

Therefore, there is the need of providing means for an accurate identification of a patient suffering from dementia of Lewy bodies, and distinguishing from Alzheimer disease, to be used in the common clinical practice.

5 SUMMARY OF THE INVENTION

The inventors have found specific alterations in BChE gene which allow determining whether a patient suffers from dementia with Lewy bodies, and distinguishing it from Alzheimer disease.

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There are documents in the state of the art that intend to find an association between BChE K variant and DLB, but as explained below, recent studies consider that there is no significant association (cf. W. Maetzler et al., *supra*). Surprisingly, the inventors of the present invention have found that specific
15 information for diagnosis of DLB is obtained with genotype of K variant in co-occurrence with the genotype of a so far unknown alteration in BChE gene, a poly-thymine region in positions 4780 to 4786. Determining these two genotypes is therefore useful to distinguish DLB from AD, these two genotypes constituting a specific genetic marker for DLB.

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The inventors have further observed that a combination of genotypes gives rise to identify a group of patients suffering from DLB, and distinguishing from AD. This combination is formed by the genotypes of the polymorphic sites at positions 3687, 4206, and 4443, the polythymine region at positions 4780 to
25 4786 in NCBI Accession Number NG_009031 (i.e. positions 3687, 4206, 4443 and 4780-4786 respectively in SEQ ID NO: 1), and the polymorphic site at position 68974 in NCBI Accession Number NG_009031 (i.e. position 934 in SEQ ID NO: 28).

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Positions of the alterations in BChE nucleotide sequence are given from the nucleotide sequence of NCBI Accession Number NG_009031 which corresponds to the promoter and the gene. This sequence was published on 31 January 2010.

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The polymorphic sites 3687, 4206, 4443 and the polythymine region at positions 4780 to 4786 are in the promoter region. For these sites, reference is made also to the SEQ ID NO: 1, which corresponds to the sequence from

nucleotide 1 to nucleotide 5040 of the complete sequence of BChE at NCBI. A possible numbering of the nucleotides sometimes used takes the transcription start as position 1 and consequently, the nucleotides upstream this position as negative positions. Transcription start position 1 corresponds to position 5001
5 in NG_0090031. The correspondence between the numbering used in this description and the "negative" one, is given herein:

A3687G corresponds to A-1314G
A4206G corresponds to A-795G
10 C4443T corresponds to C-558T
polythymine from 4780 to 4786 corresponds to -215 to -221

The polymorphic site at position 68974 is in the codifying region of NG_009031. The region from position 68041 to 7020 of NG_009031 is
15 included as SEQ ID NO: 28. Taking this region alone, the nucleotides are renumbered, so consequently, the position 68974 in the complete gene sequence becomes the position 934 in SEQ ID NO: 28. This polymorphism is associated to the change of amino acid in exon 4 of BChE resulting in the K variant. The position also used in the literature for this polymorphism is 1615
20 due to a different sequence numbering (with reference to the mRNA sequence which codifies for the mature BChE protein, without the signal peptide).

As described in the examples below, no specific association has been found between each of the five alterations in BChE gene independently evaluated
25 and DLB; but surprisingly, these alterations in combination give specific information for DLB.

In a first aspect, there is provided an *in vitro* method for the diagnosis of dementia with Lewy bodies comprising determining in a biological sample from a subject, the genotype of the following alterations in butyrylcholinesterase (BChE) gene: the polymorphic site at position 68974 in NCBI Accession Number NG_009031 (i.e. position 934 in SEQ ID NO: 28); and the poly-thymine region at positions 4780 to 4786 in NCBI Accession Number NG_009031 (i.e. positions 4780-4786 in SEQ ID NO: 1).

In a second aspect, there is provided a kit for carrying out the method as defined above which comprises the primers with sequences: SEQ ID NO: 17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, and SEQ ID NO:21.

In a third aspect, there is provided a kit for carrying out the method as defined above, which comprises the primers with sequences: SEQ ID NO: 8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, and SEQ ID NO:21

In a fourth aspect, there is provided the use of a kit as defined above, for the diagnosis of dementia with Lewy bodies.

In a fifth aspect, there is provided the use of the polymorphic site at position 68974 in NCBI Accession Number NG_009031 (i.e. position 934 in SEQ ID NO: 28), in combination with one or more alterations in BChE gene selected from the group consisting of the poly-thymine region at positions 4780 to 4786, the polymorphic site at position 3687; the polymorphic site at position 4206; and the polymorphic site at position 4443, as marker for the diagnosis of dementia with Lewy bodies, said positions with reference to NCBI Accession Number NG_009031 (i.e. positions 3687, 4206, 4443 and 4780-4786 respectively in SEQ ID NO: 1).

Accordingly, an aspect embodiment of the ~~invention~~ disclosure provides an *in vitro* method for the diagnosis of DLB comprising determining in a biological sample from a subject, the genotype of the following alterations in butyrylcholinesterase (BChE) gene, or of polymorphisms in linkage disequilibrium thereof: the polymorphic site at position 68974 and the poly-thymine region at positions 4780 to 4786.

In a particular embodiment, the method further comprises determining the genotype of the following alterations in BChE gene, or of polymorphisms in

linkage disequilibrium thereof: the polymorphic sites at position 3687, 4206, and 4443.

As it is shown in the examples below, post mortem samples of AD (n=26),
5 pure DLB (n=12), common DLB (n=24) and controls (n=23) were analyzed, as
well as clinically diagnosed samples obtained from 223 AD and 160 control
subjects. As a result, three types of genetic markers are described. First, the
co-occurrence of specific changes at two polymorphic sites is characterized by
10 the presence of one allele with 7 thymines at positions 4780 to 4786 and a
guanine for one allele and an adenine for the other allele at position 68974.
This co-occurrence is a specific marker for the differential diagnosis of DLB
versus AD. About 50% of DLB patients with a possible mis-diagnosis could be
rescued by the use of this marker.

15 Another embodiment of the ~~invention~~disclosure relates to a second genetic marker
which is a genotype combination, AAAGCC8+K+. It is constituted by the
specific genotypes of the polymorphic sites at positions 3687 (both alleles
contain an adenine at this position), 4206 (one allele contains an adenine and
the other a guanine), 4443 (both alleles contain a cytosine), 4780 to 4786 (at
20 least one of the two alleles is constituted by 8 thymines) and 68974 (at least
one of the two alleles contains an adenine). The determination of this
genotype combination in demented patients serves as differential diagnostic
marker providing the clinical diagnosis of DLB, but it may also serve as early
diagnostic marker for DLB in asymptomatic individuals.

25 Another embodiment of the ~~invention~~disclosure relates to a third genetic marker which is
a genotype combination, AAAAC+77KW. It is constituted by the specific
genotypes of the polymorphic sites at position 3687 (both alleles contain
adenine at this position), 4206 (both alleles contain adenine at this position),
30 4443 (at least one of the two alleles contains a cytosine at this position), 4780
to 4786 (both alleles are constituted by 7 thymines) and 68974 (one allele
contains an adenine and the other guanine). The determination of this
genotype combination in demented patients serves as differential diagnostic
marker providing the clinical diagnosis of DLB, but it may also serve as early
35 diagnostic marker for DLB in asymptomatic individuals.

Advantageously, within the great heterogeneity of DLB and according to the examples, the method of the invention allows to differentially detect the 45-60% of DLB cases, which otherwise would be diagnosed as AD. This percentage of patients, difficult to diagnose in the clinical practice, will receive the correct diagnostic from the beginning of the disease. The specificity for the disease is of 99%. This represents the first specific marker for DLB.

Until now, the available tools in the state of the art did not allow the specific identification of DLB in the clinical practice. In this way, when the subject was diagnosed of AD, he was submitted to therapy with neuroleptics, which is the most adequate treatment for psychotic symptoms in AD but more than 50% of DLB patients exhibit an adverse reaction to this kind of treatment causing death in many cases. The method of the ~~invention~~disclosure is of importance because it will enable the medical community to apply adequate treatment to patients suffering from DLB without the risks of an incorrect therapy. Therefore, applying the method of the ~~invention~~disclosure, diagnostic specificity for DLB is increased as well as deaths caused by adverse effects of treatment with neuroleptics will be reduced.

Furthermore, as the method of the ~~invention~~disclosure allows to specifically diagnosing patients with DLB, is it possible to have a defined group of patients to be included in a clinical trial.

By "diagnosis" in medicine it is meant the act or process of recognition of a disease or condition by its outward signs, symptoms, and underlying physiological/biochemical cause(s).

In the sense of this description, "alteration" in the BChE gene means any structural change in the nucleotide sequence considered as wild-type. Examples of alterations can include a single nucleotide polymorphism, a deletion, an insertion, a substitution or a duplication of one or more nucleotides, and a chemical modification on a nucleotide (e.g. methylation).

By "determining the genotype" in this description it is meant identifying the nucleotide in a given position.

In this description "a given nucleotide in one allele" means that the subject is heterozygote for that nucleotide in that gene, and "in both alleles", which is homozygote for that nucleotide.

5 | According to the invention disclosure, the method includes determining the alterations indicated on BChE gene, but also determining polymorphisms in linkage disequilibrium with said alterations which would give the same information. In population genetics, linkage disequilibrium is the non-random association of alleles at two or more loci, not necessarily on the same chromosome.

10 | In accordance with the diagnostic method of the present invention disclosure, the analysis of DLB would be as follows: a patient with suspected onset of dementia and/or with a non-definitive clinical-familial evaluation would be diagnosed by a genetic test determining the alterations of the BChE gene
15 | described above. In the case of detecting the DLB specific genotypes, no additional tests or trial will be needed to diagnose correctly DLB. The direct application of genotyping represents an important save of money in the daily clinical practice.

20 | The method of the invention disclosure is useful in the following suspected diagnosis: probable AD vs possible DLB; possible AD vs probable DLB; possible AD vs possible DLB; probable AD vs probable DLB; probable AD vs possible AD; possible DLB; and probable DLB. Physicians diagnose possible AD based on a full patient interview, covering personal and family medical history,
25 | combined with the outcome of any neurological, psychiatric, and lab tests conducted. Doctors are likely to expect AD when patient complains of a gradual progression of memory weakening, and when they are unable to find any other condition that could explain the memory loss. Doctors will be looking for disorders such as depression or hypothyroidism, neurological damage
30 | caused by stroke, or any medications that may be contributing to the loss of memory. An inability to uncover any contributory illness leads to the determination that AD is possible. Probable AD is a next step beyond possible Alzheimer's and means that a doctor is "relatively certain" that a patient has the disease.

35 | Advantageously, the method of the invention disclosure allows a diagnosis of DLB without the need of obtaining samples by aggressive methods like a biopsy;

and in this case a brain tissue microbiopsy. The method of the ~~invention~~disclosure, being a genetic test, is performed on any biological sample removed from the subject, as blood, since it is applicable to any cell type of the body.

5 In another embodiment, the determination of the genotype is carried out by one of the techniques selected from the group consisting of primer-specific PCR multiplex followed by detection, multiplex allele specific primer extension, a microarray-based method, and dynamic allele-specific hybridization. In a particular embodiment, it is carried out by primer-specific PCR multiplex
10 followed by detection.

The polymerase chain reaction (PCR) is the most widely used method for the *in vitro* amplification of nucleic acids. The PCR can be a real-time PCR, wherein the detection by labeled probes of the presence of the target
15 genotypes is almost instantaneous to the amplification.

The amplification of the target polymorphisms can be performed by primer-specific PCR multiplex with following detection by polyacrylamide electrophoresis or by analysis with a genetic analyzer. Alternatively, various
20 PCR reactions can be performed followed by agarose gel electrophoresis or by sequencing.

Determination of the genotype can be performed by Allele Specific Primer Extension (ASPE). This is a sequence specific enzymatic reaction technology
25 that can be used to assay multiple SNPs in a single tube. The ASPE method involves two phases, an enzymatic reaction that determines the target genotype followed by a capture on solid microsphere surface for detection. Taking advantage of the solution phase kinetics, this technique allows sequence labeled microspheres to be used for detecting new templates. This
30 is done with the help of an appropriate capture sequence attached to the allele specific oligonucleotide.

Optionally, detection may be carried out by DNA biochips/microarrays made with oligonucleotides deposited by any mechanism, by DNA biochips made,
35 with oligonucleotides synthesized *in situ* by photolithography or any other mechanism. A microarray-based method that allow multiplex SNP genotyping in total human genomic DNA without the need for target amplification or

complexity reduction can also be used for the genotyping of the BChE alterations. This direct SNP genotyping methodology requires no enzymes and relies on the high sensitivity of the gold nanoparticle probes. Specificity is derived from two sequential oligonucleotide hybridizations to the target by allele-specific surface-immobilized capture probes and gene-specific oligonucleotide-functionalized gold nanoparticle probes. The assay format is simple, rapid and robust pointing to its suitability for multiplex SNP profiling at the 'point of care'.

Furthermore, determination of the genotype can be performed by dynamic allele-specific hybridization (DASH), which represents the basis for throughput SNP genotyping in some laboratories. The core reaction principal of DASH is real-time (dynamic) tracking of allele-specific differences in the process of DNA denaturation. To achieve this, an oligonucleotide probe is first hybridized to the target DNA, a necessary component of essentially all genotyping methods. The target DNA comprises one strand of a PCR product immobilized onto a solid surface, and a single probe is used that is complementary to one of the target alleles. This assay concept was shown to be very precise (>99.9% accurate).

In a second aspect another embodiment, the present invention disclosure provides a kit for carrying out the method as defined above, which comprises adequate means for determining the genotype of the alterations in BChE gene.

In a particular embodiment, the kit comprises adequate means for carrying out amplification by primer-specific PCR multiplex.

The kit provided by the present invention disclosure can be used in a routine clinical practice to identify patients that suffer from DLB, thus differentiating said patients from other patients that suffer from AD. With the kit of the invention disclosure the clinicians will be able to apply more individualized and risk-adapted treatment strategies to patients suffering from DLB.

In another aspect embodiment, the invention disclosure relates to the use of a kit as defined above, for the diagnosis of DLB.

The present invention disclosure also refers to the use of the polymorphic site at position

68974, in combination with one or more alterations in BChE gene selected from the group consisting of the poly-thymine region at positions 4780 to 4786, the polymorphic site at position 3687; the polymorphic site at position 4206; and the polymorphic site at position 4443, as marker for the diagnosis of dementia with Lewy bodies. In a particular embodiment, the polymorphic site at position 68974 is used in combination with the poly-thymine region at positions 4780 to 4786. In another embodiment, the polymorphic site at position 68974 is used in combination with the poly-thymine region at positions 4780 to 4786, and with the polymorphic sites at positions 3687, 4206, and 4443.

The ~~invention~~disclosure also refers to a method of determining whether a subject will respond to treatment with neuroleptics, by analyzing the genotype of the above mentioned alterations in BChE gene. As the method allows determining whether a patient suffers from DLB or AD, is it possible to give the adequate treatment.

Throughout the description and claims the word "comprise" and variations of the word, such as "comprising", is not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the ~~invention~~disclosure will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the ~~invention~~disclosure. Furthermore, the present ~~invention~~disclosure covers all possible combinations of particular and preferred embodiments described herein.

The following examples and drawings are provided by way of illustration, and are not intended to be limiting of the present ~~invention~~disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the correspondence obtained for the four groups. Circles indicate the position of the different genotype combinations within the four-dimension-graph. Stars show the correspondent, genotype-combination-dependent localization of the disease and control groups. Their position clearly revealed significant differences between cDLB and controls, AD and controls, but also AD and cDLB. Whereas the control group was situated within the both dimension's negative quadrants, cLBD was situated within the