

## Vesicle-associated membrane protein 4, a positional candidate gene on 1q24-q25, is not associated with type 2 diabetes in the Old Order Amish

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### Abstract

**Objective.** The vesicle-associated membrane protein-4 (*VAMP4*) gene is an excellent type 2 diabetes (T2DM) positional candidate gene. It is located on chromosome 1q24-q25, a region of linkage to T2DM in the Amish and several other populations. *VAMP4* is expressed in liver and skeletal muscle and participates in intracellular trafficking of secreted and membrane-associated proteins.

**Design and methods.** We sequenced *VAMP4* in 20 Amish subjects. Polymorphisms in and around *VAMP4* were genotyped in 65 Amish subjects with T2DM, 64 subjects with impaired glucose homeostasis (IGH), and 126 normal glucose tolerant controls, as well as in an expanded set of 749 participants of the Amish Family Diabetes Study for whom glucose and insulin levels during an oral glucose tolerance test (OGTT) and other quantitative traits related to diabetes were available. Case-control and quantitative trait association analyses were performed.

**Results.** We found three common non-coding intragenic polymorphisms: a 23 bp insertion/deletion (I/D) in the 5' untranslated region (UTR) in exon 1 at position 73127, and G35319T and C335296T single nucleotide polymorphisms (SNPs) in the 3' UTR (NCBI Accession No. Z98751). The two 3' UTR SNPs were in complete linkage disequilibrium (LD) and both were in strong LD with the exon 1 I/D polymorphism ( $|D'|=0.82$ ). Similarly, three extragenic flanking SNPs (rs978985, rs203255, and rs1023479), showed moderate LD with the neighboring intragenic SNPs ( $|D'|=0.23-0.69$ ). None of the SNPs individually nor any of the 2-, 3-, 4-, or 5-polymorphism haplotypes were associated with T2DM or IGH. The exon 1 I/D polymorphism was not associated with significant differences in mean fasting or stimulated glucose or insulin levels during an OGTT or other diabetes-related quantitative traits in the expanded set of 749 subjects.

**Conclusion.** Variation in *VAMP4* does not significantly influence risk of T2DM or IGH in the Amish.

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**Keywords:** Type 2 diabetes; Genetics; Linkage disequilibrium mapping

### Introduction

With 16 million Americans affected [1], type 2 diabetes (T2DM) is the most common form of diabetes in the United States and worldwide. The pathogenesis of the

disease includes both genetic and environmental factors, the former supported by strong familial clustering and twin studies [2,3], and the latter by its dramatically increased incidence and prevalence over the past few decades as a result of lifestyle factors (dietary excess and physical inactivity) [4,5]. In the Old Order Amish, we have previously reported evidence for linkage of T2DM and impaired glucose homeostasis (IGH) to a locus on

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chromosome 1q21-q24 (LOD = 2.35,  $P = 0.0008$ ) [6]. Conditional analysis of chromosome 17q24 increased the LOD score on 1q21-q24 to 2.91. Linkage of this region on chromosome 1 to T2DM has been reported in at least five other populations, including Pima Indians [7], French Caucasians [8], Utah Caucasians [9], UK Caucasians [10], and Chinese [11], as well as to the quantitative trait, hemoglobin A1c [12]. Collectively, these studies provide very strong evidence for a shared diabetes susceptibility locus in this chromosomal interval. There are at least 451 genes within the approximately 30–40 Mb region defined by these collective linkages.

To identify the T2DM susceptibility gene on chromosome 1q21-q24, we and others are using both linkage disequilibrium (LD) mapping as well as positional candidate gene approaches. Positional candidate genes that have been examined to date include lamin A/C (*LMNA*) [13], apolipoprotein A2 (*APOA2*) [14], phosphoprotein enriched in astrocytes 15 (*PEA 15*) [15], potassium inwardly rectifying channel, subfamily, members 9 and 10 (*KCNJ9*, *KCNJ10*) [16,17], C-reactive protein (*CRP*) [18], solute carrier family 19 (thiamine transporter), member 2 (*SLC1A2*) [19], retinoid X receptor, gamma (*RXRG*) [20], pyruvate kinase, liver, and red blood cell (*PKLR*) [21], insulin receptor related receptor (*INSRR*) [22], among others. Only *PKLR* was found to be associated with T2DM, but the strength of the association was likely not strong enough to account for the original linkage [21].

The vesicle-associated membrane proteins (VAMP) are members of the *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) protein family. SNARE proteins are key elements in intracellular vesicle biogenesis, protein sorting, and membrane fusion [23,24]. Interaction of a specific vesicle protein (v-SNARE) with a specific target membrane protein (t-SNARE) governs efficient target recognition and fusion. Previously, the v-SNAREs, VAMP2 and VAMP3/cellubrevin, and the t-SNARE syntaxin 4 have been implicated in insulin-stimulated glucose transporter (GLUT4) trafficking [25–28]. Indeed, in the Zucker diabetic fatty rat, elevated levels of the VAMP2, VAMP3, and syntaxin 4 are associated with insulin resistance in skeletal muscle cells, which can be reversed by the insulin sensitizer, rosiglitazone [29].

VAMP4 is a recently discovered member of the SNARE family [30]. It is encoded on chromosome 1q24-q25, on the telomeric end of the T2DM linkage peak in the Amish and well within the linkage peaks of others. It is expressed in various cell types including liver and skeletal muscle. VAMP4 is preferentially located in the tubular and vesicular clathrin and non-clathrin-coated membranes of the *trans* Golgi network, with some protein noted on the *trans* side of the Golgi apparatus and the endosomes. It co-localizes with syntaxin 6, which was shown to exit the *trans* Golgi network and immature secretory granules in clathrin-coated vesicles in both endocrine and exocrine pancreatic cells [30,31].

Based on the putative role of VAMP4 in the intracellular trafficking of hormones and secretory vesicles, and of the role of VAMP proteins in glucose transporter trafficking, we hypothesized that mutations in *VAMP4* might result in decreased insulin secretion and/or increased insulin resistance, and thus increased susceptibility to T2DM. In this study, we screened *VAMP4* for sequence variation, genotyped the observed variants in subjects from the Amish Family Diabetes Study (AFDS), and performed analyses of single variants as well as multi-variant haplotypes for association with T2DM and related quantitative traits. Our findings suggest that sequence variation in *VAMP4* is unlikely to contribute risk to T2DM in our population.

## Research design and methods

### Study subjects

Subjects in this study were participants of the AFDS. Subject recruitment and phenotypic characterization have been described in detail previously [32]. Briefly, Amish probands with T2DM and their first and second degree relatives and spouses age 18 years or older were recruited into the AFDS. Phenotypic characterization was performed at the Amish Research Clinic in Strassburg, PA, or at the subjects' homes. Height and weight were measured and body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. A fasting blood sample was obtained and plasma glucose and insulin and serum lipid levels were measured as previously described [32]. An oral glucose tolerance test (OGTT) was performed in which blood samples were drawn every 30 min for 3 h following ingestion of 75 g of glucose. Total area under the curve (AUC) for the OGTT plasma glucose and insulin responses was calculated using the trapezoidal rule. The homeostatic assessment of insulin resistance (HOMA-IR) was calculated as  $\{[\text{Fasting insulin } (\mu\text{U/mL}) - 10] \times \text{fasting glucose (mmol/L)}\} / 22.5$ . Normal glucose tolerance (NGT), impaired glucose tolerance (IGT), impaired fasting glucose (IFG), and diabetes were defined based on 2003 guidelines of the American Diabetes Association [33]. The study protocol was approved by the Institutional Review Board at the University of Maryland School of Medicine, and informed consent was obtained from each study participant.

We screened for sequence variation in *VAMP4* in 20 individuals from 15 Amish families showing evidence for linkage of diabetes to chromosome 1 in our genome scan. Five subjects had normal glucose tolerance, five had impaired glucose tolerance, and 10 had T2DM. Initial genotyping of *VAMP4* polymorphisms and association analyses were performed in three groups of individuals selected as non-first degree relatives and

either having T2DM ( $n=65$ ), impaired glucose homeostasis (IGT or IFG; IGH) ( $n=64$ ), or NGT with age greater than or equal to 40 years ( $n=129$ ). To examine the relationship between *VAMP4* sequence variation and fasting and OGTT glucose and insulin levels as well as other quantitative traits related to T2DM, the larger set of 749 subjects of the AFDS who contributed to the linkage was genotyped.

#### Screening for polymorphism in *VAMP4*

Genomic DNA was extracted from whole blood using standard methods (Qiagen Maxi Preps, Valencia, CA). DNA fragments covering 1 kb of proximal promoter, all 8 exons, and all exon–intron junctions were amplified by PCR (primer sequences available on-line as Supplemental Table 1). All of the PCR reactions were performed in a total volume of 30  $\mu$ l containing 25 ng genomic DNA, 0.5  $\mu$ M of each primer, 1.5 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, and 0.4 U *Taq* DNA polymerase (Promega, Madison, WI). PCRs were performed using an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min. PCR products were visualized by separation of 3  $\mu$ l of PCR products on a 2% NuSieve agarose gel containing ethidium bromide. The remainder of the PCR products were purified by passing them through Millipore multi-screen-PCR plates (Millipore, Bedford, MA), and then were sequenced in both directions using BigDye sequencing chemistry (Applied Biosystems—Perkin–Elmer, Foster City, CA) and an ABI 3700 automated DNA sequencer. DNA sequences were analyzed for sequence variation using Sequencher and also by visual inspection.

#### Genotype analysis

##### Exon 1 insertion/deletion (I/D) polymorphism

A 293-bp DNA fragment containing the 23 bp insertion at genomic position 73245 (NCBI Accession No. Z98751) was amplified by PCR in a total volume of 30  $\mu$ l containing 2 ng genomic DNA, 0.5  $\mu$ M of each primer (forward: 5'-CGGATCGACTTCCTCGATTA-3' and reverse: 5'-CGTTTCCTTCCTCCTCACAC-3'), 1.5 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, and 0.4 U *Taq* polymerase using the same cycling conditions described above. Five microliters of the PCR products of each sample were separated on a 3% NuSieve agarose gel containing ethidium bromide. Homozygotes with the insertion (I/I) showed a single 316 bp product, homozygotes without the insertion (D/D) showed a single 293 bp product, and heterozygotes (I/D) had both products (as well as a third larger band that represents heterodimers).

##### C35296T single nucleotide polymorphism (SNP) in the 3' UTR and SNPs flanking *VAMP4*

A 645-bp DNA fragment containing the two common 3' UTR SNPs (G35319T and C35296T) was amplified by PCR in a total volume of 10  $\mu$ l containing 15 ng genomic DNA, 0.5  $\mu$ M of each primer (forward: 5'-GGAA TATT GCTTCAAACATAGCA-3' and reverse: 5'-AC CTGAG GGCAACAACATTT-3'), 1.5 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, and 0.4 U *Taq* DNA polymerase using the same conditions described above. The C35296T SNP was detected by primer extension with primer 5'-CCTG TGTAGAGTACCAAATTATAT-3' using the Perkin–Elmer Acycloprime™-Fluorescence Polarization SNP detection kit (Perkin–Elmer Life Sciences, Boston, MA).

Rs978985 and rs2032555 located 989 and 66 kb upstream, respectively, and rs1023479 located 644 kb downstream to *VAMP4* were chosen from dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). Genotype analysis was performed by primer extension using fluorescence polarization for SNP detection as described above. All PCR and primer extension sequences are available as on-line Supplemental Table 2.

#### Statistical analysis

The extent of linkage disequilibrium between the polymorphisms was estimated in a subset of 258 relatively unrelated (non-first degree) Amish individuals by maximum likelihood methods using the SNP-HAP software program (Clayton, D. <http://www-gene.cimr.cam.ac.uk/clayton/software/>). Allele and genotype frequencies for each of the polymorphisms were compared between NGT controls and both the IGH and T2DM case groups using a  $\chi^2$  test. Additional association analyses were then performed using 2-, 3-, 4-, and 5-polymorphism haplotypes using the likelihood ratio test statistic introduced by Xie and Ott [34] that compares haplotype frequencies of cases, controls and both groups combined. Haplotype frequencies were estimated using ZAPLO [35] and empirical *P*-values were derived by simulating the null distribution with 1000 replicates.

The exon 1 polymorphism was subsequently genotyped in an extended set of 749 Amish individuals to evaluate associations with levels of fasting and 2-h post glucose challenge glucose and insulin levels and other diabetes-related traits. Only nondiabetic individuals were used for these analyses since diabetes and/or its treatment may confound these quantitative trait variables. Mean concentrations of these traits were estimated using a family-based variance component approach, in which we estimated the effects of genotype (coded as a 0/1 dummy variable) on trait variability. Parameter estimates were obtained using maximum likelihood methods that accounted for the residual correlations between related family members. Serum insulin concentrations were transformed by their natural logarithms prior to analyses.

Statistical testing was performed by comparing the likelihoods (via likelihood ratio test) of models in which the genotype effects were estimated and in which they were constrained to be zero. These analyses were conducted using the SOLAR software program [36].

## Results

### Characteristics of study subjects

Selected clinical characteristics of the three groups are shown in Table 1. By selection, the NGT control group was as old or older than the IGH and T2DM case groups. However, BMI and insulin values were significantly higher in the IGH and T2DM groups compared to the NGT group consistent with the well-known association of obesity and hyperinsulinemia/insulin resistance with IGH and T2DM.

### *VAMP4* polymorphism and linkage disequilibrium analysis

To examine whether sequence variation in *VAMP4* contributes to T2DM susceptibility in the Amish, we

screened all 8 exons, exon–intron junctions as well as 1 kb of the proximal regulatory region. Three non-coding polymorphisms within *VAMP4* were identified. These variants included a 23 bp insertion/deletion (I/D) at position 73127 in the 5' UTR of exon 1 (NCBI Accession No. Z98751), a G–T substitution at position 35319 (rs15655) and a C–T substitution at position 35296 (not in current NCBI database), both in the 3' UTR (Fig. 1). The allele frequencies of the 5' UTR deletion (D), 35319T and 35296T were 0.25, 0.35 and 0.35, respectively among the 20 individuals sequenced. The two 3' UTR SNPs were only 25 bp from each other and in complete linkage disequilibrium. Thus, only the 5' UTR insertion/deletion and C35296T polymorphisms were genotyped in the case control sample set. In these samples, we found that the 5' UTR insertion/deletion, located 37,831 bp from the 3' UTR SNPs were also in strong linkage disequilibrium ( $|D'|=0.82$ ) (Table 2). To further define the interval of linkage disequilibrium, we also genotyped 3 SNPs flanking *VAMP4*, rs978985 and rs203255 (66 and 989 kb upstream, respectively), and rs1023479 (644 kb downstream). These extragenic SNPs showed moderate LD with their neighboring intragenic SNPs ( $|D'|=0.23–0.69$ ) (Table 2). Thus, *VAMP4* lies in a region of moderate to strong linkage disequilibrium in the Amish.

Table 1  
Selected characteristics of the study population

	Diabetes ( <i>n</i> = 65)	IGH ( <i>n</i> = 64)	NGT ( <i>n</i> = 129)
Age (years)	52 ± 7.8	40.4 ± 9.2	55.9 ± 11.1
Sex (M/F)	17/48	16/48	76/53
BMI (kg/m <sup>2</sup> )	29.2 ± 5.4	28.4 ± 5.4	27.5 ± 4.8
Glu 0 (mg/dL)	140.5 ± 69.8	92.4 ± 9.5	90.2 ± 7.0
Glu 120 (mg/dL)	259.6 ± 85.1	157.8 ± 12.8	106.4 ± 20.8
Ln Ins 0 (pmol/L)	—	12.1 ± 7.6	10.7 ± 3.8
Ln Ins 120 (pmol/L)	—	70.5 ± 49.0	40.7 ± 29.5

Data are means ± SD. BMI, body mass index; Glu 0, fasting plasma glucose; Glu 120, plasma glucose at 120 min during OGTT; Ln, natural logarithm; Ins 0, fasting plasma insulin; Ins 120, plasma insulin at 120 min during OGTT. For the diabetes group, Glu 0 and Glu 120 are reported for newly diagnosed diabetic subjects only, and Ins 0 and Ins 120 are not reported due to confounding influences of medication and the diabetic state.

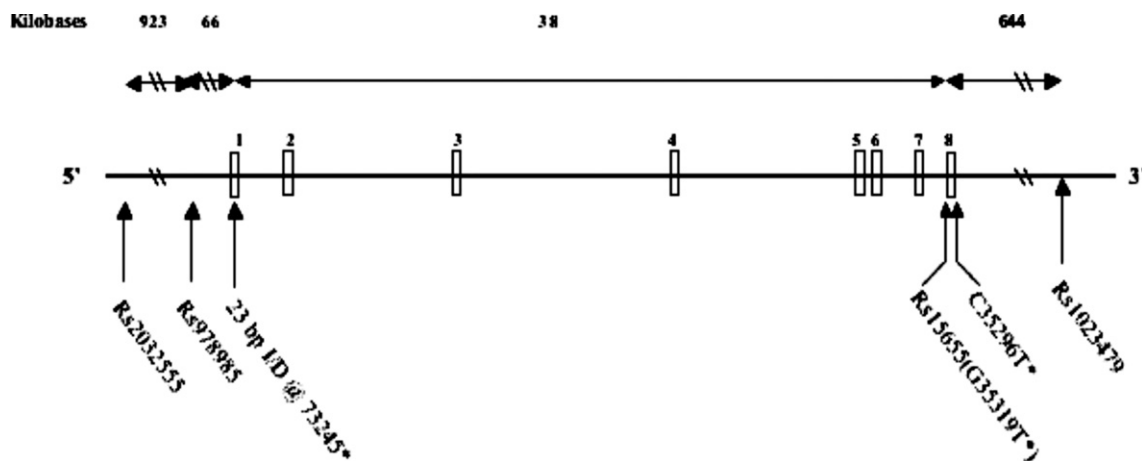


Fig. 1. Schematic of *VAMP4* on chromosome 1q24–q25. Polymorphisms are shown in their respective positions. Exons are not drawn to scale. Nucleotide positions correspond to NCBI clone Accession No. Z98751.

Table 2  
Pairwise linkage disequilibrium of *VAMP4* polymorphisms

$R^2$	D'				
	rs978985 (167923.848)	rs2032555 (168847.078)	23 bp I/D (168912.623)	C35296T (168950.456)	rs1023479 (169594.508)
rs203255	—	0.157	0.226	0.630	0.489
rs978985	0.016	—	0.691	0.684	0.452
23 bp I/D	0.008	0.472	—	0.820	0.413
C35296T	0.100	0.074	0.421	—	0.523
rs1023479	0.110	0.024	0.012	0.032	—

Nucleotide positions correspond to NCBI build 34 chromosomal positions.

### *VAMP4* polymorphism and diabetes and related quantitative traits

There was no association between either the 5'UTR I/D or C35296T polymorphisms or any of the three flanking *VAMP4* SNPs with T2DM or IGH; neither allele frequencies (Table 3) or genotype frequencies (not shown) differed between T2DM or IGH cases and controls. To examine whether other SNPs perhaps in regions of the *VAMP4* gene that was not screened for sequence variation might contribute risk to T2DM, haplotypes were constructed with rs978985 within the 5' regulatory region, the 23 bp I/D polymorphism in exon 1, and C35296T in the 3' UTR. These polymorphisms are within 1.03 Mb and span the entire *VAMP4* gene. Since these polymorphisms were in moderate LD with each other, 5 haplotypes defined 96% of those present in our Amish sample (Table 3). None of the haplotypes showed significant association with T2DM or IGH (Table 3). Similar results were obtained when using 2-, 4-, or 5-polymorphism haplotypes (data not shown).

Since it is possible that the 5' UTR 23 bp I/D polymorphism could have effects on transcription, mRNA stability, or translational efficiency, it was genotyped in an expanded set of 681 nondiabetic Amish individuals.

There was no association between this polymorphism and levels of glucose or insulin either fasting or during the OGTT, nor was there association with HOMA-IR, BMI or fasting serum lipid levels (data not shown).

### Discussion

Genome wide linkage analysis in the Amish [6], as well as in populations from several other independent genome scans [7–12] provides compelling evidence for a T2DM susceptibility gene on chromosome 1q21-q24. Although several positional candidate genes have been investigated, to date, none have been demonstrated to contain sequence variation that contribute significant enough risk for T2DM to be considered the pathogenic variant. *VAMP* proteins play key roles in insulin-stimulated trafficking of glucose transporters from the intracellular pool to the cell surface and also play an important role in hormone secretion. Thus, *VAMP4*, which resides on chromosome 1q24-q25 represents an excellent positional candidate gene for T2DM.

Our sequence analysis revealed three polymorphisms in *VAMP4*. Since we sequenced 20 Amish individuals (40 alleles), we believe that all or most of the common sequence variation in the exons, exon–intron boundaries,

Table 3  
Allele frequencies of single SNPs and 3-polymorphism haplotypes in *VAMP4*

Single polymorphisms <sup>a</sup>	Frequency			<i>P</i> -value* (T2DM vs. NGT, IGH vs. NGT)	
	Major/Minor Allele	T2DM ( <i>n</i> = 65)	IGH ( <i>n</i> = 64)		NGT ( <i>n</i> = 129)
Rs978985	C/T	0.44	0.33	0.37	0.35, 0.54
Rs2032555	C/T	0.25	0.30	0.31	0.26, 0.46
23 bp I/D (pos 73245)	I/D	0.20	0.23	0.19	0.94, 0.44
C35296T	G/A	0.25	0.29	0.29	0.64, 0.94
Rs1023479	C/T	0.29	0.18	0.22	0.22, 0.44
Three-polymorphism haplotypes <sup>b</sup>					
T–I–A		0.48	0.47	0.40	
C–I–A		0.25	0.22	0.28	
T–D–G		0.18	0.18	0.15	
T–I–G		0.08	0.06	0.12	
C–I–G		0	0.07	0.05	

<sup>a</sup> Allele frequencies shown are those of the less common (minor) allele.

<sup>b</sup> Three-polymorphism haplotypes include rs978985, 23 bp I/D in the 5' UTR [exon 1], and C35296T in the 3' UTR [exon 8]; None of the haplotype frequencies differed significantly between groups.

\* *P*-values for single SNPs are those derived by the  $\chi^2$  test.

and immediate 5' flanking region of *VAMP4* present in this population should have been detected. None of these polymorphisms nor others that flank *VAMP4* were associated with T2DM. Since we did not sequence the entirety of *VAMP4* (e.g., complete introns or distal 5' or 3' regulatory regions), it is possible that functionally important sequence variants were missed. However, our haplotype analysis also failed to detect any association with T2DM. Since *VAMP4* lies in a region of moderate to strong linkage disequilibrium, any important sequence variant not directly detected or examined would have been expected to lie on a distinct haplotype, and if present, that haplotype should have been associated with T2DM. Thus, we conclude that sequence variation in *VAMP4* is unlikely to contribute to T2DM risk in the Amish.

It is possible that with only 64 and 65 T2DM and IGH cases, respectively, and 129 controls, that we lacked adequate power to detect association of *VAMP4* polymorphisms or haplotypes. However, we do not believe this to be the case since these are the same individuals who provided evidence for linkage to this region of chromosome 1q21-q24 and case control association analyses have superior power compared to linkage analysis to detect disease genes. Furthermore, since the Amish are a genetically homogeneous founder population, we do not believe that population stratification, which can sometimes result in either false positive or false negative associations, is relevant to this study population.

The most parsimonious explanation for multiple replicated linkages to this region of chromosome 1q21-q24 in diverse populations is that it is the same variant contributing to T2DM risk. This study would thus suggest that *VAMP4* may not be contributing significant T2DM risk in other populations as well. However, it is also possible that different allelic variants of *VAMP4* may exist in other populations. As such, we cannot rule out the possibility that sequence variation in *VAMP4* might contribute to T2DM risk in these other populations. Furthermore, it is also possible that different genes within the 1q21-q24 region (which contains over 450 genes) contribute T2DM risk among populations or there may even be more than one pathogenic gene in this region within a given population.

We conclude that sequence variation in *VAMP4* is unlikely to contribute to our observed linkage to T2DM on chromosome 1q21-q24 in the Amish. This conclusion is based upon the systematic search for sequence variation and the lack of association of *VAMP4* polymorphisms or haplotypes with T2DM or its related traits. Analysis of other positional candidate genes in the region and/or a systematic linkage disequilibrium mapping effort will be required to ultimately identify the T2DM susceptibility gene on 1q21-q24 and its pathogenic variant.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ymgme.2004.09.012](https://doi.org/10.1016/j.ymgme.2004.09.012)

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