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Variation in NPC1, the gene encoding Niemann–Pick C1, a protein involved in intracellular cholesterol transport, is associated with Alzheimer disease and/or aging in the Polish population

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ABSTRACT

There is abundant evidence that cholesterol metabolism, especially as mediated by the intercellular transporter APOE, is involved in the pathogenesis of sporadic, late-onset Alzheimer disease (SLAD). Identification of other genes involved in SLAD pathogenesis has been hampered since gene association studies, whether individual or genome-wide, experience difficulty in finding appropriate controls in as much as 25% or more of normal adults will develop SLAD. Using 152 centenarians as additional controls and 120 "regular", 65–75-year-old controls, we show an association of genetic variation in NPC1 with SLAD and/or aging. In this preliminary study, we find gradients of two non-synonymous SNP's allele frequencies in *NPC1* from centenarians through normal controls to SLAD in this non-stratified Polish population. An intervening intronic SNP is not in Hardy–Weinberg equilibria and differs between centenarians and controls/SLAD. Haplotypes frequencies determined by fastPHASE were somewhat different, and the predicted genotype frequencies were very different between the three groups. These findings can also be interpreted as indicating a role for NPC1 in aging, a role also suggested by NPC1's role in Dauer formation (hibernation, a longevity state) in *Caenorhabditis elegans*.

Alzheimer disease (AD) is the most common form of dementia in the elderly, yet it is frequently misdiagnosed, perhaps since 25% of elder-onset dementia is secondary to arteriosclerosis. Approximately 5% of adults between the ages of 65 and 75 years and 25% or more of those 85 and older will develop this form of dementia [3]. It is clear that Alzheimer's dementia has genetic components and a number of genes causing familial, dominantly inherited forms have been described. These include mutations in the amyloid precursor protein gene and presenilins 1 and 2, which have been found to be associated with inherited, early onset Alzheimer's dementia (reviewed in Ref. [33]). However, these dominant familial forms only account for approximately 3% of patients with AD, and most patients with so-called sporadic late-onset AD (SLAD) are non-familial.

There have been multiple lines of evidence that strongly implicate cholesterol metabolism and the occurrence of SLAD. These include the review of approximately 60,000 patient charts which correlated use of statins for at least 2 years with a 60–73% decrease in AD [47]. There are conflicting studies with evidence for statins slowing progression but not decreasing the incidence of AD and indicating different mechanisms of action than originally proposed (reviewed in Ref. [48]). It is also apparent that, in *in vitro* models of amyloid plaque formation, various alterations of cholesterol can alter amyloid beta accumulation [1,5]. Astrocytes are the major site of synthesis of cholesterol in the brain and secrete it via the ABCA1 transporter to make HDL-like particles [45]. However, apolipoproteins E and I, instead of mostly APOA peripherally, are the predominant recipients of cholesterol from this transporter. The most important receptor for this HDL-like cholesterol in neurons is the low-density, lipoprotein-related receptor protein, LRP

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[15]. Among genes that influence the incidence of SLAD, the most important, validated in many studies, is *APOE-4* of the extracellular cholesterol transport pathway [31]. Having a single *APOE-4* allele increases the risk threefold and homozygosity for *APOE-4* increases the risk 12-fold for SLAD.

Since the availability of cholesterol for extracellular transport depends on intracellular transport, we have tested whether alterations of intracellular cholesterol movement are also important in the causation of SLAD. Since glia secrete cholesterol complexed to apolipoprotein E-containing lipoproteins [24], their ability to do so is likely to be affected by intracellular cholesterol transport. The Niemann–Pick C1 protein has been shown to affect intracellular cholesterol transport (reviewed in Refs. [11,34,39]) and we have recently shown that astrocyte-only expression of Npc1 in *Npc1*$^{-/-}$ mice greatly delays neuronal degeneration [51]. Although *NPC1* variation has not been studied in SLAD [www.Alz.Gene.org], variation in the functionally-related *NPC2* has been studied (*Npc2*$^{-/-}$ is pathologically similar to *Npc1*$^{-/-}$ and the combined deficiency of Npc1 and Npc2 did not significantly alter disease onset in either of the single mouse models [38]). A strong association of SNPs in *NPC2* with SLAD was found in one population but not in several others [46]. We have studied associations of SNPs in NPC1 with SLAD and used an important additional control: centenarians, since the probability that they have avoided developing Alzheimer disease is high.

Polish populations were studied in accordance with the declaration of Helsinki, the local Ethics Committees approved of the studies, and informed consent was obtained from individuals or guardians before the investigation. For Centenarians, the preliminary data obtained from the interview, medical examination and blood tests show that about 30% of the 150 examined Polish subjects are in relatively good health, and are able to interact actively with their environment, care for themselves and can face everyday life events (more fully described in Ref. [26]). The randomly chosen, 72 control patients for the centenarians were 65 years old and consisted of 22% males, 78% females. Clinical diagnosis of probable AD was made for SLAD according to the NINCDS-ADRDA criteria [25]. Dementia and memory deficits in geographically matched control subjects ($n = 48$, average age 72.2 ± 7.4, 28.3% males, 71.7% females) were excluded by neuropsychological testing, consisting of the "Consortium to Establish a Registry for Alzheimer's Disease" (CERAD) neuropsychological test battery and the "mini mental status examination".

A total of 368 individuals were typed for three SNPs in this study: 96 Alzheimer affected subjects, 152 centenarians and 120 control individuals. For Niemann–Pick C SNP rs1805081, a non-synonymous coding polymorphism, genomic DNA was amplified using a standard PCR protocol as described in Ref. [44]. Amplification of the 316 bp region was carried out using the primers rs1805081_digestF 5′ ATG CTC CAA AAA ACA CAA GC 3′ and rs1805081_digestR 5′ CAG TGG GCT TTT CTT TGA GTT T 3′ in a reaction volume of 25 μL consisting of 30–50 ng DNA, 0.25 mM dNTPs, 0.25 μM sense and anti-sense primers, 2.5 μL 10X BIOLASE PCR buffer, 2.5 μL 25 mM magnesium and 1 U Taq DNA polymerase (BIOLASE). Thermocycling conditions consisted of an initial denaturation step of 95 °C for 3 min, followed by 32 cycles of 95 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s, with a final extension step of 72 °C for 6 min. PCR products were digested with 5 U *NcoI* restriction endonuclease (New England Biolabs). While *NcoI* does not cut the C allele, the T allele is cut to yield products of 213 bp and 103 bp and was visualized on 2.5% agarose gel stained with ethidium bromide.

For Niemann–Pick C SNP rs1631685, an intronic polymorphism, genomic DNA was amplified using a standard PCR protocol as described in Ref. [44]. Amplification of the 444 bp region was carried out using the primers rs1631685_digestF 5′ GTG TGA TAC ATG ACA CTG TGT TAG CGA C 3′ and rs1631685_digestR 5′ TTG TAT TTT CAG TAG AGA TGG GGT TTC G 3′ in a reaction volume of 25 μL consisting of 30–50 ng DNA, 0.25 mM dNTPs, 0.25 μM sense and anti-sense primers, 2.5 μL 10X BIOLASE PCR buffer, 2.5 μL 25 mM magnesium and 1 U Taq DNA polymerase (BIOLASE). Thermocycling conditions were as for rs1805081 except the annealing temperature was 58 °C. PCR products were digested with 10 U *NdeI* restriction endonuclease (New England Biolabs). While *NdeI* does not cut the C allele, the T allele is cut to yield products of 265 bp and 179 bp and was visualized on 2.5% agarose gel stained with ethidium bromide.

For Niemann–Pick C SNP rs1788799, a non-synonymous coding polymorphism, genomic DNA was amplified using the allele specific PCR protocol as described in Refs. [27,30], using the primers 1788799forG 5′ GAA GCC TGC GAC AGC TTT TC 3′ for allele G, 1788799forC 5′ GAA GCC TGC GAC AGC TTT TG 3′ for allele C, and 1788799rev 5′ ACT TCG TTC AGC AGT GAA GG 3′ as anti-sense for both. Amplification of the 171 bp region was carried out in a reaction volume of 25 μL consisting of 30–50 ng DNA, 0.2 mM dNTPs, 0.5 μM sense and anti-sense primers, 2.5 μL 10X BIOLASE PCR buffer, 2.5 μL 25 mM magnesium and 1 U Taq DNA polymerase (BIOLASE). Thermocycling conditions were at for rs1631685 but with 35 cycles. PCR products were run on a 1.5% agarose gel stained with ethidium bromide. This procedure was followed for both sets of allele specific primers.

There were two groups of controls ascertained separately for the centenarians and SLAD. They had a similar sex distribution but were somewhat older for the SLAD group. These were pooled since they were not different by χ^2-tests of allele frequencies ($p > 0.05$). The centenarians, controls, and SLAD patients were compared for allele frequencies of the three SNPs studied, Table 1. Rs18050810 (A: histidine, G: arginine at amino acid 215, a non-conserved amino acid; here studied as C, T on a non-coding strand) shows the highest frequency of the C allele in centenarians and lower values sequentially in controls and SLAD. The centenarians are significantly different from SLAD ($p = 0.007$). All three groups are in Hardy–Weinberg equilibria. Rs1631685 showed a lower frequency of the C allele in centenarians than in both controls and SLAD, which had comparable allele frequencies. The centenarian/SLAD difference was significant at $p = 0.026$. The allele frequencies were not at Hardy–Weinberg equilibria in controls (disequilibria only contributed to by the centenarian controls which had not been screened to rule out dementia) or SLAD. Rs1788799 (C: isoleucine, G: methionine at amino acid 642, a non-conserved amino acid) shows a decline in frequency of the C allele from centenarians through controls to SLAD. The centenarians are different from SLAD for allele frequencies ($p = 0.017$) but not different from controls. Rs1788799 was in Hardy–Weinberg equilibria in all three groups. These differences are unlikely to be due to stratification of the Polish population since they, like other Eastern Europeans, show little population substructure [43].

HapMap shows that there is strong linkage disequilibrium across the NPC1 gene [hapmap.org/cgi-perl/gbrowse/hapmap_B35/]. Haplotype analysis was performed using fastPHASE 1.0.1 [36]. While this program is one of the best for this purpose, it assumes Hardy–Weinberg equilibria and a few common haplotypes [32]. Thus, especially since the Hardy–Weinberg equilibria condition is not met for rs1631685, we consider the results more provisional than those from allele frequencies. All possible eight haplotypes were found of which three had minor frequencies of 1.6% or less. Of note, 15/21 of these rare haplotypes were found in the centenarians (X^2, p 0.001). The major haplotypes, accounting for 97.2% of the total, were analyzed in the three groups, Table 2. There was very little difference in the frequency of the three

Table 1
Statistical analysis of allele frequencies and Hardy–Weinberg equilibria for three SNPs in NPC1 between centenarians, controls and SLAD patients*.

SNP	Population	Allele 1	Allele 2	% Allele 1	Fit to Hardy–Weinberg	Chi-Square	
rs18050810							
	Centenarians	149 (C)	143 (T)	51.0	$p = 0.511$	0.074	0.007
	Controls	102 (C)	134 (T)	43.2	$p = 0.138$		
	SLAD	64 (C)	118 (T)	35.1	$p = 0.207$	0.095	
rs1631685							
	Centenarians	133 (C)	153 (T)	46.5	$p = 0.485$	0.006	0.026
	Controls	138 (C)	98 (T)	58.5	$p = 0.005$		
	SLAD	106 (C)	80 (T)	57.0	$p = 0.042$	0.759	
rs1788799							
	Centenarians	220 (C)	74 (G)	74.8	$p = 0.460$	0.146	0.017
	Controls	166 (C)	74 (G)	69.2	$p = 0.267$		
	SLAD	123 (C)	67 (G)	64.7	$p = 0.326$	0.331	

* For each SNP, and in each group, there were several individuals who could not be typed.

Table 2
Major NPCI haplotype frequencies predicted by fastPHASE 1.0.1 in centenarians, controls and SLAD.

	CCC	CTT	CCT	GCT	CTC
Centenarians	21(8.6%)	37(15.2%)	41(16.8%)	53(21.8%)	91(37.4%)
Controls	16(6.7%)	13(5.4%)	51(21.3%)	73(30.5%)	86(36%)
SLAD	7(3.6%)	20(10.5%)	37(19.3%)	65(34%)	62(32.4%)

most abundant predicted haplotypes in the three groups. However, centenarians, as they were for the three rarest genotypes, are different from controls for the frequency of the first four haplotypes ($p = 0.042$) and significantly so for the first two ($p = 0.016$).

Genotypes were then assigned to individuals using the predicted haplotypes. The frequencies of the most abundant genotypes (of 22 found from the possible 28) were analyzed in the three groups (Table 3). The frequency distributions were significantly different than those expected if there was only one population: Pearson's χ^2, $p \leq 0.001$.

There have been hundreds of genetic studies of SLAD, both for individual genes and genome-wide searches (updated at www.Alz.Gene.org). The Niemann–Pick C1 region on chromosome 18 has not shown a peak of association in these studies. In contrast, the Niemann–Pick C2 (NPC2) gene is located in a region of chromosome 14q with a genome-wide association linkage signal and variations in it were found to be significantly associated with SLAD in some European populations but not others [46]. Deficiency of NPC2 creates a nearly identical pathology to the deficiency of NPC1, although it alters lysosomal cholesterol trafficking while NPC1 affects a late endosomal pathway. The shared pathology has many features of SLAD with neurofibrillary tangles and altered tau [2,7]. It is now being recognized that SLAD, like NPC1, has endosomal abnormalities [28,42].

Table 3
Common NPCI genotype frequencies in centenarians, controls and SLAD.

Genotype	Group			
	Centenarians	Controls	SLAD	Total
CCC–CTC	6	15	4	25
CCT–CCT	8	7	6	21
CCT–CTC	20	23	9	52
CCT–CTT	6	2	1	9
CCT–GCT	8	13	15	36
CTC–CTC	24	10	10	44
CTC–CTT	14	2	12	28
CTC–GCT	22	24	16	62
CTT–GCT	6	7	5	18
GCT–GCT	4	14	14	32
Total	118	117	92	327

Our inclusion of centenarians as an "extreme" control supports the association of variation in NPC1 with SLAD and/or aging. As expected, with the paucity of SLAD, there is abundant evidence that centenarians have a lower frequency of APOE4 [23,35]. Although centenarians have mostly avoided SLAD, some demented centenarians meet criteria for the disease [37]. Even non-demented centenarians develop some neurofibrillary tangles, but it is in different brain regions than those areas first involved in SLAD [16]. Some amyloid deposits also develop in non-demented centenarians, e.g. in the visual cortex [20]. SLAD in the very old and centenarians shows distinctive distributions of plaques, tangles and nerve death [10,13,14]. Finally, dementia in centenarians is more frequently associated with alternative pathologies, such as argyrophilic grain disease [6].

Our findings of an enhanced difference in allele frequencies for *NPC1* between centenarians and SLAD as compared to controls and SLAD and the analysis of provisional predicted haplotypes showing a difference between centenarians and controls (while genotypes were different between the three groups) may indicate a role for *NPC1* variation in aging. It is of note that deficiency of the two homologues of *NPC1* (*npc-1* and *npc-2*) results in *Caenorhabditis elegans* inappropriately entering the Dauer, hibernation phase [40]. Hibernation may be thought of as enhancing longevity and mutations in the Dauer-formation pathways affect longevity in *C. elegans* [12,17,18,22,29] and mammals [19,41]. Allelic variation in one of these genes, *SIRT1*, was not found to be associated with human longevity [9]. While the *npc-1*, *npc-2* deficiency functions in a steroid hormonal pathway [21] and Npc1 deficiency does not appear to limit somatic steroid synthesis in mice [49], the link between the Dauer-formation pathway and longevity involves insulin-signaling and general metabolism [4,18,29] which NPC1 possibly influences through its effects on liver metabolism [8]. A goal of future work will be to determine if rs18050810, T allele; rs1631685, T allele and/or rs1788799, G allele are associated with decreased or increased NPC1 function, it is likely that it will be the former since aged NPC1 heterozygous mice (NPC1±) show neurodegeneration [50].

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