enrollment [22] (table 2). The relative proportion of subjects on anti-diabetic and anti-lipid medications was sim-

ilar between the two groups. While the proportion of T2DM patients treated with insulin was similar between the groups (36.3 vs. 40.5%; $p = 1.0$), subjects within the high HDL ratio subset initiated insulin treatment somewhat later than patients in the complement (63 (10) vs. 61 (12) years; $p = 0.032$).

In addition to the high HDL ratio result around 80 cM, there was an interesting cluster of results distal to this locus. OSA in F1 also identified 33 families (6.7% of families) with the lowest fasting insulin showing strongly increased evidence for linkage at 128 cM near D6S1569 (fig. 2, $MLS = 3.45$; 1-LOD support 123–138 cM; uncorrected $p = 0.017$). The low fasting insulin OSA result for F2 ($MLS = 3.87$; uncorrected $p = 0.088$) maximized proximal to the F1 result at 102 cM between D6S268 and D6S474. The joint analysis yielded an MLS of 4.23 (uncorrected $p = 0.062$) at 98 cM near D6S268. Although these OSA results were not statistically significant after correcting for multiple comparisons, they overlapped with a cluster of insulin-related QTL linkage results making them of greater interest. QTL linkage analysis in non-diabetic spouses and the offspring of F1 subjects with T2DM (fig. 2) yielded a relatively strong signal for fasting insulin ($MLS = 2.65$ at 127 cM between D6S1009 and D6S1569) and a more modest signal for 2-hour insulin from the oral glucose tolerance test ($LOD = 1.55$ at 129 cM). Thus, we observed a series of insulin-related results pointing to the same region of chromosome 6q.

Discussion

The identification of susceptibility genes for complex diseases is complicated by etiologic heterogeneity that not only reduces statistical power within a study, but may, in part, account for variability in results observed across studies [17]. There are statistical approaches to dissect heterogeneity that improve power to detect linkage [17, 18, 20, 30]. Given that disease classification is often defined by assigning threshold values of a continuous trait, it is possible consideration of disease-related phenotypes can provide important clues regarding the underlying heterogeneity. Methods to assess heterogeneity typically require a priori stratification of the data [18, 30] or do not consider quantitative trait information [17]. We introduced OSA [19, 21] as an alternative approach that attempts to take advantage of disease-related phenotype information. The principle behind OSA is that subjects with similar disease-related phenotype values may also have similar genetic predisposition and therefore be ge-

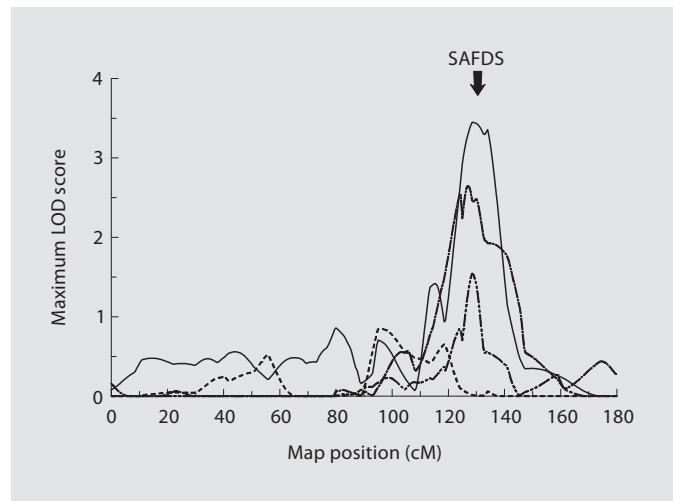


Fig. 2. Cluster of Results on Chromosome 6q. (---) T2DM linkage analysis result based on all F1 families. (—) Low fasting insulin OSA result comprising 33 F1 families. (- - -) QTL linkage analysis for fasting insulin in unaffected spouses and offspring of F1 affected index cases and siblings. (- · -) QTL linkage analysis for 2-hour insulin from the OGTT in unaffected spouses and offspring of F1 affected index cases and siblings. ‘SAFDS’ indicates location of fasting insulin QTL result from the San Antonio Family Diabetes study, maximizing near D6S403 (24).

netically more homogeneous. Therefore, the method attempts to reduce heterogeneity within the sample by selecting families that have similar disease-related phenotype values, thus there is no requirement for a priori stratification of the data.

We applied OSA to two independent genome-wide scans in the FUSION study [4, 23]. A series of consistent high HDL ratio subsets across both F1 and F2 at ~80 cM on chromosome 6 were observed. This result was of interest given it yielded the strongest improvement in the evidence for linkage ($MLS = 7.91$; $p = 0.0006$) in the joint F1 and F2 OSA. It should be noted that the final subset size in the joint F1 and F2 analysis (105 families) is not the sum of families in the independent F1 (93 families) and F2 (141 families) analyses. This is due to the fact that interdigitation of F1 and F2 families can result in slight differences in familial ranking by mean quantitative trait. Also, OSA analysis maximizes the overall evidence for linkage with each addition of a family. Therefore, given the slight differences in familial ranking and subsequent group of families included in the subset, the maximum evidence for linkage could occur at different values of the δ parameter. In fact, the F1 and F2 maxima were at δ val-

ues of 0.459 and 0.354, while the maximum for the joint F1 and F2 analysis occurs at $\delta = 0.525$. Thus, 56 F1 families and 49 F2 families contribute to the joint F1 and F2 OSA result.

In the case of our chromosome 6 analyses we observed a clear phenotype profile was observed where individuals in the high HDL ratio subset were leaner, had lower fasting glucose, insulin, and C-peptide concentrations, and had a lower-risk lipid profile compared to the complement set of affected subjects. The T2DM patients within the high HDL subset do not appear to follow the stereotypic T2DM with concomitant obesity (cf. [31]). Despite having similar age at onset and duration of T2DM as the complement, their diabetes appears to be of a milder form; hyperglycemia in the presence of lower adiposity, which in turn results in a more favorable cardiovascular risk profile. T2DM in these subjects may not be due to the primary effects of obesity inducing insulin resistance and dyslipidemia, but perhaps due to a primary β -cell defect. These findings point to a form of T2DM that may be milder and associated with less hyperinsulinemia and insulin resistance than the form(s) affecting the complement set of individuals.

Consistent with the healthier lipid profile, individuals in the high HDL ratio subset had a marginally lower proportion of individuals whose hospital discharge records revealed a history of clinical coronary artery events (23.8 vs. 30.1%; $p = 0.051$) and a significantly lower proportion of individuals with history of cerebrovascular events (13.0 vs. 20.5%, $p = 0.007$). Thus, the evidence suggests a subset of patients with T2DM that may also have a unique genetic predisposition. These differences were observed despite the fact that both groups of T2DM patients (F1 and F2) had similar age-at-onset and duration of disease. Finally, among F1 families in which we have phenotypic data on the offspring of affected individuals, the offspring from families within the high HDL ratio subset show a similar differences in phenotypic profile compared to the offspring from the complement set of families (data not shown). The presence of a distinct phenotypic pattern may be evidence for a disease susceptibility variant(s) that has a strong physiologic effect.

Families were selected based on a given disease-related quantitative trait. One might assume that individuals within the selected subset will, by default, have an overall phenotypic profile distinct from the complement set of individuals. In fact, the correlation between HDL ratio and other lipid measurements was predictably high, but the correlation with non-lipid phenotypes was rather modest (cf. table 1), although many times statistically sig-

nificant partially owing to substantial sample size. Thus, while the differences in lipid profile may be a function of the underlying correlation among those traits, the differences in other phenotypes are unlikely to be completely driven by the underlying correlation.

Our experience with OSA across the genome does not support the assumption of predetermined phenotypic differences. When we observe a significant OSA result and compare phenotypes between affected individuals within the subset versus the complement set of affected individuals there is a predictable difference in the trait used to select the subset. However, typically there is not a difference in the overall phenotypic profile. For example, the next most significant genome-wide OSA result using the combined F1 and F2 families was observed on chromosome 17 where families with low HDL ratio showed evidence for linkage (LOD = 2.37; uncorrected $p = 0.006$). When phenotypes were compared between individuals within the subset versus those in the complement set, there was a predictable pattern of significant differences in lipid values and a significant difference in BMI, but no differences in the other phenotypes (data not shown). The extent to which any overall phenotypic pattern can be attributed to selection upon a single trait will depend upon the strength of the correlation among the traits, which is in part due to the physiologic link among those traits. Identification of loci linked to or associated with such correlated traits may identify a specific physiological subtype of diabetes.

Another possibility that would explain the differences in phenotype pattern is if the treatment regimen for T2DM patients within the subset was substantially different from the complement. However, examination of medication use at the time of enrollment into the study shows similar proportions of patients on anti-diabetic therapies and lipid-lowering agents (cf. table 2). Furthermore, there was no difference in the proportion of patients treated by lifestyle alteration alone (12 vs. 11%). An alternative explanation is that subjects within the subset observed greater treatment compliance or responded more favorably to treatment. Unfortunately, we do not have accurate data to assess these possibilities.

Given the increased evidence of linkage was observed using a lipid-related trait, an alternative possibility is that the locus underlying the high HDL ratio subset is directly related to cholesterol metabolism that could secondarily affect glucose metabolism and alter susceptibility to T2DM. Naoumova et al. studying two cohorts with familial combined hyperlipidemia (FCHL) observed evidence for linkage to FCHL-related traits in the same re-

gion of chromosome 6q [32]. Similarly, Canizales-Quinteros et al. noted that in large kindred with heterozygous familial hypercholesterolemia linked to chromosome 1p32, ten family members with elevated HDL cholesterol or hyperalphalipoproteinemia had delayed or no atherosclerotic complications [33]. When additional linkage analysis was performed to hyperalphalipoproteinemia, evidence for linkage to a 7.3 cM region of chromosome 6q was observed. The results from both these studies fall within our 1-LOD support for the high HDL ratio result and suggest a variant directly related to HDL cholesterol or cholesterol metabolism may lie within this linkage region.

Our second set of results combined overlapping signals from OSA and QTL linkage analyses all related to fasting or 2-hour insulin concentrations in our F1 sample. It is important to note that OSA is not analogous to QTL linkage analysis, as the statistic for OSA is still linkage to T2DM and the phenotype data are only used as a metric of similarity to identify relatively homogeneous subsets [19, 21]. Although the low fasting insulin OSA result is not statistically significant, the fact these overlapping signals are all related to insulin concentration is of interest and suggests the possibility of an insulin-related locus. Furthermore, the San Antonio Family Diabetes Study (SAFDS) observed QTL linkage signals for both fasting glucose [25] and insulin [26] in the identical region of chromosome 6q (cf. fig. 2).

In summary, we applied ordered subset analysis to two independent samples of Finnish affected sibling pair families and identified two regions on chromosome 6q with evidence for a type 2 diabetes susceptibility variant. The first region was identified by a series of overlapping results based on families with relatively high HDL ratios.

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This result constituted the strongest improvement in the evidence of linkage in the joint F1 and F2 analysis among all traits examined. Subjects within the subset had lower BMI and less of the dyslipidemia commonly associated with diabetes and insulin resistance than the complement of families with lower HDL ratios, suggesting a form of T2DM that is less dependent upon obesity and, perhaps, insulin resistance. The second region was identified within the F1 families alone and consisted of both OSA and QTL linkage results related to insulin concentrations. Evidence for a fasting insulin QTL was also observed in the San Antonio Family Diabetes Study [25, 26] in the same region. These results suggest there may be two loci on chromosome 6q that confer susceptibility to a possibly milder form of T2DM and may exert their effects by directly altering lipid metabolism, or through their impact on β -cell function.

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