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(54) **PROCEDE PERMETTANT DE DETERMINER LA PROPENSION
A DEVELOPPER TARDIVEMENT LA MALADIE
D'ALZHEIMER**
(54) **METHOD FOR DETERMINING PROPENSITY FOR
DEVELOPING LATE-ONSET ALZHEIMER'S DISEASE**

(57) L'invention concerne un procédé permettant de déterminer la propension ou la prédisposition d'un sujet humain à développer tardivement la maladie d'Alzheimer. Ce procédé consiste à déterminer la présence chez ce sujet humain d'un gène 1) d'une variante k de la butyrylcholinestérase, et d'un gène 2) de l'apolipoprotéine E4, la présence du gène 1) ou des gènes 1) et 2) susmentionnés indiquant la propension ou la prédisposition à développer tardivement la maladie d'Alzheimer.

(57) A method is provided for determining the propensity or predisposition of a human subject to develop late-onset Alzheimer's disease by the presence in a human subject of a gene (1) for butyrylcholinesterase K variant, and a gene (2) for apolipoprotein E4, the presence of the gene (1) or the presence of both of the above genes (1) and (2) indicating a propensity or predisposition to develop late-onset Alzheimer's disease.

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(54) Title: METHOD FOR DETERMINING PROPENSITY FOR DEVELOPING LATE-ONSET ALZHEIMER'S DISEASE		
(57) Abstract A method is provided for determining the propensity or predisposition of a human subject to develop late-onset Alzheimer's disease by the presence in a human subject of a gene (1) for butyrylcholinesterase K variant, and a gene (2) for apolipoprotein E4, the presence of the gene (1) or the presence of both of the above genes (1) and (2) indicating a propensity or predisposition to develop late-onset Alzheimer's disease.		

METHOD FOR DETERMINING PROPENSITY FOR
DEVELOPING LATE-ONSET ALZHEIMER'S DISEASE

Field of the Invention

5 The present invention relates to a method for
determining the propensity or predisposition of a human
subject to develop late-onset Alzheimer's disease through
the presence of a gene for butyryl-cholinesterase K variant
or the presence of genes for both butyrylcholinesterase K
10 variant and apolipoprotein E4.

Background of the Invention

 Alzheimer's disease is a progressive
neurodegenerative disorder which causes irreversible damage
15 to brain cells leading to dementia and ultimately death.
It is characterized by formation of amyloid plaques and
neurofibrillary tangles in the brain. Currently, it is
primarily diagnosed by exclusion of other known causes of
dementia. Diagnosis at an early stage prior to
20 irreversible changes is practically non-existent.

 In order for therapeutic intervention to be
significantly effective in treating Alzheimer's disease, it
will have to be administered very early on prior to such
irreversible changes. Accordingly, a biological marker
25 which could flag patients who have a propensity or
predisposition to developing Alzheimer's disease and thus
could be a guide to early diagnosis would be a most-
welcomed addition to the diagnostician's armamentarium.

 Butyrylcholinesterase (BChE) (located on chromosome
30 3) is expressed in most human tissues (1), yet its function
is unknown. BChE activity in the brain increases with age
over 60 years and is elevated in Alzheimer's disease (AD)
(2,3). Histochemically reactive BChE is associated with
amyloid plaques and neurofibrillary tangles and with
35 amyloid angiopathy in AD (4-8).

 A large number of human genetic variants of BChE are
known, two of the more common variants being the atypical

allele (referred to as the A variant) and the K variant (BCHE-K). The A variant has an Asp70Gly mutation and is rare (0.5% allelic frequency), while the K variant has a point mutation at nucleotide 1615 (GCA→ACA) which changes
5 alanine 539 to threonine and the catalytic activity is reduced by a third (9). BCHE-K is thought to have an allelic frequency of around 12% in Caucasians (9-11).

Until now, there has not been any known connection or association of AD with either the A variant or the K
10 variant.

That the gene for apolipoprotein E4 (APOE ε4 allele) is a risk factor for AD is well established (12, 13). In fact, the likelihood of a carrier of APOE ε4 allele developing AD is 7-fold greater than a control who does not
15 carry such gene.

Description of the Invention

It has now been found that the gene for butyrylcholinesterase K-variant is about twice as common in
20 patients with late-onset AD as compared to controls (without taking into account the presence of the APOE ε4 gene), while the A variant had little or no connection with AD. Thus, a human subject carrying the gene for BCHE-K has a propensity or predisposition to develop late-onset AD
25 which is about twice that of a human who does not carry such gene.

Furthermore, surprisingly and unexpectedly, it has been found that the presence of both the genes for BCHE-K and for APOE ε4 is about 30 times as common in patients
30 with late-onset AD as compared to controls. Thus, there is an unexpected synergy between the genes for BCHE-K and APOE ε4 in late-onset confirmed AD.

In accordance with the invention, a method is provided for determining the propensity or predisposition
35 of a human subject to develop late-onset Alzheimer's disease (AD), which includes the step of detecting the presence or absence of a gene for butyrylcholinesterase K

variant in a human subject. The presence of the gene for butyryl-cholinesterase K variant indicates an increased propensity or predisposition of the human subject to develop late-onset Alzheimer's disease as compared to a human control who does not carry the gene.

In fact, it has been found that a human subject carrying the gene for butyrylcholinesterase K variant (BCHE-K) (or a nearby gene on chromosome 3) has at least a 2-fold increase in risk or predisposition to develop late-onset Alzheimer's disease than a human control who does not carry the gene for BCHE-K (without regard to the presence of the apolipoprotein E4 gene).

In addition, in accordance with the present invention, a method is provided for determining the propensity or predisposition of a human subject to develop late-onset Alzheimer's disease, which includes the steps of detecting the presence or absence in a human subject of a gene for butyrylcholinesterase K variant, and detecting the presence or absence in the same human subject of a gene for apolipoprotein E4, the presence of both the genes for butyrylcholinesterase K variant and apolipoprotein E4 indicating an increased propensity, predisposition or susceptibility to develop late-onset Alzheimer's disease as compared to a human control who carries neither of these 2 genes or only one of the genes.

Surprisingly and unexpectedly it has been found that a synergy exists between the genes for butyrylcholinesterase K variant and apolipoprotein E4 in late-onset confirmed Alzheimer's disease. Thus, a human subject carrying both of the genes will have at least a 30-fold increase in propensity, predisposition or susceptibility to develop late-onset Alzheimer's disease as compared to a human control who does not carry either of the genes.

In addition, a human subject carrying both of the genes will have at least a 7-fold increase in propensity, predisposition or susceptibility to develop late-onset

Alzheimer's disease as compared to a human control who carries only the gene for apolipoprotein E4.

The term "late-onset Alzheimer's disease" as employed herein refers to the onset of Alzheimer's disease (the patient displays recognized clinical symptoms
5 indicating Alzheimer's disease) after the age 65.

The terms "apolipoprotein E4" and "APOE ε4" are used interchangeably to refer to the ε4 allele of apolipoprotein E.

10 The terms "butyrylcholinesterase K variant" and "BCHE-K" are used interchangeably to refer to the gene for the K variant of butyrylcholinesterase located at 3q26.1-q26.2 which serves as a predictor or marker for Alzheimer' disease by itself or in conjunction with the gene for
15 apolipoprotein E4. BCHE-K may or may not be implicated in a causal relation to the development of late-onset Alzheimer's disease, and which may, in fact, involve a gene closely located to BCHE-K on chromosome 3, for example, the gene for transferrin at 3q21-q25.

20 The terms "propensity", "predisposition" or "susceptibility" are used interchangeably with reference to developing Alzheimer's disease.

In carrying out the methods of the invention, a biological source of DNA, such as a blood sample, is taken
25 from human subjects and controls and PCR (polymerase chain reaction) methods used to detect the presence of the genes for (1) butyrylcholinesterase K variant (using the method of Jensen, F.S. et al (1996), Detection of the plasma K variant by PCR using an amplification-created restriction
30 site, Hum. Hered. 46, 26-31), and (2) apolipoprotein E4 (using the method of Wenham, P.R. et al Apolipoprotein E genotyping by one-stage PCR, Lancet 337, 1158-1159).

The biological source of DNA which may be used to detect the presence of the above genes includes any
35 suitable cells readily available through scrapings and the like and those available through biopsy, such as blood cells (including platelets), skin cells, oral mucosal

cells, nasal mucosal cells, muscle cells, bone cells, or neurons, with blood cells being preferred.

Example

5 The following clinical tests were carried out to determine if there is synergy between the genes for butyrylcholinesterase K variant and apolipoprotein E4 in late-onset confirmed Alzheimer's disease.

10 Patients and Methods

 282 Caucasians from the Oxford region of England recruited into the Oxford Project to Investigate Memory and Aging (OPTIMA) (38) were examined. 88 were autopsy confirmed CERAD (39) 'definite or probable' AD cases (74
15 late-, i.e. over 65y, and 14 early-onset), 29 were pathologically confirmed cases of other dementias (12 vascular, 5 Parkinson's, 5 non-specific neurodegeneration, 3 Pick's, 1 each of progressive supranuclear palsy, normal pressure hydrocephalus, Huntington's, glioma), 61 were
20 living cases diagnosed 'possible AD' or 'probable AD' by the National Institutes of Neurology and Communicative Disorders - Alzheimer's disease and Related Disorders Association Work Group (NINCDS/ADRDA) (40) criteria (31
late- and 30 early-onset) and 104 were controls without
25 cognitive impairment with CAMDEX (41) scores >80 (14 had died and had no AD pathology on necropsy). Subjects were genotyped blind to diagnosis, using blood samples, by PCR methods for the atypical and K variants of BCHE (42) and for APOE (43).

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Results

 After 189 subjects had been genotyped, three control subjects out of 115 and three AD cases out of 74 were identified as heterozygotes for the atypical BCHE allele.
35 Thus, no association was found between histopathologically confirmed AD and the atypical allele of BCHE. Two out of the six atypical BCHE heterozygotes were carriers of

BCHE-K.

Altogether 282 Caucasian subjects were genotyped for BCHE-K and for APOE alleles. BCHE-K was more common in late-onset AD cases than in early-onset AD cases, other
5 dementias or controls (Table 1). For subjects aged over 65, the allelic frequency of BCHE-K was 0.09 in 104 controls and 0.17 in 74 confirmed AD cases, giving an odds ratio of AD for BCHE-K of 2.15 (Table 2) (without taking
10 account whether the subjects were carriers of APOE ϵ 4 gene). Most of this association appeared to be due to subjects over 75y old, for whom the frequency of BCHE-K was 0.08 in 68 controls and 0.19 in 36 confirmed AD cases (X^2 with Yates' correction = 4.7; $p = 0.03$), giving an odds ratio of AD of 2.7 (Table 2). Although there were
15 insufficient cases for this trend with age to reach significance, the data for the >75y subgroup as well as for all subjects >65y are shown in Tables 2-5.

Taking account of an individual's carrier status for APOE ϵ 4 produced striking results. In subjects >65y
20 without APOE ϵ 4, the allelic frequency of BCHE-K was 0.14 in 22 confirmed AD cases and 0.11 in 72 controls. There was thus no association of BCHE-K with late-onset AD in subjects lacking an APOE ϵ 4 allele. In APOE ϵ 4 carriers, however, BCHE-K allelic frequencies in subjects aged >65y
25 were 0.18 in 52 confirmed AD cases and only 0.03 in 32 controls; in subject aged >75y the frequencies were 0.23 in 24 confirmed AD cases and 0.02 in 22 controls. These results gave odds ratios of confirmed AD of 6.9 and 12.8 for BCHE-K in APOE ϵ 4 carriers aged >65y and >75y,
30 respectively (Table 2). For comparison, Table 3 shows the odds ratios of confirmed AD for APOE ϵ 4 in BCHE-K carriers. It can be seen that an individual's BCHE-K status markedly influences the strength of the effect of APOE ϵ 4. Table 4 shows the striking differences between cases and controls
35 in the proportions of subjects who carry both BCHE-K AND APOE ϵ 4 alleles.

All these findings indicated an interaction between BCHE-K and APOE ε4, which is confirmed by logistic regression analysis. Several logistic regression models were fitted to the data and it was found that a parsimonious model with only APOE ε4, BCHE-K and their interaction gave an adequate fit (residual deviance: 5.5 on 4 degrees of freedom). In this model, APOE ε4 and its interaction with BCHE-K were significantly associated with AD, but BCHE-K by itself was not.

In order to examine further the strength of the interaction between BCHE-K and APOE ε4, Table 5 shows the observed odds ratios of confirmed AD for subjects with different combinations of these two genes, taking subjects who have neither gene as the reference. In subjects over 65y, the odds ratio in those who only carry BCHE-K was 1.1 and in those with only APOE ε4 it was 3.8. This gave a predicted odds ratio of 4.2 in subjects with both genes, on the assumption of independent effects of the two genes, compared with the observed value of 30.2. In subjects over 75y the predicted odds ratio was 1.7, but the observed odds ratio was 36. Thus, there appears to be a strong synergy between these two alleles in late-onset AD.

It was also found that in 108 subjects (>65y) with dementia all with autopsy diagnosis, 71 subjects with confirmed AD, the presence of APOE ε4 (without BCHE-K) gave 67% sensitivity and 45% false-positives for AD; the presence of BCHE-K (without APOE ε4) gave 32% sensitivity and 16% false-positives for AD; and the presence of both BCHE-K and APOE ε4 gave 25% sensitivity and 3% false-positives for AD. The NINCDS criteria gave 94% sensitivity, but 43% false positives, while the NINCDS criteria and both alleles (BCHE-K and APOE ε4) gave 47% sensitivity and 5% false-positives for AD.

Thus, it is seen that adding BCHE-K as a risk factor to APOE ε4 allele greatly decreases the false-positive rate in diagnosis of AD.

Discussion

That APOE ϵ 4 is a risk factor for AD is well established (12, 13). The results shown herein are consistent with the hypothesis that BCHE-K is associated with and is a marker or predictor for further increase in the risk of late-onset AD in APOE ϵ 4 carriers. It is theorized that the effect could be due to linkage disequilibrium with another gene on chromosome 3, conceivably transferrin, whose C2 variant was reported to be more common in 20 clinically diagnosed AD cases than in the general population (14). BCHE is at 3q26.1-q26.2 (15), while the gene for transferrin is at 3q21-q25 (16). On the other hand, the effect on late-onset AD appears to be mediated by BCHE-K itself.

15

Biological plausibility

There are various potential interactions between BChE and apolipoprotein E (apoE). Both proteins are produced in quantity in the liver and secreted into the circulation (1, 17). Not only apoE, but probably also BChE, interact with lipoproteins (18, 19). Both proteins are found in astrocytes (20, 21) and in other glia, BChE in oligodendrocytes (22) and apoE in microglia (23). Both proteins occur in AD in plaques, intra- and extracellular neurofibrillary tangles and in association with amyloid angiopathy (7, 8, 24, 25). Furthermore, both BChE and apoE have been implicated in certain models of neurite growth (26-31). In the case of apoE, there are differences between E3 and E4 isoforms (29-31) and, in the case of BChE, it has been suggested that the nature of the attached sugar residues may be important (26-28). It should be noted that the K variant of BChE has an additional threonine residue at position 539, but whether this is a potential O-glycosylation site (or, indeed, a phosphorylation site) remains to be shown. Furthermore, a threonine residue has a high propensity for β -sheet formation (32). In lysozyme, conversion of an isoleucine

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residue to threonine causes amyloidosis (33), as does conversion of an alanine residue to threonine in transthyretin (34).

5 Implications for clinical genetics

Approximately 6% of the Caucasian population carry genes for both BCHE-K and APOE ε4, assuming respective allelic frequencies of 0.12 (9-11) and 0.15 (17, 35), while 56% of such population carry neither gene. Thus, several
10 millions of the elderly who carry both these genes are at high risk of developing AD, especially when aged over 75y. The ability to identify such at-risk individuals could serve as a means for targeting subjects for early testing for AD before irreversible damage and possibly for early
15 chemotherapeutic intervention.

Table 1. Allelic frequencies of BCHE-K and APOE ε4

	Number of Subjects	F:M ratio	Mean age#	BCHE-K allele frequency	APOE ε4 allele frequency
Control >65y	104	1.26	78.1	0.09	0.16
All LOAD cases	105	1.50	80.9	0.16*	0.40**
All EOAD cases	44	1.10	65.9	0.09	0.42**
Confirmed LOAD	74	1.39	81.4	0.17*	0.41**
Confirmed EOAD	14	2.50	67.8	0.07	0.54**
Confirmed other dementia	29	0.81	76.8	0.10	0.19

LOAD and EOAD are respectively late-onset (>65y) and early-onset Alzheimer's disease. #Ages were at death, if status confirmed, but at last clinical assessment for living subjects. Each of the above groups was in exact Hardy Weinberg equilibrium. *p < 0.03, **p < 0.0001 (X² with Yates' correction) v. controls.

Table 2. Odds ratios of confirmed late-onset Alzheimer's disease for BCHE-K alleles

5

Subjects	Controls	Cases	Odds ratio#	95% C.I.
All>65y	104	74	2.15	1.1-4.25
All>75y	68	36	2.7	1.1-6.8
ApoE ε4 carriers>65y	32	52	6.9	1.65-29
ApoE ε4 carriers>75y	22	24	12.8	1.9-86

10 *Ages in Tables 2-5 were at onset for late-onset AD cases and for controls at death, if confirmed, but at last clinical assessment if living. #These odds ratios were based on alleles. The equivalent odds ratios for BCHE-K based on carriers were: 2.3, 2.6, 7.9, 15.0.

Table 3. Odds ratios of confirmed late-onset Alzheimer's disease for APOE ε4 alleles

15

Subjects	Controls	Cases	Odds ratio	95% C.I.
All>65y	104	74	3.6	2.2-5.9
All>75y	68	36	2.8	1.4-5.5
BCHE-K carriers>65y	17	23	12.3	2.6-58
BCHE-K carriers>75y	11	12	15.0	1.95-115

20

Table 4. Proportions of controls and confirmed late-onset AD cases with both BCHE-K and APOE ε4 alleles

Subjects	Proportions with both alleles		
	Controls	Cases	p*
All>65y	2/104 (2%)	18/74 (24%)	<0.0001
All>75y	1/68 (1%)	10/36 (28%)	<0.0001
APOE ε4 carriers, >65y	2/32 (6%)	18/52 (35%)	0.007
APOE ε4 carriers, >75y	1/22 (5%)	10/24 (42%)	0.009

* χ^2 with Yates' correction.

10 **Table 5.** Odds ratios of confirmed late-onset AD, taking subjects who had neither APOE ε4 nor BCHE-K as the reference

APOE ε4	BCHE-K	Controls	Cases	Odds ratio	95% C.I.
Age>65y					
-	-	57	17	Reference	
-	+	15	5	1.1	NS
+	-	30	34	3.8	1.8-8.1
+	+	2	18	30.2	8.4-108
Age>75y					
-	-	36	10	Reference	
-	+	10	2	0.7	NS
+	-	21	14	2.4	0.8-7.3
+	+	1	10	36.0	6.1-211

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What is Claimed is:

1. A method for determining the propensity or predisposition of a human subject to develop late-onset Alzheimer's disease, which comprises detecting the presence or absence of a gene for butyrylcholinesterase K variant in a human subject, the presence of the gene for butyrylcholinesterase K variant indicating an increased propensity or predisposition of the human subject to develop late-onset Alzheimer's disease as compared to a human subject who does not carry said gene.
2. The method as defined in Claim 1 wherein a human subject carrying the gene for butyryl-cholinesterase K variant has a propensity or predisposition to develop late-onset Alzheimer's disease at least about twice that of a human control who does not carry said gene.
3. A method for determining the propensity or predisposition of a human subject to develop late-onset Alzheimer's disease, which comprises detecting the presence or absence in the human subject of a gene for butyrylcholinesterase K variant, and detecting the presence or absence in the human subject of a gene for apolipoprotein E4, the presence of both the genes for butyryl-cholinesterase K variant and apolipoprotein E4 indicating an increased propensity or predisposition to develop late-onset Alzheimer's disease as compared to a human subject who carries neither of said genes or only one of said genes.
4. The method as defined in Claim 3 wherein the human subject carrying both the genes for butyrylcholinesterase K variant and apolipoprotein E4 has a propensity or predisposition to develop late-onset Alzheimer's disease at least about 30 times greater than that of a human control who does not carry either of said genes.
5. The method as defined in Claim 3 wherein a human subject carrying both the genes for butyrylcholinesterase K variant and apolipoprotein E4 has a propensity or

predisposition to develop late-onset Alzheimer's disease at least about 7 times greater than that of a human control, who carries the gene for apolipoprotein E4, but not the gene for butyrylcholinesterase K variant.

5 6. The method as defined in Claim 3 wherein the false-positive rate in diagnoses of Alzheimer's disease where both of said genes are carried by the subject is greatly decreased as compared to false-positive rate where only one of said genes is carried by the subject.

10 7. The method as defined in Claim 6 wherein the false-positive rate in diagnoses of Alzheimer's disease where both of said genes is carried by the subject is about 5% or less.

15 8. The method as defined in Claim 3 including the steps of obtaining biological sample of DNA from the subject and subjecting the sample to analytical methods to determine the presence or absence of either or both of said genes.

20 9. The method as defined in Claim 8 wherein the biological sample is a sample of blood cells, skin cells, cells of the oral mucosa or nasal passage, muscle cells, bone cells or neurons.

 10. The method as defined in Claim 9 wherein the cell sample is a sample of blood cells.

25 11. The method as defined in Claim 10 wherein the blood cells are subjected to PCR methods to determine the presence or absence of either or both of said genes.