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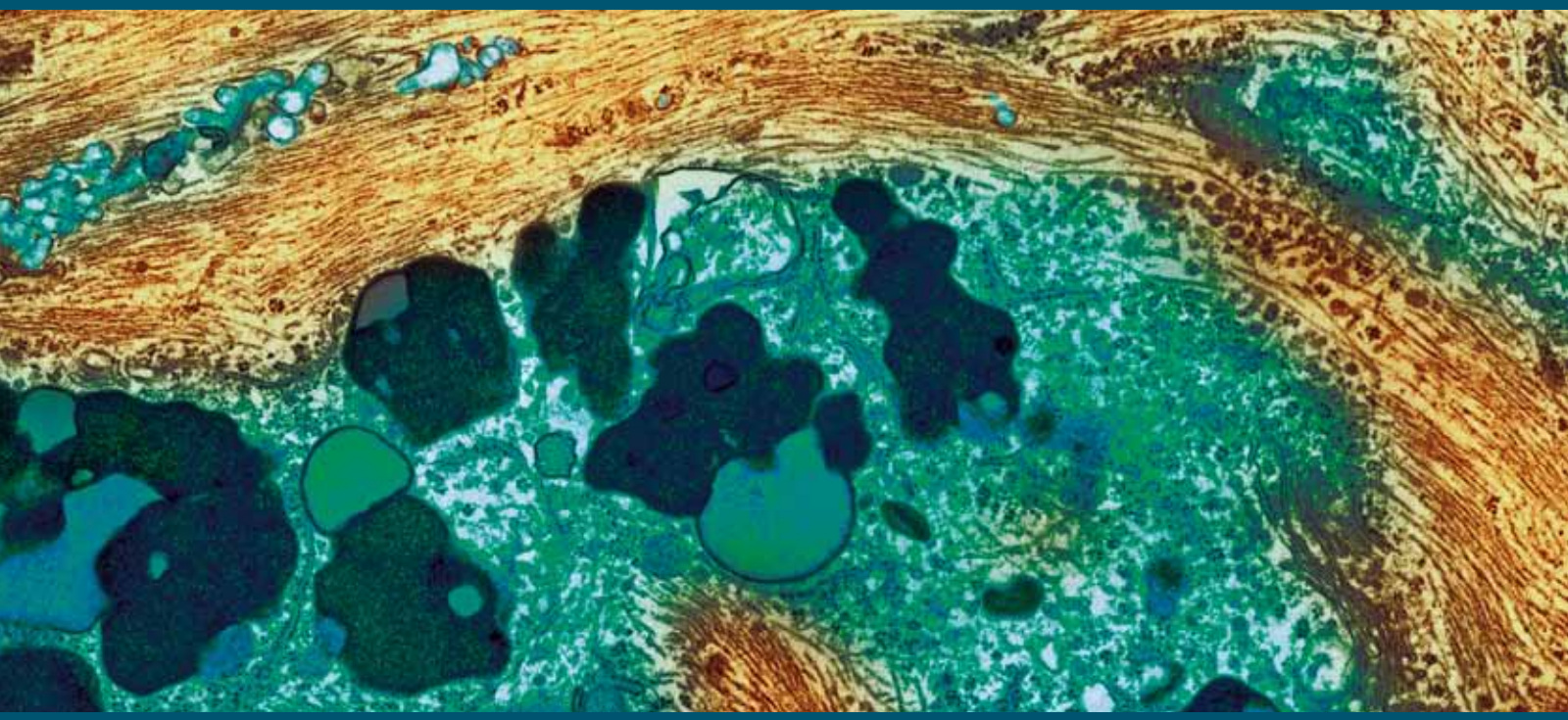
Early Clinical and Molecular Detection of Alzheimer's Disease

Guest Editors: Benedetta Nacmias, Christiane Reitz, and Thomas Arendt



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Editorial

Early Clinical and Molecular Detection of Alzheimer's Disease

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Alzheimer's disease (AD) is the most frequent neurodegenerative disease in the elderly. It is characterized by progressive impairment in multiple cognitive domains, in particular memory, leading to severe impairment in daily living followed by progressive physical deterioration and death. The neuropathological hallmark of AD is the presence of cortical intracellular neurofibrillary tangles (NFT) and extracellular β-amyloid (Aβ) plaques, which leads to synapse dysfunction, neuronal cell loss, and subsequent brain atrophy. In the past few decades the knowledge of the key pathogenic mechanisms of the disease has improved, but it remains far from being fully understood. Traditionally, the diagnosis of AD has been based on clinical symptoms, and accuracy studies of the current clinical criteria conducted in referral have shown high sensitivity for AD. However, identification of the disease, in particular in the early stages, remains difficult as pathological alterations may be apparent several years before the clear-cut clinical picture.

There is growing evidence that the use of biomarkers will increase our ability to identify AD earlier in the disease process and with higher accuracy. In addition, biomarkers will help elucidate the underlying biological and molecular changes, improve the detection of patients suitable for specific treatments, research studies or drug trials, and will contribute to a better management of the disease in the clinical practice. In this special issue on early clinical and molecular detection of AD, we have invited manuscripts that specifically address issues related to early and improved diagnosis of AD.

The first two articles address the usefulness of current biological and neuroimaging markers for the diagnosis of AD, including genetic variation, plasma, serum and CSF biomarkers, brain atrophy measures, functional MRI measures, and amyloid burden evaluated by PiB compound. The third paper reevaluates the usefulness of common clinical rating scales for AD, frontotemporal dementia, and vascular dementia using factor analysis. In the fourth paper, the sociocultural impacts of novel molecular technologies for the early diagnosis of AD are addressed. The paper outlines three steps to assess sociocultural impacts. First, conceptual analysis of the ideas underlying technological developments shows how these technologies redraw the boundary between AD and normal ageing and between biological and social approaches of ageing. Second, scenarios are designed depicting different possible futures of AD diagnosis and societal ways to deal with ageing. Finally, the paper reviews the possibilities for deliberation on the potential sociocultural impacts.

Manuscripts five and six are original research studies assessing the effect of specific genetic variations on AD and AD endophenotypes. While the study by Swaminathan et al. explores the effect of copy number variation (CNV) derived by genome-wide screening in the ADNI cohort on AD and MCI, the study by Tedde et al. explores the effect of genetic variation in *CDKN2A* and *CDKN2B* on AD. Finally, the last paper assesses the effect of siRNAs silencing on gene expression. While commonly studies use RNA interference (RNAi) to investigate the effect of silencing of specific genes on their expression and effect on cell metabolism, the current paper takes this technology further by assessing the effect of allele-specific silencing of a particular mutant allele, in this case mutation L392V in *PSEN1*.

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Review Article

Biomarkers of Alzheimer's Disease: From Central Nervous System to Periphery?

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Alzheimer's Disease (AD) is the most frequent form of dementia and represents one of the main causes of disability among older subjects. Up to now, the diagnosis of AD has been made according to clinical criteria. However, the use of such criteria does not allow an early diagnosis, as pathological alterations may be apparent many years before the clear-cut clinical picture. An early diagnosis is even more valuable to develop new treatments, potentially interfering with the pathogenetic process. During the last decade, several neuroimaging and cerebrospinal fluid (CSF) parameters have been introduced to allow an early and accurate detection of AD patients, and, recently, they have been included among research criteria for AD diagnosis. However, their use in clinical practice suffers from limitations both in accuracy and availability. The increasing amount of knowledge about peripheral biomarkers will possibly allow the future identification of reliable and easily available diagnostic tests.

1. Introduction

Autopsy data show that neuropathological features of AD are associated with subtle cognitive changes among nondemented subjects, thus suggesting the presence of a "preclinical Alzheimer's disease" [1, 2]. Moreover "*in vivo*" data based on amyloid PET ligands suggest that accumulation of neuropathologic damage lasts about 20 years before clear-cut clinical manifestations of the disease [3].

Currently, the diagnosis of AD is made according to clinical criteria by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS)-Alzheimer's Disease and Related Disorders Association (ADRDA) [4], which have limitations in terms of sensitivity and specificity and, above all, do not allow an early diagnosis of the disease.

In the last few years an extensive search for sensitive and specific biochemical and neuroradiological biomarkers of AD has been performed. Such markers would also help to better identify Mild Cognitive Impairment (MCI) patients at higher risk of conversion to AD. Based on the results of such studies, research criteria for AD diagnosis have been proposed which include neuroimaging and cerebrospinal fluid (CSF) parameters [5].

The main aim of such efforts is to diagnose AD when neurological damage might still be reversible. This would be highly valuable in the research setting, as treatments, which are currently under study to potentially interfere with the pathogenetic process of the disease, will probably show their full efficacy only if administered during the prodromal, or even preclinical, phase of AD. Consistently with this idea, the first immunization trial against beta-amyloid in full-blown AD patients showed that, although the treatment was effective in reducing neuropathological changes, it was not able to modify the clinical course of the disease [6].

Therefore, while subtle cognitive changes are highly prevalent among older subjects [7] and neuroradiological and CSF biomarkers, as discussed below, are not optimal diagnostic tools in term of availability, accuracy, and invasiveness (as in the case of lumbar puncture), the identification of reliable and easily accessible peripheral biomarkers will be of great interest in the setting of AD.

2. Neuroradiological Markers

Among neuroimaging parameters, the presence of atrophy, detected with MRI in specific areas of the brain, has been proposed as an early manifestation of AD [8]. Indeed, a good correlation seems to exist between hippocampus atrophy and the extension of neuropathological lesions in AD brains at autopsy [9]. It has been reported also that a visual determination of medial temporal lobe atrophy is a reliable instrument to discriminate AD and MCI from normal controls and to predict AD conversion in MCI patients [10]. On the other hand, other studies have reported that cross-sectional measurements of brain atrophy have limited diagnostic accuracy among older subjects [11]. This is consistent with data that show an overlap between AD- and ageing-associated atrophy in hippocampus and entorhinal cortex [12].

Among nuclear medicine techniques, the evaluation of cerebral blood flow by SPECT (Single Photon Emission Computed Tomography) has a well-established sensitivity in identifying AD, showing hypoperfusion of temporoparietal regions and posterior cingulus [13]. Brain metabolism evaluation by PET (Positron Emission Tomography) with fluor-deoxyglucose has shown an even greater sensitivity and a greater spatial resolution, allowing the study of smaller areas of the brain, such as the hippocampus, of great clinical interest in AD [14]. Several studies have demonstrated that PET has a sensitivity of more than 90%, even in the early phases of the disease, suggesting that it may be able to differentiate AD from age-related cognitive impairment [15]. Moreover, PET proved to be a reliable tool for the identification of MCI patients bound to become AD, with a diagnostic accuracy which has been proposed to be better than SPECT and MRI in a recent meta-analysis [16]. On the other hand, a recent study found no evidence that FDG-PET is more sensitive than MRI to quantify the degeneration present in preclinical and mild AD, in specific brain regions [17]. The authors of this paper suggest that hippocampal volume, measured by MRI, is probably the best trade-off between accuracy and convenience for detection of early AD.

In the last few years some "*in vivo*" PET ligands for AD lesions have been identified, such as [(11)C]PIB (Pittsburgh Compound B) that binds specifically beta-amyloid, and [(18)F]FDDNP (2-(1-{6-[(2-[F-18]fluoroethyl) (methyl) amino]-2-naphthyl} ethylidene)malononitrile) that binds both neurofibrillary tangles and beta-amyloid plaques. Their ability to differentiate AD patients from control subjects has been demonstrated [18, 19]. Longitudinal studies have established that the pathological changes identified with these molecules may occur in preclinical stages of the disease and may be detected earlier than atrophic changes and hypometabolism recognized by FDG-PET [20]. On the other hand, PIB deposition has been shown in about 20% of normal elderly subjects as well [21] and seems to proceed at the same rate both in cognitively intact and in cognitively impaired subjects [22]. These data were confirmed in a Japanese study, which found no difference in PIB retention pattern among very mild, mild, and moderate AD. An AD-like pattern of PIB deposition was also found in 48% of MCI and 18% of healthy control subjects [23]. Nevertheless, higher PIB binding has been related to progression to very mild dementia, independently of age, in 23 out of 159 not cognitively impaired subjects, confirming that PIB retention must not be considered a benign process [24]. Moreover, both PIB and FDDNP retention have been found to correlate with different cognitive domains in AD, MCI, and cognitively normal subjects. In particular an increased FDDNP binding was specifically associated with episodic memory impairment, while increased PIB retention was associated with a broader range of cognitive impairment [25].

3. CSF Biomarkers

CSF biomolecular markers of AD have been extensively investigated in recent years. Among them, an increased concentration of total and hyperphosphorylated tau protein and a reduction of amyloid β peptide Aβ42 have been reported in CSF of AD patients [26], with the combination of the two markers capable of further improving the diagnostic accuracy to a sensitivity and specificity of nearly 90% [27]. CSF markers accurately predict the risk of AD conversion in MCI patients, after a 5-year follow-up [28, 29]. Other authors have shown a strong relation between CSF Aβ42 and PET with 11C-PIB during prodromal and early phases of AD, demonstrating that CSF measurements actually parallel the neuropathological changes that occur inside the brain [30].

Despite their diagnostic accuracy, determination of CSF Aβ42 and tau levels has several limitations: circadian variability of their concentration [31], lack of standardization (use of different techniques or different protocols among different laboratories) [26], inadequacy of these markers to accurately discriminate between AD and other types of dementia, such as vascular and Lewy body dementia [27]. Moreover laboratories able to conduct such determinations are not widespread, and lumbar puncture is a relatively invasive procedure. A large-scale multicenter study, aimed at evaluating CSF Aβ42 and total and hyperphosphorylated tau-protein as predictors of AD in MCI patients, found that these CSF biomarkers are able to identify incipient AD with satisfactory accuracy (sensitivity 83%; specificity 72%; negative predictive value 88%; positive predictive value 62%), but with inferior power than what is reported by single-center studies, because of a great intersite assay variability [32]. The accompanying editorial suggests that the use of CSF markers, although advised in a research setting, should not yet be included in clinical practice [33].

Other CSF surrogate markers of brain amyloid deposition have been proposed. Like Amyloid Precursor-Protein (APP), Amyloid Precursor like Protein (APLP) undergoes a metabolic processing by secretases. Higher levels of APLP-1-derived peptides have been identified in CSF of AD patients, both in familiar and in sporadic forms [34].

Moreover, lower levels of Sortilin-related receptor (SORL1, also known as SorLA or LR11) have been identified in CSF of AD patients compared with cognitively normal controls and have been proposed as a diagnostic biomarker

for AD [35]. During the last few years, SORL1 has been identified as a facilitative factor of intraneuronal APP redistribution inside the Golgi, increasing its processing in the nonamyloidogenic pathway, while SORL1 deficit has been associated to an increased production of Aβ fragment [36]. In neuropathologic AD samples a reduction of SORL1 protein, compared to controls, was observed [37], that was inversely related to amyloid plaques and neurofibrillary tangles [38].

Finally, some studies have focused on products of oxidative stress to discriminate AD from control subjects (see also below): higher CSF and plasma isoprostanes level may represent a marker of oxidative damage in AD [39] and MCI [40] subjects, compared to controls. Other studies have identified lower level of antioxidants, in particular superoxide dismutase (SOD), in CSF of subjects with neurodegenerative diseases, including AD [41].

4. Peripheral Biomarkers

Lately, several Authors have directed their efforts in identifying AD biomarkers in plasma or serum, but the results are still inconclusive. A longitudinal study has found that high plasma levels of Aβ42 were associated with an increased risk of developing AD in subjects without dementia. Besides, conversion to AD was associated with a decrease of plasma Aβ42 levels and of plasma Aβ42/Aβ40 ratio [42]. This biphasic trend might be interpreted as follows: higher levels of Aβ42 are linked to an increased risk, while its subsequent decline might reflect compartmentalization of the peptide in the brain. This interpretation is consistent with a recent study that found an increased risk of dementia after 5 years in subjects with high plasma levels of Aβ42 [43]. On the other hand, a previous study found an increased risk of transition from cognitive normality to MCI or dementia in subjects with low plasma Aβ42/Aβ40 ratio [44]. Moreover, a longitudinal study showed that low plasma levels of Aβ42 and Aβ42 in AD patients were significantly associated with a more rapid functional and cognitive decline [45].

Other putative plasma biomarkers include molecules involved in the inflammatory response. In particular, higher level of soluble CD40 (sCD40) is found in plasma of AD patients compared with age-matched controls [46] and is able to predict the risk of conversion to AD in a sample of MCI patients [47]. Moreover, the expression of CD40 cognate ligand, CD40L, is upregulated in AD patients and is associated with an increased cognitive decline over the following 2 years [48]. These data are consistent with autopsy and animal studies that show an enhanced expression of CD40 and CD40L on astrocytes of AD brains [49].

In agreement with the involvement of inflammation in the pathogenesis of AD, recently an algorithm based on the values of several serum proteins, many of whom are related to inflammation, demonstrated 80% sensitivity and 91% specificity in discriminating AD from controls; the addition of gender, age, education, and ApoE status to the prediction algorithm increased sensitivity and specificity to 94% and 84%, respectively [50]. In a previous study with a similar approach a different set of plasma signaling proteins was identified, which was able to correctly differentiate AD from controls (cognitively normal and other dementia) with 90% sensitivity and 88% specificity; moreover the same algorithm showed 91% sensitivity and 72% specificity in predicting AD development in a small sample of MCI subjects after 2–6 years [51]. Interestingly, the panel of 18 proteins which allowed dementia prediction in the cited study were involved in hematopoiesis and inflammation, leading the authors to hypothesize an impairment in macrophage function in AD subjects, possibly related to a decreased Aβ clearance from brain [52].

Other authors have studied the influence of Brain-Derived Neurotrophic Factor (BDNF), a potential neuroprotective agent, on neuron survival and function, and found that its level is significantly higher in serum of AD and MCI patients, compared with healthy subjects, independently of disease severity, treatment with antidepressant or cholinesterase inhibitors [53]. These data are partially consistent with another study showing an increase of BDNF concentration in mild AD, compared with controls (the result being interpreted as a compensatory mechanism), with a subsequent decline in later stages of the disease [54]. On the contrary, another research has found decreased BDNF levels in MCI subjects, compared with cognitively normal controls [55].

A different approach is the search for possible AD biomarkers in peripheral cells, based on the hypothesis that modifications of signal transduction, oxidative metabolism or APP metabolism that are present in neurons, may be found in peripheral tissues as well [56].

Peripheral tissues would constitute an easier model to study the pathogenesis of AD and to identify biomarkers of the disease. Until now, several peripheral tissues have been employed in AD research, including peripheral blood mononuclear cells (PBMCs), platelets, and fibroblasts. Each model has advantages and disadvantages, with fibroblasts being particularly useful, due to high stability under physiological and pharmacological stress [57]. Unlike PBMCs and platelets, fibroblasts behavior becomes independent from circulating molecules as soon as the cells are propagated *in vitro*. On the other hand, fibroblasts will age in culture, making it more difficult to interpret the results obtained with this model. Moreover, PBMCs and platelets are more accessible and may be a better model when techniques such as cytofluorimetry are employed.

One possible peripheral cell AD biomarker is represented by the PKC intracellular signaling system. In cerebral tissues of AD patients, PKC protein level, activity, and intracellular translocation are altered compared to control brain tissues [58]. In fibroblasts of AD patients, a reduced PKC activity has been described [59]. Moreover, inflammatory stimuli, such as bradykinin (BK), determine in fibroblasts of AD patients a PKC-mediated phosphorylation of extracellular signal-regulated kinases (ERKs) 1/2, which is not detected in fibroblasts of age-matched healthy controls [60]. A phospho-ERK1/phospho-ERK2 index, before and after BK stimulation, has been proposed as AD biomarker, being able to discriminate not only between AD and healthy subjects,

but also between AD and non-AD dementia [61]. This index was validated in a sample which included autopsy-confirmed cases, demonstrating higher sensitivity and specificity for diagnosing AD compared with clinical criteria, especially within the first 4 years from the onset of the disease [62]. Adding to the validity of this result, another study has observed increased levels of phosphorylated ERK1/2 in CSF of patients with neurodegenerative conditions (AD, frontotemporal dementia, and MCI), suggesting that these kinases are released into CSF in parallel with tau and phospho-tau proteins [63].

In a different research line, a conformational modification of p53 protein, associated with an alteration of its transcriptional activity, has been described in skin fibroblasts isolated from AD patients. This protein misfolding, which can be induced in non-AD fibroblasts by low concentrations of Aβ peptide [64], results in an increased resistance of the cells to p53-mediated apoptosis; therefore, its involvement in the early phases of amyloid deposition has been hypothesized and its possible use as a biomarker of early AD proposed [65]. The same authors have developed a cytofluorimetric test on PBMC that quantitatively evaluates the amount of altered p53 present within the cell. Such test has a sensitivity and a specificity comparable to routine CSF biomarkers in identifying AD, but only in patients under 70 years of age. In 70+ subjects, the amount of conformationally altered p53 increases, independently of the presence of AD; however, older AD patients still display increased amount of altered p53 compared to age-matched healthy controls [66]. Moreover the same mutant form of p53 was found to predict MCI conversion to AD after two years with good specificity and satisfactory sensitivity [67].

More recently, the same Authors have described an increase of membrane CD44 expression in lymphocytes of patients with AD, in comparison with healthy subjects. CD44 is an adhesion molecule involved in the immune response even inside the central nervous system, and its increase seems to parallel the rise of unfolded p53 in AD lymphocytes [68].

Another research approach is related to the study of APP metabolism in platelets, based on data showing functional similarities between platelets and neurons. In particular, it has been shown that platelets isolated from AD patients have a different ratio of APP isoforms, with a lower amount of high molecular weight APP, compared to cognitively intact subjects. The "APP ratio" of high and low molecular weight isoforms is able to accurately discriminate between AD patients and normal controls [69] and to predict poor cognitive prognosis in MCI subjects at 2-year follow-up [70]. This test was found to be highly reproducible, with the main limitation being its sensitivity to pharmacological treatments (e.g., cholinesterase inhibitors, antiplatelet agents) [71].

Finally, several studies have shown increased markers of oxidative stress in brain from AD and MCI patients, compared to controls [72, 73]. Oxidative stress can result from diminished levels of antioxidants, even if reactive oxygen species levels are unchanged. A significant decrease of superoxide dismutase (SOD) has been observed in AD and MCI patients, compared with controls, both in plasma [74] and in specific brain areas [75]. In a separate study, the authors found not only a decrease of SOD and glutathione levels, but also an increase of lipid peroxidation markers in serum of AD patients, compared to an age-matched control group [76]. However, such studies, although adding useful information on the pathogenetic process of the disease, do not seem to provide results specific enough to justify their use as diagnostic tools.

5. Conclusions

During the last several years, our knowledge about possible biomarkers of AD has increased, paralleling the development of new therapeutic approaches. CSF and neuroimaging biomarkers seem to be the most promising; however limitations regarding their reliability, diffusion, as well as costs, still remain. In this perspective, the availability of peripheral biomarkers, less invasive, more readily accessible, and possibly cheaper, would be of great value. Results in this field are promising, and some of these biomarkers might become available in the clinical setting soon.

However, due to the multifactorial nature of AD pathogenesis, it seems unlikely that a single marker may prove to be the ultimate diagnostic tool. More likely, a combination of peripheral biomarkers, along with extensive clinical and neuropsychological assessment, might be able to suspect cases of prodromal AD, among the vast number of subjects with subjective or mild cognitive impairment, to be classified as "high risk" and to be subjected to more invasive and/or expensive procedures of functional neuroimaging and CSF analysis. More research is needed to validate this approach.

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Review Article

The Usefulness of Biological and Neuroimaging Markers for the Diagnosis of Early-Onset Alzheimer's Disease

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The recent proposed criteria for Alzheimer's Disease (AD) have strongly claimed the usefulness of biological and neuroimaging markers for early identification AD. Cerebrospinal fluid (CSF) Tau/Abeta ratio, hippocampal atrophy, posterior cingulate, and neocortical associative area hypometabolism, or amyloid burden evaluated by PiB compound, held the premises to increase diagnostic accuracy in the preclinical disease stages. Despite many efforts to identify subjects at risk of developing AD, less attention has been paid to presenile AD diagnosis. A few data are already available in early onset AD, mainly obtained in cases of monogenic disorder. In this paper, we discuss the current literature on the role of biological and neuroimaging markers in presenile AD.

1. Introduction

Alzheimer's Disease (AD) is the most common form of neurodegenerative dementia, with an incidence that increases with age [1].

Typically, the core clinical features of AD are memory impairment, related to early tissue damage within hippocampus and medial temporal lobes [2]. However, AD is rather clinically heterogeneous and there has been increasing awareness of atypical AD forms, which are characterized at disease onset by either language deficits, behavioural disturbances, or visuospatial abnormalities [3–7]. AD may be termed late-onset AD when the onset of symptoms is at 65 years or later, and early-onset AD (EOAD) with symptoms beginning before 65 years of age. The atypical presentations are more frequent in EOAD than in late-onset disease. Notwithstanding, the differential diagnosis between EOAD and psychiatric disturbances, metabolic disorders and even other neurodegenerative dementias associated with an early disease onset, namely, frontotemporal dementia (FTD), corticobasal degeneration, and posterior cortical atrophy, is of particular importance in light of the emergence of potential disease modifying therapies in the future.

Recent proposed consensus criteria for AD have underlined the role played by biological and neuroimaging markers for disease diagnosis [8]. Accordingly, cerebrospinal fluid (CSF) Tau/Abeta ratio, hippocampal atrophy as measured by MRI, posterior-cingulated and neocortical-associative area hypometabolism as detected by FDG-PET, and amyloid burden evaluated by PiB compound have been claimed as the fingerprints of preclinical AD.

However, in spite of the wide body of literature on the accuracy of biological and neuroimaging markers in identifying subjects at risk of developing AD, much less attention has been devoted to EOAD. Different reasons may be addressed for this: first, presenile AD is a rare disorder as compared to late-onset disease, thus being difficult to collect a reasonable sample of patients to draw reliable results; second, in most of cases, investigators, considering EOAD to be genetically based, give too much attention to genetics determinants and less attention has been paid to biomarkers.

The aim of the present work is to review the available data on the role of biological and neuroimaging tools that might be helpful in carefully detecting EOAD and to discuss the need of future clinical trials to define diagnostic algorithms for EOAD.

2. Genetic Diagnosis in Early-Onset Alzheimer's Disease

The majority of AD cases are sporadic buta relatively small number of cases (25%), present familial aggregation (familial AD, FAD). FAD is typically associated with EOAD (60% of EOAD is familial), with 13% appearing to be transmitted as a pure genetic, autosomal dominant trait [9].

In the last years, mutations in three genes have been described as the cause of some familial forms of EOAD, and these cases with known pathogenetic mutations are named monogenic AD.

Firstly, in 1991, mutations within the "*Amyloid Precursor Protein gene* (*APP*)" on chromosome 21q21.3 were firstly reported, and more than 32 pathogenetic mutations have been described so far (http://www.molgen.ua.ac.be/ADMutations). APP mutations account for less than 5% of autosomal dominant EOAD [9]. The *APP* gene encodes for a polypeptide of 770 amino acids that may be involved in nuclear signaling [10]. It has been shown that APP protein can be processed by at least two separate pathways, one involves α-secretases cleavage and the other requires sequential proteolysis by β- and γ-secretases to generate A$\beta_{40–43}$ peptides [11]. A$\beta_{42\text{-}43}$ were considered the more neurotoxic isoforms and were found increased in the brains of AD patients [12]. Interestingly, pathogenetic mutations within *APP* gene are clustered very close the secretase cleavage sites, thus exerting a direct effect on APP processing [13].

Families with *APP* mutations show variable age of onset ranging from 31 to 60 years and are clinically defined by EOAD frequently associated with cerebral amyloid angiopathy [14]; some cases have been reported with atypical clinical presentation at symptom onset, resembling Dementia with Lewy Bodies or epilepsy [15, 16].

Some years later to the identification of APP mutations, pathogenetic variations within "*Presenilin 1gene* (*PSEN1*)" on chromosome 14q24.3 were identified as key to disease pathogenesis. *PSEN1* mutations represent the most common cause of autosomal dominant EOAD (18–50%), and more than 180 mutations have been identified [17, 18].

PSEN1 gene encodes a transmembrane protein of 476 amino acids that acts as catalytic for the γ-secretase complex. The majority of the known mutations are missense substitutions and show almost complete penetrance by the age 60 years [19].

PSEN1 mutations have been associated with a wide range in age of onset, ranging from very early-onset (before age 30 years) to cases with onset later than 80 years old [18, 20, 21]. It has been demonstrated that carrying *PSEN1* mutations is a negative prognostic factor on survival as compared to *PSEN2* mutations or sporadic late-onset AD [22].

Phenotypic heterogeneity is common in patients with *PSEN1* mutations. Although the majority of known *PSEN1* genetic variations are associated with classical EOAD, several cases have been reported to have atypical features, namely, myoclonus and seizures [23, 24], extrapyramidal signs [23], behavioural and psychiatric symptoms, early aphasia [23, 25], visual agnosia [26], cerebellar ataxia [27, 28], and spastic paraparesis [23, 29].

Conversely, the third gene responsible for autosomal dominant-inherited disorder, that is, "*Presenilin 2* (*PSEN2*)" maps on chromosome 1q31-42, and it does not represent a common cause of EOAD. Only 14 pathogenic mutations have been described so far [30, 31]. *PSEN2* gene encodes for a transmembrane protein of 448 amino acids that, as PSEN1, plays a role as a catalytic core of the γ-secretase complex.

The clinical phenotype associated with *PSEN2* mutations is still not clearly defined, as a few cases are available. Up to now, 7 out of 14 mutations are likely to be pathogenetic [22] and they have been associated with AD with age at onset ranging from 39 to 75 years.

Some mutations were associated with atypical or distinctive clinical features: A85V mutation was described in a family with dementia and parkinsonism and with a clinical diagnosis of Dementia with Lewy Bodies in one member; T122R mutation was reported in subjects with frontotemporal dementia-like phenotype [32], whilst N141I mutation was associated with seizures in 32% of carriers [30–33]. Twelve patients carrying N141I mutation underwent neuropsychological testing and memory loss and impairment in verbal fluency, spatial perception deficits, acalculia, and executive dysfunctions were reported.

In summary, EOAD accounts for 1–5% of AD cases [34]. It has been shown that mutations within *APP*, *PSEN1,* or *PSEN2* (see Table 1) can explain up to 71% of the autosomal dominant transmission pattern in FEOAD [35]. The screening for known pathogenetic mutations, in patients with appropriate phenotype and familial EOAD with autosomal dominanttransmission pattern, should be considered useful in clinical practice, but it should be accompanied by neurogenetic counselling and undertaken only after full consent and by specialist centres.

It is however true that the wide spectrum of clinical symptoms of monogenic AD, beyond memory disturbances, claims for the need of biological and neuroimaging markers that can be of help in suggesting DNA sequencing. Moreover, most of EOAD cases are not explained by known Mendelian mutations, and also in these cases there is a need for biomarkers for an accurate diagnosis.

3. Biological and Neuroimaging Markers in Detecting Early-Onset Alzheimer's Disease

The recent published criteria for AD have strongly supported the usefulness of biological and neuroimaging markers for improving diagnostic accuracy in preclinical stages [9].

It is well established that reduced CSF Abeta42 levels and increased CSF Tau levels are the signature of AD. In the same way, hypometabolism of posterior cingulate cortex and temporoparietal regions and hippocampal atrophy are the neuroimaging hallmark of the disease [8].

However, the usefulness of these markers in EOAD has been poorly tested, and no reliable large sample size studies in EOAD patients compared to other neurodegenerative dementias are available yet.

Table 1: Monogenic forms of Early-Onset Alzheimer's Disease (EOAD).

Gene	Locus	N° mutations	Clinical presentation
APP	21q21.3	32	Onset from 31 to 60 years. EOAD frequently associated with cerebral amyloid angiopathy
PSEN1	14q24.3	>180	Onset before age 30 years to cases with onset later than 80 years old. Classical EOAD, several cases with atypical features: myoclonus and seizures, extrapyramidal signs, behavioural and psychiatric symptoms, early aphasia, visual agnosia, cerebellar ataxia, and spastic paraparesis.
PSEN2	1q31-42	14	Onset from 39 to 75 years. Atypical features: dementia and parkinsonism, frontotemporal dementia-like phenotype, seizures.

APP: amyloid precursor protein; PSEN: presenilin.

3.1. Cerebrospinal Fluid for EOAD Diagnosis. Sporadic and FAD share common pathogenetic mechanisms, as neurotoxic forms of amyloid appear to be elevated in both cases. Thus, it can be hypothesised that CSF analysis might be of help in EOAD as well. The current findings on CSF markers are based on case reports and small samples of EOAD mutation carriers, namely with *PSEN1* genetic variations.

To date, more than 180 mutations in *PSEN1* have been detected (http://www.molgen.ua.ac.be/ADMutations). It has been reported that pathogenetic *PSEN1* genetic variations lead to significant increases in plasma Abeta42 levels and massive amyloid deposition in the brain [36, 37]. A role of *PSEN1* mutations in Tau pathology has also been suggested by the finding of tau hyperphosphorylation in *PSEN1* transgenic mice [38].

In a recent work, it has been demonstrated that in a case of presenile dementia, spastic paraparesis, and frontal executive function impairment, CSF analysis facilitated DNA diagnosis. Increased CSF total Tau levels and decreased CSF Abeta42 dosage distinguished this familial EOAD from Creutzfeldt Jacob disease and frontotemporal dementia [39]. A causative *PSEN1* L424R mutation was subsequently identified [39].

In the same view, a CSF pattern resembling that found in late-onset AD pointed towards genetic analysis in a case of *PSEN1* Q223R mutation [40]; the patient presented familial early-onset dementia at the age of 35 years along with spastic paraplegia and behavioural symptoms.

Interestingly, CSF biomarkers have been demonstrated useful not only in distinguishing EOAD from other neurodegenerative dementias, but also in predicting AD-related mutations in cases with clinical and skin biopsy features suggestive for storage disease [41]. A patient with progressive cognitive decline associated with delusions, myoclonus, and seizure and with no family history for dementia was diagnosed as cereidolipofuscinosis; once CSF analysis was carried out and AD-like pattern reported, de-novo *PSEN1* P117L mutation was identified and the diagnosis of EOAD made.

Up to now, no extensive data on CSF analysis in *PSEN2* carriers have been performed, as rare causes of EOAD. However, in a case of *PSEN2* N141I mutation, the CSF pattern was comparable to sporadic AD.

3.2. Functional Neuroimaging for EOAD Diagnosis. If in both EOAD and in sporadic late-onset AD, CSF data are almost comparable, different findings may be obtained when neuroimaging markers are considered.

Little is known about the usefulness of neuroimaging in the differential diagnosis with other neurodegenerative dementias. In fact, it has been widely demonstrated that the cognitive pattern of EOAD is different from late-onset AD, in the former the neocortical functions are more affected. Accordingly, EOAD and late-onset AD differ in their typical topographic patterns of brain atrophy [42].

Indeed, in monogenic EOAD cases, neuroimaging reports usually show the pattern of sporadic AD, involving temporoparietal areas, but extra brain regions not commonly observed in typical AD cases are described. It has been demonstrated that patients with EOAD due to *PSEN1* mutations (i.e., His163Tyr) showed greater hypometabolism not only in the posterior cingulate but in frontal cortex as well [43] and thalamic hypometabolism was found in EOAD case carrying *APP* Val717Ile mutation [44].

Some authors evaluated the role of PiB-compound, a Positron Emission Tomography (PET) tracer binding amyloid, in early diagnosis of EOAD. In a *PSEN1* His163Tyr case, PiB binding was comparable to sporadic AD, but slightly higher striatal levels were detected [43]. The same findings were obtained evaluating ten *PSEN*1 mutation carriers, with intense and focal PiB retention in the striatum [45]. Moreover, in most *PSEN1* mutation carriers, there also were increases in PiB retention compared to controls in cortical brain areas, but these increases were not as great as those observed in sporadic AD subjects [45].

Another interesting work aimed at evaluating PiB distribution in EOAD in seven *PSEN1* mutation carriers and one *APP* mutation carrier [46]: the authors demonstrated that all mutation carriers had high PiB retention in the striatum, with some also having cortical PiB retention in ventrofrontal and posterior cingulate/precuneus areas. The striatal pattern

of PiB retention was similar in the *PSEN1* and *APP* mutation carriers [46].

These studies suggested that the pattern of Abeta deposition in FAD differs from that in sporadic AD, with higher striatal and somewhat lower cortical PiB retention in FAD.

3.3. Structural Neuroimaging for EOAD Diagnosis. Studies on structural neuroimaging have strongly supported the usefulness of hippocampal measures in early diagnosis of AD. From pathological and MRI studies, the hippocampus is known to be severely affected in established AD [47, 48]. Hippocampal volumes have consistently been shown to be reduced by as much as 40% in patients with clinically diagnosed AD, the extent of atrophy correlating with disease severity [49]. Hippocampal atrophy has been introduced in current proposed criteria for preclinical diagnosis of AD [8, 50].

In EOAD, the available data seem to suggest that evaluating hippocampal atrophy may be of help.

In a case of *PSEN1* S170F mutation, cortical brain atrophy, particularly within hippocampus, frontal and temporal cortex, was reported [51]. However, as demonstrated, patients with EOAD showed greater neocortical atrophy at the temporoparietal junction, while the patients with lateonset AD showed greater hippocampal atrophy [52]. Taken together, these results argue that hippocampus evaluation is a signature in EOAD as in late-onset disease.

3.4. Biological and Neuroimaging Markers in Presymptomatic Subjects with EOAD. A few studies have estimated the importance of biological and neuroimaging markers in presymptomatic subjects with known mutations. As, in these, casesgenetic testing is exhaustive, the role of markers has not been fully elucidated. Indeed, the assessment of biological and neuroimaging markers in presymptomatic subjects carrying pathogenetic mutations might be of help in defining the time of conversion. Diagnosis in subjects carrying pathogenetic mutations represents a very delicate aspect with even profound ethical implications that should be treated cautiously and only in the frame of controlled research protocols.

The usefulness of CSF biomarkers has been tested on six presymptomatic subjects with pathogenetic mutations in the *PSEN1* gene [53], and CSF Abeta42 levels were found to be significantly lower than age-matched control group. No other data are still available.

Studies in presymptomatic carriers employing imaging techniques like PET and single photon emission computed tomography have revealed regional abnormalities in cerebral glucose metabolism [54] and brain blood-flow [55] prior to the development of clinically significant impairment. Likewise, very early pathological changes in terms of amyloid deposition have been shown in a study of young presymptomatic *PSEN1* carriers by using 11C-PiB-PET [45]. A recent study carried out in presymptomatic and mildly affected *PSNE1* carriers revealed significantly greater thalamic retention than sporadic AD [56]. Moreover, a few individuals with *PSNE1* mutations showed increased cerebellar 11C-PiB retention suggesting that this region may not be as suitable as a reference region in FAD [56].

Finally, volumetric measurement of hippocampus showed asymmetrical atrophy in seven presymptomatic *APP* Val717Ile carriers [52].

4. Conclusions

A few efforts have been addressed to elucidate the usefulness of biological and neuroimaging markers in EOAD. Studies involving larger numbers of individual are required in order to determine whether and which marker abnormalities are consistently detected at early disease stage in young onset dementia. Up to now, no clear-cut conclusions might be drawn.

Preliminary data suggest that CSF markers, such as Abeta and Tau levels, may be considered as a helpful tool in the diagnosis of EOAD, as the reported changes are comparable to those detected in sporadic late-onset AD. Hippocampal measurement seems to be early affected in EOAD, thus to be considered in diagnostic workup. Conversely, more data on functional abnormalities in EOAD are required, as peculiar pattern of hypometabolism/amyloid accumulation occurs in the available EOAD cases associated with monogenic mutations.

The assessment of biomarkers in EOAD should be recommended in order to increase diagnostic accuracy in those cases with atypical presentation and/or familial aggregation of the disease. Biological and neuroimaging markers may be of help to suggest DNA sequencing of known causative genes or to support diagnosis of EOAD in those cases without *APP, PSEN1,* and *PSEN2* mutations.

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Research Article

A Factor Analytic Approach to Symptom Patterns in Dementia

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Previous publications have shown a high diagnostic sensitivity and specificity of three short clinical rating scales for Alzheimer's disease (AD), frontotemporal dementia (FTD), and vascular dementia (VaD) validated against neuropathological (NP) diagnoses. In this study, the aim was to perform an exploratory factor analysis of the items in these clinical rating scales. The study included 190 patients with postmortem diagnoses of AD ($n = 74$), VaD ($n = 33$), mixed AD/VaD ($n = 31$), or FTD ($n = 52$). The factor analysis produced three strong factors. Factor 1 contained items describing cerebrovascular disease, similar to the Hachinski Ischemic Score. Factor 2 enclosed major clinical characteristics of FTD, and factor 3 showed a striking similarity to the AD scale. A fourth symptom cluster was described by perception and expression of emotions. The factor analyses strongly support the construct validity of the diagnostic rating scales.

1. Introduction

Dementia is a clinical syndrome with a marked variety of aetiology, clinical profile, severity, and clinical course. The differential diagnosis between various clinical and aetiological subtypes may be difficult, and so far no single diagnostic approach or biomarker has fully solved these problems. Few clinical symptoms and signs are pathognomonic of dementia or a specific type of dementia. It is mostly the symptom constellation, the timing of appearance, and the clinical progression that lead to a diagnostic conclusion [1]. A positive diagnosis of dementia is often made comparatively late in the disease process and for this reason most clinical investigations are performed on patients in an advanced stage or retrospectively on patients with organic dementia defined postmortem.

Relevant to the present study, most factor analyses of symptoms in dementia have been carried out for descriptive purposes and less for the construction of diagnostic rating scales. A conventional factor analysis of 78 symptoms in early onset dementia resulted in 14 clinically meaningful factors [2]. Three factors contained symptoms of severe dementia, three factors described mood changes or delusions, five factors described personality changes and impaired control of emotional expressions, and three factors described various motoric dysfunctions. The factors showed specific relationships with regional Cerebral Blood Flow (rCBF) and psychometric testing [2, 3]. In another study, factor analysis of 16 symptoms of the Brief Psychiatric Rating Scale (BPRS) in 87 geropsychiatric patients resulted in five clinical dimensions: withdrawn depression, agitation, cognitive dysfunction, hostile suspiciousness, and psychotic distortion [4]. Petrovic et al. [5] identified four symptom clusters based on factor analysis of the Neuropsychiatric Inventory (NPI) in patients with dementia: psychosis, psychomotor, mood liability, and instinctual factors. Another factor analysis of ten NPI items in probable AD resulted in three subsyndromes: mood, psychotic, and frontal [6], and a factor analysis of the 12 item NPI showed the presence of four behavioural subsyndromes called hyperactivity, psychosis, affective symptoms, and apathy [7]. Thus, so far few factor analytic studies of dementia symptoms have focused on differential diagnostic issues. Björkelund et al. presented a systematic review of 30 studies of the Organic Brain Syndrome (OBS) scale for description of delirium and dementia [8]. Factor analysis of the 53 clinical items of the OBS scale revealed three factors

describing different types of disorientation and nine factors describing different cognitive and emotional disturbances, and neurological symptoms.

Our previous publications from the Lund Longitudinal Dementia Study have introduced two short diagnostic rating scales, one for recognition of Alzheimer's disease (AD), the AD scale, and the other for diagnosis of primary degenerative frontotemporal dementia (FTD), the FTD scale [9]. Differential diagnostic screening with these two rating scales and the Hachinski Ischemic Score (HIS) scale [10] has been evaluated against postmortem neuropathological (NP) diagnoses to analyze their feasibility for antemortem clinical diagnosis of AD, vascular dementia (VaD), mixed AD/VaD, and FTD [11]. The sensitivity and specificity of the AD scale were 0.80 and 0.87, respectively, of the FTD scale 0.93 and 0.92, respectively, and of the HIS score (VaD diagnosis) 0.69 and 0.92, respectively. Cases with mixed AD/VaD generally presented a combination of high AD and ischemic scores [11]. However, no analysis of the individual items was performed. Therefore, we present results from a principal component factor analysis of the individual items of the AD, FTD, and HIS scales (Table 5). The factor analyses were used to identify clinical dimensions of dementia and to confirm the construct validity of the clinical rating scales. Furthermore, the relationship between different items and NP diagnoses was studied as also the possibility to modify and improve the clinical rating scales as diagnostic tools.

2. Material and Methods

This study was based on a prospective longitudinal clinical work-up with a final postmortem NP examination. The study covers the time period from the late 1960s and onwards and includes consecutive patients with symptoms of dementia referred to the Psychogeriatric and Psychiatric Departments of the University Hospital in Lund. The patients and other informants were interviewed and the neuropsychiatric symptoms and signs of the HIS, AD, and FTD scales were evaluated and scored by a psychiatrist with experience in the dementia field. The 30 items and scores of the three rating scales are presented in (Table 5). Exclusion criteria were chronic psychosis and epilepsy, severe somatic disease, severe head trauma, addiction, stroke with remaining gross focal neurological symptoms, and conditions that did not allow the application of the three clinical rating scales. All patients fulfilled DSM III and ICD-10 criteria for a dementia syndrome [12, 13]. The NP diagnoses were based on standardized NP procedures and criteria recently published [11]. The age characteristics and NP diagnoses are shown in Table 1. The factor analytic study was based on 190 cases (77 male and 113 female) deceased in the years 1967–2007 with an NP diagnosis of AD, FTD, VaD, or mixed AD/VaD and with a complete diagnostic scoring. Patients with other NP diagnoses or incomplete scoring were not included.

The average age at onset in the total material was 64.6 ± 12.3 years (range 30–92 years) and differed significantly between all the four major NP groups (ANOVA followed by Student-Newman-Keuls test, Table 1). The mean age at death was 73.6 ± 11.35 years (range 34–97 years) with significant group differences, except between AD and VaD (74.7 and 76.5 years, resp.). The mean duration of illness was 8.9 ± 5.3 years (range 1–26 years). The mean duration was similar in the AD and FTD groups (10.4 and 9.1 years, resp.). Only AD corresponded with a significantly longer duration compared with VaD and mixed AD/VaD.

2.1. Diagnostic Rating Scales. The three diagnostic rating scales, HIS scale, AD scale, and FTD scale, and their thirty clinical items (Table 5) have been presented in a previous publication as also the validation of the three diagnostic scores against NP diagnoses [11]. The 30 items were selected for the purpose of differential diagnosis of dementia diseases. In this paper, the factor analysis was performed of the diagnostic items scored in the 190 patients with NP diagnoses.

2.2. Factor Analytic Approach. In order to detect clusters of clinical symptoms and signs, the item scores were subjected to conventional factor analysis using the principal component method with varimax rotation [14]. Factor analysis is a construct validity tool aiming at identifying underlying clinical dimensions. The validity of a symptom cluster has been defined as the common variance of the factor and the construct validity is studied by comparison with other constructs [15]. Factors with an eigenvalue exceeding 1.0 and an interpretable constellation of items are usually considered of interest for the clinical description. The factor structure will be described by the symptoms with factor loadings in the rotated factor matrix, which are considered as "significant" (at the 1% level), although there is no accepted standard error of factor loadings [16]. Factor loadings of 0.30 or greater are judged as significant in most textbooks [17–19]. The simple structure idea is further corroborated by a pattern of zero factor loadings [20]. There are different opinions in terms of sample size in factor analysis. Hatcher [21] recommended that the number of subjects should be five times the number of variables, (which in this study means 150) or at least 100, while Hutcheson and Sofroniou [22] recommended 150–300 subjects.

2.3. Statistical Analysis. Factor analysis was performed with Stat View version 5.0.1. SAS Institute Inc. We performed a principal component analysis of the 30 items included in the rating scales, using an orthotran varimax procedure. Factors with eigenvalues greater than 1.9 were selected in the three-factor solution. Factor loadings with higher values (i.e., minimum 0.25) were included when they contributed to a clinically meaningful interpretation pattern.

3. Results

There was a marked variation of the prevalence of diagnostic scale items for the NP groups (Table 2).

Factor analysis of the 30 items scored in the 190 patients resulted in several factors with eigenvalues exceeding 1.0. We will first present the three-factor solution with eigenvalues of 5.2, 3.7, and 1.9 (Table 3). All three factors were clearly

Table 1: Age at onset, age at death, duration of illness (mean ± SD (range)), and gender characteristics in 190 patients with neuropathological dementia diagnosis.

(a)

NP diagnosis	*n* (%)	Male/Female	Age at onset (years)	Age at death (years)	Duration of illness (years)
AD	74 (35.4)	20/54	64.2 ± 10.2 (44–88)	74.7 ± 8.7 (59–93)	10.4 ± 4.9 (1–21)
FTD	52 (24.9)	23/29	54.7 ± 10.9 (30–84)	63.8 ± 11.5 (34–85)	9.1 ± 5.2 (1–26)
VaD	33 (15.8)	19/14	69.5 ± 9.9 (53–89)	76.5 ± 9.0 (58–93)	7.1 ± 6.4 (1–26)
Mixed AD/VaD	31 (14.8)	15/16	77.0 ± 6.5 (64–92)	84.3 ± 6.3 (71–97)	7.4 ± 4.0 (1–15)
Total	190 (100)	77/113	*64.6 ± 12.3 (30–92)*	*73.6 ± 11.5 (34–97)*	*8.9 ± 5.3 (1–26)*

(b)

Group comparisons (difference (95% CI))	Age at onset[#]	Age at death[##]	Duration of illness[###]
AD versus FTD	9.5 (5.7–13.3)*	10.9 (7.3–14.4)*	1.3 (−0.5–3.1)
AD versus VaD	−5.2 (−9.5–−1.0)*	−1.8 (−5.5–1.8)	3.3 (1.0–5.6)*
AD versus mixed AD/VAD	−12.7 (−16.7–−8.8)*	−9.6 (−13.1–−6.2)*	2.9 (1.0–4.9)*
FTD versus VaD	−14.7 (−19.4–−10.1)*	−12.7 (−17.4–−8.0)*	2.0 (−0.5–4.5)
FTD versus mixed AD/VaD	−22.2 (−26.5–−17.9)*	−20.5 (−25.0–−16.1)*	1.7 (−0.5–3.8)
VaD versus mixed AD/VaD	−7.5 (−11.7–−3.3)*	−7.8 (−11.7–−3.9) *	−0.4 (−3.0–2.3)

[#] ANOVA $F_{3,184} = 36$ ($P < .001$)
[##] ANOVA $F_{3,186} = 34$ ($P < .001$)
[###] ANOVA $F_{3,184} = 4$ ($P < .007$)
* Significant difference ($P < .05$) Student-Newman-Keuls test.

interpretable and clinically relevant with several items with strong factor loadings explaining 35.9% of the total variance. The majority of items were unique, that is, mainly correlating to a single factor.

3.1. The Three-Factor Solution. The first and strongest factor was comprised of eight items with positive factor loadings (0.47–0.75): "history of stroke", "stepwise progression", "focal neurological symptoms", "abrupt onset", "focal neurological signs", "evidence of associated arteriosclerosis", "history of hypertension", and "fluctuating course". Furthermore, there was one item, "slow progression" with a high negative factor loading (−0.81) and four items with moderately negative factor loadings (−0.25 to −0.43): "dyspraxia, dysphasia, and dysgnosia", "early loss of insight", "early spatial disorientation", and "early amnesia for remote events". Thus factor 1 in several aspects agreed with the structure and scoring of the HIS scale with the exception of "relative preservation of personality" and "nocturnal confusion".

Factor 2 (Table 3) included eight items with positive factor loadings (0.26–0.67): "echolalia, late mutism, amimia", "early signs of disinhibition", "early loss of insight", "progressive reduction of speech", "Klüver-Bucy syndrome", "stereotypy of speech", "logorrhoea", and "irritability, dysphoria", all of them present in the FTD scale. Four items showed negative factor loadings: "relative preservation of personality" (−0.47), "dyspraxia, dysphasia, dysgnosia" (−0.58), "early spatial disorientation" (−0.72), and "early amnesia for remote events" (−0.67). Thus the structure of the second factor agrees with the symptom pattern described in the original FTD scale with the exception of "confabulation".

Finally, factor 3 (Table 3) contains eleven items with positive factor loadings (0.27–0.63): "dyspraxia, dysphasia, dysgnosia," "epileptic seizures of late onset," "increased muscular tension," "myoclonic twitchings," "early spatial disorientation," "early amnesia for remote events," "confabulation," "logoclonia," "nocturnal confusion," "irritability, dysphoria," and "emotional incontinence". Seven of these items belong to the AD scale. However, two other items, "irritability-dysphoria" and "confabulation", belong to the FTD scale, and the two items "nocturnal confusion" and "emotional incontinence" belong to the HIS scale. There was no clinical item with an important negative factor loading in factor 3.

The three-factor solution based on the clinical scoring of 190 patients with NP diagnosis of AD, VaD, mixed AD/VaD, and FTD showed striking similarities to the three previously established short clinical rating scales. Only two of the 30 items, "depression" and "somatic complaints", did not show any factor loading above 0.25 or below −0.25.

Table 2: Prevalence of clinical items (in percent) in four neuropathologically diagnosed dementia groups, AD ($n = 74$), FTD ($n = 52$), VaD ($n = 33$), and mixed AD/VaD ($n = 31$).

	AD	FTD	VaD	Mixed AD/VaD
Slow progression	96	92	24	76
Early loss of insight	43	75	15	41
Early amnesia for remote events	77	8	15	66
Early spatial disorientation	77	2	21	72
Dyspraxia, dysphasia, dysgnosia (all symptoms present to some extent)	84	10	35	66
Logoclonia (stuttering-like speech disturbance)	14	2	0	7
Logorrhea (voluble speech)	8	15	0	7
Progressive reduction of speech	42	79	56	21
Epileptic seizure of late onset	23	6	12	17
Increased muscular tension	57	17	35	24
Myoclonic twitchings	19	0	0	14
Klüver-Bucy syndrome (hyperorality, hypersexuality, utilization behaviour)	8	37	9	4
Early signs of disinhibition	16	79	24	10
Irritability, dysphoria	37	52	35	41
Confabulation, spontaneous	32	14	24	31
Stereotypy of speech	3	25	0	0
Echolalia, late mutism, amimia (during the course)	10	56	3	0
Abrupt onset	10	2	74	21
Stepwise progression	4	6	74	38
Fluctuating course	27	6	79	66
Nocturnal confusion	26	4	15	28
Relative preservation of personality	41	2	62	28
Depression	18	37	44	14
Somatic complaints	27	31	47	31
Emotional incontinence	32	19	47	10
History of hypertension	15	8	65	31
History of stroke	12	4	77	45
Evidence of associated atherosclerosis	18	10	62	41
Focal neurological symptoms	10	14	74	31
Focal neurological signs	15	10	56	41

3.2. The Four-Factor Solution. To test the possibility of additional clinical dimensions for the description and classification of dementia, a four-factor solution was also calculated. This resulted in four strong factors with eigenvalues 5.2, 3.7, 1.9, and 1.6, accounting for 41.2% of the unrotated and rotated clinical variance. Positive factor loadings greater than 0.25 corresponding to $P < .01$ are shown in Table 4.

There were strong similarities between the first three factors of the four-factor solution and the factors of the three-factor solution. All four factors were interpretable as clinically meaningful. The new fourth factor described an interesting clinical dimension including five rather unique items with positive factor loadings, "depression" (0.72), "somatic complaints" (0.55), "emotional incontinence" (0.42), "irritability, dysphoria" (0.40), and "progressive reduction of

Table 3: A Three-factor analysis of 30 clinical items scored in 190 patients with a neuropathological diagnosis of AD, FTD, VaD, and mixed AD/VaD. Factor loadings ≥+0.25 are in bold. Factor loadings ≤ −0.25 are set in italic.

	Factor 1	Factor 2	Factor 3
History of stroke	**0.70**	−0.04	0.03
Stepwise progression	**0.72**	0.05	−0.07
Focal neurological symptoms	**0.75**	0.20	0.00
Abrupt onset	**0.75**	0.02	−0.03
Focal neurological signs	**0.58**	0.07	0.14
Evidence of associated atherosclerosis	**0.49**	−0.12	0.05
History of hypertension	**0.47**	−0.06	0.07
Fluctuating course	**0.52**	−0.21	−0.07
Depression	0.19	0.19	−0.18
Somatic complaints	0.16	−0.04	−0.09
Relative preservation of personality	0.12	*−0.47*	−0.13
Slow progression	*−0.81*	−0.14	0.11
Echolalia, late mutism, amimia (during the course)	−0.13	**0.58**	−0.05
Early signs of disinhibition	0.00	**0.68**	−0.17
Early loss of insight	*−0.25*	**0.40**	0.16
Progressive reduction of speech	0.06	**0.46**	0.00
Klüver-Bucy syndrome (hyperorality, hypersexuality, utilization behaviour)	−0.09	**0.49**	0.03
Stereotypy of speech	−0.11	**0.41**	−0.11
Logorrhea (voluble speech)	−0.13	**0.27**	0.11
Irritability, dysphoria	0.10	**0.26**	**0.27**
Dyspraxia, dysphasia, dysgnosia (all symptoms present to some extent)	*−0.30*	*−0.58*	**0.52**
Epileptic seizure of late onset	0.00	0.03	**0.60**
Increased muscular tension	0.00	−0.09	**0.58**
Myoclonic twitchings	−0.09	−0.04	**0.63**
Early spatial disorientation	*−0.32*	*−0.72*	**0.31**
Early amnesia for remote events	*−0.43*	*−0.67*	**0.32**
Confabulation, spontaneous	−0.03	−0.06	**0.35**
Logoclonia (stuttering-like speech disturbance)	−0.13	0.01	**0.41**
Nocturnal confusion	0.03	−0.17	**0.39**
Emotional incontinence	0.23	0.08	**0.36**
Eigenvalue	**5.2**	**3.7**	**1.9**
Variance %	**17,3**	**12,2**	**6,4**

Table 4: A four-factor analysis of 30 clinical items scored in 190 patients with neuropathological diagnosis of VaD, AD, mixed AD/VaD and FTD. Factor loadings ≥0.25 are in bold. Factor loadings ≤ −0.24 are set in italic.

	Factor 1 Vascular	Factor 2 Frontal	Factor 3 Alz.type	Factor 4 Mood
History of stroke	**0.71**	−0.04	0.02	−0.03
Stepwise progression	**0.67**	0.03	−0.07	0.14
Focal neurological symptoms	**0.74**	0.20	0.00	0.01
Abrupt onset	**0.75**	0.02	−0.04	−0.04
Focal neurological signs	**0.64**	0.09	0.12	−0.17
Evidence of associated atherosclerosis	**0.49**	−0.12	0.04	0.00
History of hypertension	**0.48**	−0.06	0.06	−0.03
Fluctuating course	**0.49**	−0.22	−0.07	0.08
Depression	0.04	0.11	−0.11	**0.72**
Somatic complaints	0.01	−0.10	−0.05	**0.55**
Relative preservation of personality	0.04	*−0.49*	−0.13	0.21
Slow progression	*−0.79*	−0.13	0.11	−0.04
Echolalia, late mutism, amimia (during the course)	−0.11	**0.58**	−0.03	−0.04
Early signs of disinhibition	0.00	**0.68**	−0.14	−0.01
Early loss of insight	−0.18	**0.42**	0.16	−0.18
Progressive reduction of speech	0.01	**0.44**	0.04	**0.25**
Klüver-Bucy syndrome (hyperorality, hypersexuality, utilization behaviour)	0.13	**0.48**	0.06	0.16
Stereotypy of speech	−0.07	**0.42**	−0.10	−0.09
Logorrhea (voluble speech)	−0.02	**0.30**	0.10	*−0.33*
Irritability, dysphoria	0.00	0.22	**0.31**	**0.40**
Dyspraxia, dysphasia, dysgnosia (all symptoms present to some extent)	−0.23	*−0.56*	**0.48**	−0.17
Epileptic seizure of late onset	−0.00	0.03	**0.62**	0.11
Increased muscular tension	0.01	−0.09	**0.59**	0.10
Myoclonic twitchings	0.00	0.00	**0.62**	−0.19
Early spatial disorientation	*−0.31*	*−0.71*	**0.28**	−0.04
Early amnesia for remote events	*−0.38*	*−0.65*	**0.29**	−0.14
Confabulation, spontanteous	0.05	−0.03	**0.34**	−0.21
Logoclonia (stuttering-like speech disturbance)	−0.10	0.02	**0.41**	−0.04
Nocturnal confusion	0.02	−0.17	**0.39**	0.06
Emotional incontinence	0.12	0.05	**0.39**	**0.42**
Eigenvalue	**5.2**	**3.7**	**1.9**	**1.6**
Variance %	**17.3**	**12.2**	**6.4**	**5.3**

Table 5: Rating scales for differential diagnosis of dementia.

Alzheimer's disease scale		Frontotemporal dementia scale		Hachinski Ischemic Score, HIS	
Symptom/item	Score	Symptom/item	Score	Symptom/item	Score
Slow progression	1	Slow progression	1	Abrupt onset	2
Early loss of insight	1	Early loss of insight	2	Stepwise progression	1
Early amnesia for remote events	2	Early signs of disinhibition	2	Fluctuating course	2
Early spatial disorientation		Irritability, dysphoria	1	Nocturnal confusion	1
(impaired sense of locality)	2	Confabulation spontaneous	1	Relative preservation of personality	1
Dyspraxia, dysphasia, dysgnosia, *(all*		Logorrhea, (*voluble speech)*	1	Depression	1
symptoms present to some extent)	2	Progressive reduction of speech	1	Somatic complaints	1
Logoclonia, (*stuttering-like speech*		Stereotypy of speech	1	Emotional incontinence	1
disturbance)	2	Echolalia, late mutism, amimia, *(at*		History of hypertension	1
Logorrhoea, (*voluble speech)*	1	*least two of three symptoms during the*		History of strokes	2
Progressive reduction of speech	1	*course)*	2	Evidence of associated	
Epileptic seizure of late onset	1	Klüver-Bucy syndrome,		atherosclerosis	1
Increased muscular tension	2	(*hyperorality, hypersexuality*		Focal neurological symptoms	2
Myoclonic twitchings	1	*utilization behaviour)*	1	Focal neurological signs	2
Klüver-Bucy syndrome, (*hyperorality,*					
hypersexuality, utilization behaviour)	1				
Total score		Total score		Total score	
Max score 17		Max score 13		Max score 18	

speech" (0.25). Together these five items highlight the clinical importance of a symptom pattern described by emotional feelings and expressions (Figure 1).

Figure 1 shows the mean number of patients (in percent) within each diagnostic group, scoring on each individual item within the respective factor. The vascular, frontal, and Alzheimer type factors showed specific relationship to the respective NP diagnoses, while the symptoms of the mood factor were found in all four NP groups.

4. Discussion

In an earlier publication from our prospective longitudinal study of dementia conditions, three diagnostic rating scales with thirty clinical items were validated against NP diagnoses of dementia. The results showed satisfactory specificity and sensitivity of the rating scales for diagnosis of AD, FTD, VaD, and mixed AD/VaD. The aim of the present study was to further elucidate the structure of the rating scales by factor analysis of the clinical items that were used in the diagnostic process. The scoring was based on direct observations as well as on information from the patient and other informants. This information is also crucial for estimation of the patients' premorbid personality, emotional behaviour, social competence, cognitive profile, education, and clinical changes over time. There are limitations but

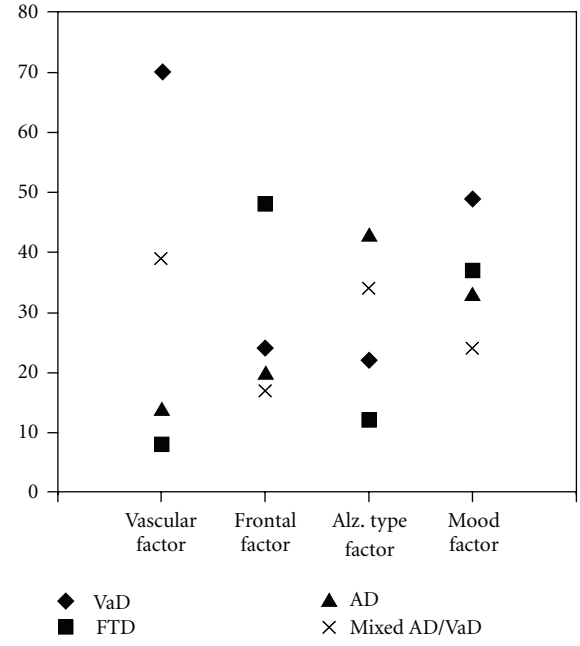

Figure 1: The X-axis depicts the individual factors obtained in the 4-factor analysis presented in Table 4. The points in the graph show the mean number of patients (in percent) within each diagnostic group, scoring on each individual item within the respective factor.

Table 6: Correlations between 30 clinical items in 190 patients with dementia.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	1	.179	.271	.22	.183	.142	.159	.101	.045	.082	.126	.116	.13	−.056	.069	.154	.226	−.733	−.572	−.429	−.022	−.16	−.058	−.078	−.095	−.39	−.5	−.346	−.597	−.352
2	.179	1	−.081	−.081	−.135	.041	.174	.128	.046	−.009	.06	.193	.343	.062	.116	.198	.263	−.169	−.275	−.372	.007	−.367	−.11	−.096	.047	−.131	−.291	−.372	−.234	−.141
3	.271	−.081	1	.554	.479	.172	−.047	−.21	.084	.182	.175	−.21	−.447	−.071	.104	−.149	−.287	−.132	−.163	−.137	.1	.19	−.169	−.036	−.01	.029	−.111	−.026	−.27	−.056
4	.22	−.081	.554	1	.543	.089	−.162	−.273	.202	.095	.175	−.239	−.447	−.114	.177	−.266	−.367	−.081	−.088	.061	.182	.235	−.097	.054	.06	−.02	−.015	.022	−.125	−.031
5	.183	−.135	.479	.543	1	.179	−.048	−.148	.236	.303	.242	−.204	−.357	.059	.151	−.224	−.295	−.12	−.181	.031	.178	.125	−.221	−.123	.003	.007	−.043	.04	−.151	.018
6	.142	.041	.172	.089	.179	1	.143	.057	.117	.182	.126	.175	−.105	.028	.082	−.002	.077	−.038	−.047	−.075	.145	−.052	−.023	−.097	.014	−.007	−.162	.071	−.112	−.107
7	.159	.174	−.047	−.162	−.048	.143	1	−.006	.029	−.035	.096	.082	.225	−.022	.085	.192	.234	−.157	−.119	−.15	−.092	−.168	−.141	−.006	−.065	−.083	−.138	−.055	−.092	−.086
8	.101	.128	−.21	−.273	−.148	.057	−.006	1	.094	.006	−.043	.211	.23	.025	−.061	.17	.375	−.114	−.099	−.158	−.092	−.116	.189	.042	−.013	−.086	−.06	−.001	.024	.025
9	.045	.046	.084	.202	.236	.117	.029	.094	1	.131	.412	.064	−.086	.092	.191	−.07	.013	−.04	−.02	−.047	.107	.053	−.026	.053	.154	.033	−.021	−.026	.017	−.006
10	.082	−.009	.182	.095	.303	.182	−.035	.006	.131	1	.275	−.081	−.198	.095	.083	−.143	.01	−.02	−.022	−.055	.184	.036	.03	−.034	.196	.027	.014	−.019	.024	.118
11	.126	.06	.175	.175	.242	.126	.096	−.043	.412	.275	1	.013	−.078	.059	.101	−.028	−.068	−.079	−.089	−.095	.037	−.107	−.155	−.068	.035	−.099	.011	−.034	−.026	.064
12	.116	.193	−.21	−.239	−.204	.175	.082	.211	.064	−.081	.013	1	.255	.241	−.112	.31	.272	−.183	−.125	−.229	−.084	−.166	.139	−.166	−.073	−.137	−.187	−.224	−.15	−.108
13	.13	.343	−.447	−.447	−.357	−.105	.225	.23	−.086	−.198	−.078	.255	1	.152	.021	.287	.409	−.177	−.119	−.191	−.129	−.299	.046	−.061	.045	−.195	−.198	−.18	−.038	−.125
14	−.056	.062	−.071	−.114	.059	.028	−.022	.025	.092	.095	.059	.241	.152	1	−.042	.073	−.032	−.011	.06	−.007	.169	−.093	.122	−.001	.209	−.047	−.037	.002	−.052	−.082
15	.069	.116	.104	.177	.151	.082	.085	−.061	.191	.083	.101	−.112	.021	−.042	1	.009	−.041	−.003	−.054	.04	.139	−.037	−.052	−.037	.036	.096	−.017	.085	−.038	.004
16	.154	.198	−.149	−.266	−.224	−.002	.192	.17	−.07	−.143	−.028	.31	.287	.073	.009	1	.102	−.151	−.158	−.221	−.086	−.201	.043	−.118	−.098	−.12	−.175	−.133	−.083	−.077
17	.226	.263	−.287	−.367	−.295	.077	.234	.375	.013	.01	−.068	.272	.409	−.032	−.041	.102	1	−.254	−.234	−.316	−.16	−.31	.002	−.082	−.045	−.123	−.234	−.178	−.138	−.128
18	−.733	−.169	−.132	−.081	−.12	−.038	−.157	−.114	−.04	−.02	−.079	−.183	−.177	−.011	−.003	−.151	−.254	1	.523	.415	.028	.253	.008	.06	.075	.401	.512	.328	.551	.362

Table 6: Continued.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
19	−.572	−.275	−.163	−.088	−.181	−.047	−.119	−.099	−.02	−.022	−.089	−.125	−.119	.06	−.054	−.158	−.234	.523	1	.481	−.023	.221	.127	.086	.105	.34	.534	.354	.429	.331
20	−.429	−.372	−.137	.061	.031	−.075	−.15	−.158	−.047	−.055	−.095	−.229	−.191	−.007	.04	−.221	−.316	.415	.481	1	.161	.254	.037	.067	.131	.263	.369	.308	.255	.277
21	−.022	.007	.1	.182	.178	.145	−.092	−.092	.107	.184	.037	−.084	−.129	.169	.139	−.086	−.16	.028	−.023	.161	1	.061	−.097	.002	.102	.057	.033	.151	−.024	.019
22	−.16	−.367	.19	.235	.125	−.052	−.168	−.116	.053	.036	−.107	−.166	−.299	−.093	−.037	−.201	−.31	.253	.221	.254	.061	1	.067	.082	−.033	.138	.203	.143	.084	.024
23	−.058	−.11	−.169	−.097	−.221	−.023	−.141	.189	−.026	.03	−.155	.139	.046	.122	−.052	.043	.002	.008	.127	.037	−.097	.067	1	.296	.119	−.037	.043	.035	.159	−.017
24	−.078	−.096	−.036	.054	−.123	−.097	−.006	.042	.053	−.034	−.068	−.166	−.061	−.001	−.037	−.118	−.082	.06	.086	.067	.002	.082	.296	1	.242	.111	.075	.194	.058	.024
25	−.095	.047	−.01	.06	.003	.014	−.065	−.013	.154	.196	.035	−.073	.045	.209	.036	−.098	−.045	.075	.105	.131	.102	−.033	.119	.242	1	.161	.127	−.039	.082	.071
26	−.39	−.131	.029	−.02	.007	−.007	−.083	−.086	.033	.027	−.099	−.137	−.195	−.047	.096	−.12	−.123	.401	.34	.263	.057	.138	−.037	.111	.161	1	.388	.268	.229	.189
27	−.5	−.291	−.111	−.015	−.043	−.162	−.138	−.06	−.021	.014	.011	−.187	−.198	−.037	−.017	−.175	−.234	.512	.534	.369	.033	.203	.043	.075	.127	.388	1	.366	.495	.516
28	−.346	−.372	−.026	.022	.04	.071	−.055	−.001	−.026	−.019	−.034	−.224	−.18	.002	.085	−.133	−.178	.328	.354	.308	.151	.143	.035	.194	−.039	.268	.366	1	.322	.341
29	−.597	−.234	−.27	−.125	−.151	−.112	−.092	.024	.017	.024	−.026	−.15	−.038	−.052	−.038	−.083	−.138	.551	.429	.255	−.024	.084	.159	.058	.082	.229	.495	.322	1	.526
30	−.352	−.141	−.056	−.031	.018	−.107	−.086	.025	−.006	.118	.064	−.108	−.125	−.082	.004	−.077	−.128	.362	.331	.277	.019	.024	−.017	.024	.071	.189	.516	.341	.526	1

(1) Slow progression, (2) Early loss of insight, (3) Early amnesia of remote events, (4) Early spatial disorientation, (5) Dyspraxia, dysphasia, dysgnosia, (6) Logoclonia, (7) Logorrhoea, (8) Progressive reduction of speech, (9) Epileptic seizure of late onset, (10) Increased muscular tension, (11) Myoclonic twitchings, (12) Klüver-Bucy syndrome, (13) Early disinhibition, (14) Irritability, dysphoria, (15) Confabulation, (16) Stereotypy of speech, (17) Echolalia, (18) Abrupt onset, (19) Stepwise progression, (20) Fluctuating course, (21) Nocturnal confusion, (22) Relative preservation of personality, (23) Depression, (24) Somatic complaints, (25) Emotional incontinence, (26) History of hypertension, (27) History of stroke, (28) Evidence of associated atherosclerosis, (29) Focal neurological symptoms, and (30) Focal neurological signs.

also advantages of the long-term design of this study. There might be certain difficulties to standardize the diagnostic process, both the clinical and the histopathological aspects. In fifteen items, the evaluation was based on the patient's medical history as well as on clinical observations. Twelve items relied on history mainly, and for three items (increased muscular tension, evidence of associated arteriosclerosis, and focal neurological signs) the scoring was almost exclusively based on observations.

An additional limitation to be considered is the sample size. The 190 cases were considered representative of patients referred for clinical examination and diagnosis of dementia disease [11]. The mean age at onset was fairly low probably due to the comparatively large number of FTD cases. Moreover, there was a wide range of the disease duration compared to other studies of postmortem verified dementia. During the time span of the NP examinations in the present study, the procedures and the classification of dementia have developed and changed. The advent of immunohistochemistry in the 1980–90 supplemented the basic neuropathological observations made during the 20 years antedating the mentioned histotechnical advances. Basically these innovations confirmed the originally observed changes rather than adding new features. Still, however, AD, VaD, mixed AD/VaD, and FTD have been the predominant NP diagnoses similar to those in other large studies [23]. Patients with AD pathology probably include cases with additional histopathological presence of dementia with Lewy bodies.

The clinical dimensions were attained and studied with conventional factor analysis. We are contented with the first three factors with high eigenvalues and meaningful clinical constructs based on unique items and "significant" factor loadings. Factor 1 with a strong similarity to the original HIS contained items describing risk factors, clinical course, symptoms, and signs associated with cerebrovascular disease [24]. Factor 2 presented a cluster of clinical features associated with brain dysfunction predominantly involving frontal and frontotemporal brain areas. It has a striking similarity to the consensus on clinical criteria for FTD [25] and frontotemporal lobar degeneration (FTLD) [26]. Finally, the third factor contained cognitive, executive, and neurological symptoms related to hippocampal, temporoparietal, and subcortical structures often involved in AD. Although none of these symptoms are unique for AD, they may strongly contribute to the diagnostic reliability, when appearing in a specific constellation. The factor analyses strongly support the construct validity of the three diagnostic rating scales. Finally the factor analysis also revealed a new symptom cluster characterised by perception and expression of emotions. The rating scales and the factor solutions are recommended for clinical as well as research centre settings.

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Review Article

May "Mitochondrial Eve" and Mitochondrial Haplogroups Play a Role in Neurodegeneration and Alzheimer's Disease?

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Mitochondria, the powerhouse of the cell, play a critical role in several metabolic processes and apoptotic pathways. Multiple evidences suggest that mitochondria may be crucial in ageing-related neurodegenerative diseases. Moreover, mitochondrial haplogroups have been linked to multiple area of medicine, from normal ageing to diseases, including neurodegeneration. Polymorphisms within the mitochondrial genome might lead to impaired energy generation and to increased amount of reactive oxygen species, having either susceptibility or protective role in several diseases. Here, we highlight the role of the mitochondrial haplogroups in the pathogenetic cascade leading to diseases, with special attention to Alzheimer's disease.

1. Introduction

The mitochondrion is a membrane-enclosed cytoplasmic organelle, which has evolved from a primitive aerobic bacteria by means of a symbiotic relationship that started 1.5 billions years ago [1]. These organelles range from 0.5 to 10 micrometers (μm) in diameter and are composed of compartments devoted to specific functions. These regions include the outer membrane, the intermembrane space, the inner membrane, the cristae, and the matrix. Mitochondria are described as "cellular power plants" because they produce most of the cell's supply of adenosine triphosphate (ATP) [2], by means of the oxidative phosphorylation (OXPHOS) machinery, which comprises electron transport chain (ETC) and ATP synthase (complex V). The ETC provides the cell with the most efficient energetic outcome in terms of ATP production. It consists of four multimeric protein complexes (complex I to IV) located in the inner mitochondrial membrane together with complex V [3].

Furthermore, mitochondria are key regulators of cell survival and death. ETC dysfunction leads to reduced ATP production, impaired calcium buffering, and increased generation of reactive oxygen species (ROS) [4]. Mitochondria have their own DNA, the mitochondrial DNA (mtDNA), represented by a circular molecule of 16.5 kb without introns, constituting of a heavy chain (H) and a light chain (L) [3, 5]. mtDNA carries 37 genes: 22 encoding for mitochondrial transfer RNAs (tRNAs) (for the 20 standard amino acids, plus an extra gene for leucine and serine), 2 for ribosomal RNAs (rRNAs) and 13 encoding for polypeptides subunits of complexes of the respiratory chain system, 7 of them belonging to complex I or NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), 1 to complex III or cytochrome c reductase, 3 to complex IV or cytochrome c oxidase (COX I, COX II and COX III), and 2 to complex V or ATP synthase (ATPase6 and ATPase8). These subunits are assembled together with nuclear-encoded subunits. The remaining mitochondrial proteins, including all the complex II subunits, are encoded by nuclear DNA. One mitochondrion can contain two to ten copies of mtDNA. Mitochondrial genetics differs from Mendelian genetics in three major aspects: maternal inheritance, heteroplasmy, and mitotic segregation. Mitochondria are inherited in humans via the female line [3, 5], transmitted as a nonrecombining unit by maternal inheritance [6]. Furthermore, human mtDNA is characterized by a much greater evolutionary rate

than that of the average of nuclear genes. Thus, its sequence variation has been generated by the sequential accumulation of new mutations along radiating maternal lineages. Therefore, mtDNA contains a "molecular record" of the human migrations. We can theoretically follow the transmission of mtDNA from the original "ancestor mother", the "mitochondrial Eve" [7], by identifying common polymorphisms that have been accumulated with time. These common polymorphisms describe classes of continent-specific genotypes, the haplogroups, evolved from the same ancestor, which can be detected by restriction fragment length polymorphism (RFLP) analysis. "Mitochondrial Eve" probably lived in Africa about 200,000 years ago and phylogeographic studies allowed to identify the mtDNA haplogroups tree and the mtDNA migration route (see Figure 1). Because the process of molecular differentiation is relatively fast and occurred mainly during and after the recent process of dispersal into different parts of the world, haplogroups usually tend to be restricted to particular geographic areas and populations.

The basal branching structure of mtDNA variation in most parts of the world is now well understood [6]. The major branches of the tree were usually restricted geographically, some to sub-Saharan Africans, others to East Asians and yet others to Europeans and Near Easterners [8]. African haplogroups fall into seven major families (L0, L1, L2, L3, L4, L5, L6). About 85000 years ago, probably in the Horn of Africa, changes in climate, the glacial interstadial phase 21, triggered the rising of many descendant haplogroups from the root of haplogroup L3, the first multifurcation node, probably because of some colonization event or local population growth [6, 9, 10]. Non-African mtDNA (excluding migrations from Africa within the past few thousand years) descend from L3 and belong either to the M or N superclades. Haplogroup N soon gave rise to haplogroup R. In the Indian subcontinent and in Southeast Asia there is the richest basal variation in the three founder haplogroups M, N, and R, and this suggests a rapid colonization along the southern coast of Asia, about 60000 years ago [6]. Over 30 subclades of the haplogroup M are present in Asia. Haplogroups A, B, C, D, and X have been found in the Americas, coming mainly from Asia. The expansions northwards occurred later, about 45000 years ago when technology and climatic conditions enabled the exploration of the interior of Eurasia. One of the more marginal extensions eventually led to the peopling of Europe [6].

In Europeans and Near Easterners (who share a rather recent common ancestor), nine different mitochondrial haplogroups have been identified (H, I, J, K, T, U, V, W, X). The variation in the basal European mtDNA haplogroups dates to about 45000 years ago [6]. European mtDNA variation is surprisingly impoverished in the number of independent basal lineages, compared with the South Asian mtDNA variation, maybe for the peripheral role that the pioneer migration into the Near East and subsequently Europe must have had in the broader "Out-of-Africa" scenario. Complete mtDNA sequencing and the increasing number of samples analyzed allowed subdividing haplogroups in smaller groups identifying younger branches on the mtDNA evolution tree. Therefore, subhaplogroups classification is continuously evolving [6].

2. Mitochondrial Haplogroups and Medicine

It has been supposed that genetic polymorphisms within the mitochondrial genome might lead to impaired energy generation and to increased amount of ROS, causing subtle differences in the encoded proteins and, thus, minimum changes in mitochondrial respiratory chain OXPHOS activity and free radical overproduction. Increased production of ROS damages cell membranes and further accelerates the high mutation rate of mtDNA. The mtDNA is particularly susceptible to oxidative damage. Because of its vicinity with ROS source (ETC) and because it is not protected by histones and it is inefficiently repaired, mtDNA shows a high mutation rate. Accumulation of mtDNA mutations enhances normal ageing, leads to oxidative damage, and causes energy failure and increased production of ROS, in a vicious cycle. This could predispose to diseases or modify longevity in individual or in a population sharing the same mtDNA genotype. Maybe the opposite could be also true for different polymorphism(s), which could be protective.

Several studies correlate mitochondrial haplogroups with disease's susceptibility or confer them protective roles. In a study of 2008, conducted on 114 healthy Spanish males, Marcuello et al. [11] found that haplogroups J presents with lower VO(2max) (oxygen consumption) than non J variants. J has been related with a lower efficiency of ETC, diminished ATP, and ROS production. Furthermore, the lower ROS production associated to J could also account for the accrual of this variant in elderly people consequent to a decreased oxidative damage [11]. The H haplogroup seemed to be responsible for the difference between J and non-J, and this group also had significantly higher mitochondrial oxidative damage than the J haplogroup, suggesting that ROS production is responsible for the higher VO(2max) found in this variant [12]. In agreement with these results, VO(2max) and mitochondrial oxidative damage were positively correlated. Supporting the hypothesis that J haplogroup may impair the OXPHOS coupling, Pierron et al. [13] demonstrated that J haplogroup was markedly underrepresented among the A3243G mutation carriers. This mutation on the tRNALeu gene (UUR) is one of the most common mtDNA mutation. The phenotypic expression of this mutation is quite variable, ranging from mild to severe clinical phenotypes (MELAS). The authors, in order to explain the epidemiology of haplogroup J and A3243G mutation speculated that this association is lifethreatening, and therefore lethal to the embryo or germ line [13].

One of the most common manifestation of some mitochondrial disease is the presence of retinal pigmentary changes, which are similar to those observed in age-related maculopathy (ARM). Jones et al. [14] demonstrated an association between haplogroup H and a reduced prevalence of this disease, after adjusting for known ARM risk factors.

Haplogroup H1 may be protective for ischemic stroke; in fact, it was found to be significantly less frequent in stroke patients than in controls, when comparing each clade against

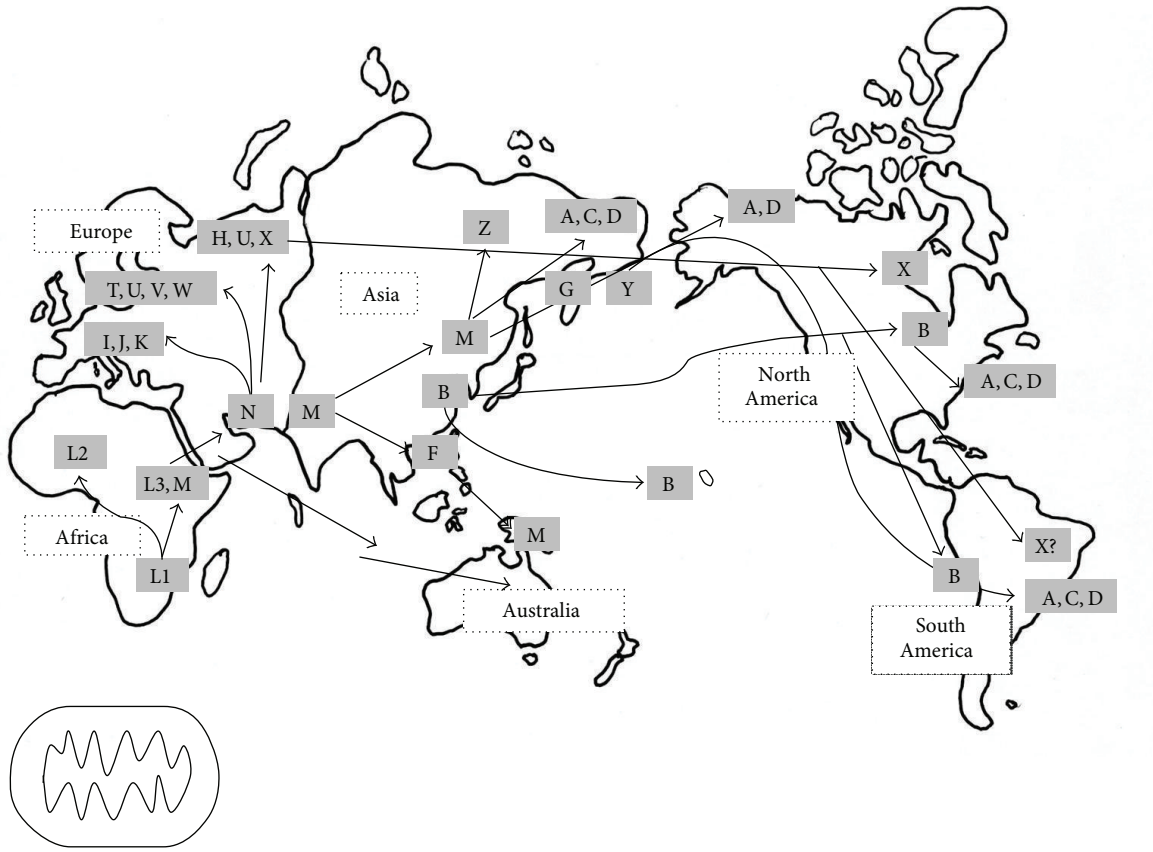

Figure 1: MtDNA haplogroup migration patterns. The map shows the migration patterns of the main mtDNA haplogroups.

all other haplogroups pooled together, in a paper of Rosa et al. [15] focusing on 534 ischemic stroke patients and 499 controls.

In a prospective study of intensive care patients in Newcastle-upon-Tyne conducted by Baudouin et al. [16], haplogroup H seemed to confer an increased chance of long-term survival after sepsis than non-H haplogroups (primarily the closely-related haplogroups J and T) maybe for the linkage between mitochondrial dysfunctions and sepsis-induced multiple organ failure demonstrated by Protti and Singer [17]. Two possibilities have been proposed: haplogroup H might protect through greater heat generation (because of higher electron transport rates or looser coupling) [16], or through greater ROS production (because of tighter coupling and raised protonmotive force), which could reduce bacterial infection [18, 19]. Haplogroup R also was a strong independent predictor for survival advantage in severe sepsis with an important impact on long-term clinical outcome [20]. Haplogroup H has been associated with high spermatozoa motility, haplogroup T, instead, with asthenozoospermia, showing significant differences in their OXPHOS performance [21]. However Pereira et al. [22] did not confirm these findings demonstrating no systematic differences between haplotypes from asthenozoospermic samples and those from population surveys. Haplogroup T could be related, instead, with a lower capacity to respond to endurance training, appearing as a marker negatively associated with the status of elite athletic endurance [23].

Within the Western European, haplogroup J, was associated with 80% decrease in the risk of progression to neuroretinal disorder [24]. Haplogroups U and K were independently associated with a higher prevalence of age-related hearing loss as described by Manwaring et al. [25] on 912 patients of the Blue Mountains Eye Study cohort. mtDNA haplogroup U, in mitochondrial patients carrying mtDNA single macrodeletion, is associated with a higher relative risk of developing pigmentary retinal degeneration, short stature, dysphasia-dysarthria and cardiac conduction defects, modulating the clinical expression of mitochondrial encephalomyopathies due to mtDNA macrodeletions [26]. mitochondrial dysfunctions have long been hypothesized to be involved in tumorigenesis. Supporting such idea, changes in the number, shape, and function of mitochondria but also mutations in both the noncoding and coding regions of the mtDNA have been reported in various types of cancers [27–29]. In a recent study conducted in China, Fang et al. [30] found that macrohaplogroup M and its subhaplogroup D5 had an increased frequency in breast cancer patients relative to controls but haplogroup M was decreased in the metastatic group; macrohaplogroup N are more likely to exhibit metastatic tumors. Haplogroup D4a was associated with an increased risk of thyroid cancer, while

Table 1: A complete list of published researches focusing on mtDNA haplogroups and neurodegenerative diseases.

	Reference	Number of patients	Number of controls	Haplogroups that increase risk	Haplogroups that reduce risk	Sample's region of origin
Parkinson's disease	Van der Walt et al. [40]	609	340	None	J in females, K in individuals older than 70 years	Europe
	Autere et al. [42]	238	183	supercluster JTIWX increase risk both in Parkinson disease and in Parkinson with dementia	None	Finland
	Pyle et al. [41]	455	447	None	cluster UKJT	United Kingdom
	Ghezzi et al. [43]	620	1486	None	K	Italy
	Latsoudis et al. [44]	224	383	None	trend for haplogroups J, T, U and I and the supercluster of haplogroups UKJT to be slightly underrepresented in PD patients	Crete
	Takasaki, [79]	96	96	M7b2, B4e, and B5b	None	Japan
	Simon et al. [45]	168 families	895	None	None	USA (non Hispanic Caucasian)
Amyotrophic lateral sclerosis	Mancuso et al. [46]	222	151	None	I	Italy
	Chinnery et al. [47]	504	493	None	None	United Kingdom
Friedreich's ataxia	Giacchetti et al. [48]	99	48	None	No. However, patients with haplogroup U have a delay of 5 years in the disease onset and a lower rate of cardiomyopathy	Italy
Huntington's disease	Mancuso et al. [49]	51	181	None	None	Italy
	Arning et al. [50]	404	48	haplogroup H associated with a lower age of onset	None	Germany
Multiple sclerosis	Kalman et al. [51]	77	84	K and J	None	Caucasian
	Otaegui et al. [52]	unknown	unknown	unknown	trend for haplogroups JT to be slightly underrepresented in PD patients with multiple sclerosis and multiple sclerosis with optic neuritis	Basque country
	Yu et al. [53]	>2500	>2500	J, 13708A variant	None	Europe (Norway, Spain, Germany, Sardinia, Finland)
	Ban et al. [54]	994	1506	trend for haplogroup U to be overrepresented in patients with multiple sclerosis	None	United Kingdom
	Ghabaee et al. [55]	52	None	Haplogroup A associated with lower age of onset and haplogroup H associated with optic nerve involvement	None	Iran

Table 1: Continued.

	Reference	Number of patients	Number of controls	Haplogroups that increase risk	Haplogroups that reduce risk	Sample's region of origin
Alzheimer's disease	Chagnon et al. [74]	69	83	J	T	Quebec, Canada
	Carrieri et al. [77]	213	179 age-matched and 210 individuals aged more than 100 years	None	K and U seem to neutralize the risk effect of the APOE $\varepsilon 4$ allele	Italy
	Van der Walt et al. [75]	989	328	U in males	U in females	Europe
	Pyle et al. [41]	185	447	None	None	United Kingdom
	Elson et al. [78]	145	128	None	None	United Kingdom and United States (European descent)
	Mancuso et al. [56]	209	191	None	None	Italy
	Fesahat et al. [76]	30	100	H and U	None	Iran
	Takasaki, [79]	96	96	G2a, B4c1 and N9b1	None	Japan
	Maruszak et al. [80]	222	252	HV	None	Poland
	Tanaka et al. [84]	153	129	np956-965 poly-c insertion and 856A>G variant	None	Japan
	Santoro et al. [81]	936	776	H5, especially in subjects younger than 75 years old	None	Italy

no significant correlation has been detected between any mtDNA haplogroups and colorectal cancer. These evidences suggest that mitochondrial haplogroups may have a tissue-specific, but also population-specific and stage-specific role in modulating cancer development [30].

3. Haplogroups and Aging

In 1992, Harman [31] first proposed the "mitochondrial-free-radical hypothesis", a mitochondrial theory of aging, supposing that accumulation of damage to mitochondria and mtDNA leads to aging of humans and animals and affects longevity. The observation that mitochondrial function declines and mtDNA mutation increases in tissue cells in an age-dependent manner supported his theory. Age-related impairment in the respiratory enzymes not only decreases ATP synthesis but also enhances production of ROS through increased electron leakage in the respiratory chain [31]. The association between longevity and mtDNA haplogroups has been long reported [32–34]. This association is probably population-specific, being evident in centenarians from northern Italy (haplogroup J increase the individual chance to attain longevity) but not in those from southern Italy [34]. In nonagenarians/centenarians from Ireland, Finland, and Japan, mtDNA haplogroups and longevity are also associated. In a recent investigation on longevity in a Japanese population, haplogroup D4b2b, D4a, and D5 have been found associated with centenarians (over 100 years-of-age) and haplogroup D4a with semisupercentenarians (over 105 years-of-age) [35], while another variant (mt9055A, haplogroup UK) was found to be significantly more frequent in French centenarians [36]. A higher frequency of the J haplogroup and a significantly high frequency of three mtDNA polymorphisms (150T, 489C, 10398G) have also been reported in Finnish long-lived subjects [37]. Castri and colleagues [38] found that the 5178A mutation in haplogroup D is associated with decreased longevity, whereas the 150T mutation is associated with increased longevity in 152 subjects from Costa Rica [38].

4. Haplogroups and Neurodegeneration

Oxidative "free radical" damage is therefore involved in normal aging, but it is also generally viewed as an etiological cause of tissue degeneration, and ROS damage has been reported within specific brain regions in many neurodegenerative conditions [39]. Basing on the evidence that mtDNA variations lead to different OXPHOS performance, than to different oxidative stress profile, the association between haplogroups and neurodegenerative disorders has been long investigated (see Table 1). Van der Walt et al. [40] observed that haplotypes J and K reduced the incidence of Parkinson's disease (PD) by 50%. A further analysis revealed that the SNP at 9055A of *ATP6* (which defines haplogroup K) reduced the risk in women, and the SNP at 13708A of *ND5* gene was protective in individuals older than 70 years (haplogroup J) [40]. The cluster UKJT was associated with a 22% reduction in risk for PD but not with risk of Alzheimer's disease (AD), confirming that the association with PD was disease-specific and not a general effect seen in

all neurodegenerative diseases [41]. The supercluster JTIWX, indeed, increased the risk of both PD and PD with dementia. This cluster was associated with a twofold increase in nonsynonymous substitutions in the mtDNA genes encoding complex I subunits [42]. In a large cohort of 620 Italian PD patients, haplogroup K was associated with a lower risk for PD [43]. In contrast to these evidences, Latsoudis et al. [44] did not observe mtDNA haplogroups that predisposed to PD in 224 PD patients and 383 controls from Crete. A recent study of 2010 [45] conducted on 168 multiplex PD families in which the proband and one parent were diagnosed with PD and 895 controls, in order to investigate the potential contribution of mtDNA variants or mutations to the risk of PD, found no significant differences in the frequencies of mitochondrial haplogroups or of the 10398G complex I gene polymorphism in PD patients compared to controls, and no significant associations with age of onset of PD. Mitochondrial haplogroup and 10398G polymorphism frequencies were similar in probands having an affected father as compared to probands having an affected mother.

To investigate if specific genetic polymorphisms within the mtDNA could act as susceptibility factors and contribute to the clinical expression of sporadic amyotrophic lateral sclerosis (ALS), our group genotyped predefined European mtDNA haplogroups in 222 patients of clear Italian origin with sporadic ALS and 151 matched controls [46]. Mutations on the entire *SOD1* gene were excluded. The frequency of haplogroups I resulted lower in ALS cases than in controls. Age of onset, severity, and neurological system involved in the disease did not associate with haplogroups. In a comparison developed to test what makes this haplogroup I different from the other haplogroups tested, we found highly significant difference in 16391A and 10034C alleles. In accordance with the described study, mtDNA polymorphisms might contribute to motor neuron degeneration, possibly interacting with unknown genetic or environmental factors [46]. However, this finding was not confirmed by Chinnery et al. [47], who studied large UK cohort of 504 ALS patients and 493 controls and found no evidence that mtDNA haplogroups contribute to the risk of developing ALS.

As far as regard Friedreich's ataxia (FA), Giacchetti et al. [48] studied 99 FA patients and 48 control individuals, all from southern Italy revealing that patients with haplogroup U class had a delay of 5 years in the disease onset and a lower rate of cardiomyopathy. However, no significant difference was found in the frequency distribution of haplogroups between patients and controls [48].

Huntington's disease (HD), as FA, is a trinucleotide expansion disorder, caused by a CAG expansion in the IT15 gene. On the basis of the Giacchetti's report [48], we wanted to determine whether mtDNA polymorphisms play the role of "modifier genes" in HD. In this work, we have genotyped 51 patients with HD and 181 matched controls. Only HD subjects with a similar number of triplets (49.3 ± 5.3, range 45–60) were enrolled. The frequency of the haplogroups and haplogroup clusters did not differ between the two groups, and we did not observe correlation with gender, age of onset, and disease status. Over the last 3 years, patients have undergone prospective evaluations, with a control every 6 months. Different functional scales have been performed. No significant difference was observed between different haplogroups and haplogroup clusters in the cognitive or motor progression of the disease. Therefore, our study did not support the association between mtDNA haplogroup(s) and HD [49]. However, in a recent study, Arning and colleagues demonstrate a significantly lower age at onset in patients carrying the most common haplogroup H [50].

Several lines of evidence suggest that mitochondrial genetic factors may influence susceptibility to multiple sclerosis (MS). Large scale screening of the mtDNA revealed no pathogenic mutations but an association between haplogroups K and J with MS in Caucasians [51]. Another study reported that none of the haplogroups were associated with the disease. There seemed to be a protective trend in the "JT + the protective allele of UCP2" combination [52]. More recently, Yu and colleagues [53] observed that the frequency of haplogroup J was higher in patients than in controls. 13708A variant itself seemed to explain this association and might represent a susceptibility allele for MS [53]. In the same year a study of Ban et al. [54] performed in order to explore this hypothesis further, resequenced the mitochondrial genome from 159 patients with MS and completed a haplogroup analysis including a further 835 patients and 1,506 controls. Haplogroup analysis identified a trend towards an overrepresentation of superhaplogroup U. In addition they also found modest evidence of an association with variations in the nuclear gene NDUFS2 [54]. In 2009, Ghabaee and coauthors studied mtDNA haplogroups, age, gender, clinical disability, course of the disease, and presenting symptoms of 52 MS patients. The prevalent mutations were J, L, and T haplogroups. Haplotype A was more prevalent in patients with younger age of onset and high proportion of haplogroup H was associated with optic nerve involvement. No motor symptoms were seen in haplogroup H patients [55].

5. Haplogroups and Alzheimer's Disease

AD is a brain neurodegenerative disorder named for German physician Alois Alzheimer, who first described it at a scientific meeting in November 1906, presenting the case of "Frau Auguste D." a 51-year-old woman brought to see him in 1901 by her family. Approximately 2-3% of AD cases are early onset and familial, with autosomal dominant inheritance. Mutations in three genes are known to cause familial AD: mutations in the gene of amyloid precursor protein (APP), which is leaved sequentially by b- and c-secretases, and mutations in genes of presenilins 1 and 2 (PS1 and PS2), one or other of which is a component of each c-secretases complex. Although these specific mutations have been associated with the relatively rare forms of familial AD, the causes of the much more frequently occurring sporadic AD remain unknown, and the mechanisms leading to neuronal death are still unclear [56]. Most forms of sporadic late-onset AD have probably a complex aetiology due to environmental and genetic factors, which taken alone, are not sufficient to develop the disease. Presently, age is

the major risk factor for sporadic AD, while the genetic major risk factor is recognized in the presence of allele $\varepsilon 4$ of apolipoprotein E (ApoE4) [57]. However, in the majority of late-onset AD patients, the casual factors are still unknown and genetics factor probably interact with environmental factors or with other pathologic or physiologic conditions to exert the pathogenic effect, such as chronic inflammation [58], excitotoxicity damage [59], oxidative stress [60], and diminished brain metabolism [61]. To date, the "amyloid-β cascade" hypothesis, first proposed in 1992 [62], remains the main pathogenetic model of AD. However, although this cascade is potentially viable in familial AD cases with mutation in APP and PS genes, its role in the sporadic AD is unclear [63].

Although the pathogenic mechanisms of neurodegeneration in AD are not clarified, in the past 15 years, numerous studies have been performed in order to better understand the possible involvement of mitochondria, accumulating evidences suggesting that mitochondrial dysfunction and oxidative stress occur in brain and peripheral tissues of AD patients and supporting the idea that mitochondria may trigger the abnormal onset of neuronal degeneration and death in AD [56].

In order to study the importance and potential causes of mitochondrial dysfunction, the cytoplasmic hybrid ("cybrid") technique, a technique in which mitochondria/mtDNA from human AD and control platelets is transferred to culturable cells depleted of endogenous mtDNA [64], has been applied. The resulting AD cybrids showed a transferred COX defect and revealed a number of consequences that recapitulate the pathology observed in AD brain [65–67]. Remarkably, AD cybrid lines had increased intracellular amyloid accumulation and increased amyloid secretion [65], as well as an increased proportion of morphologically abnormal mitochondria [68]. Trimmer et al. [67] observed that AD cybrid lines show a bioenergetic defect that becomes more severe with passage in culture. AD cybrids also showed elevated spontaneous death with apoptotic nuclear morphology [69], increased cytoplasmic cytochrome c levels and caspase-3 activity [65], and elevated cleavage of caspase substrate [69] not observed in non-AD cybrids. Again, the spontaneous alterations in cell death and cell-death pathways observed spontaneously in AD cybrids can be reproduced by exposing non-AD cybrids to exogenous aggregated beta amyloid or oxidative stress [69, 70]. Despite these reports, a number of technical issues have confounded cybrid studies [71, 72]. However, these studies on cybrid models reinforce the concept that primary mtDNA changes could be responsible for the "mitochondrial features" of sporadic AD and could be the origin of the increased oxidative stress and Aβ deposition found in sporadic AD brain.

With the aim of better clarifing the involvement of mitochondria in the pathogenesis of AD, it has studied the possible association of mitochondrial haplogroups and AD susceptibility. As already discussed, inherited European mitochondrial haplogroups may be related to longevity [37, 73], as well as to neurodegeneration, AD risk, and, thus, death in Caucasians. In a paper of Chagnon et al. [74], haplogroup T is described underrepresented in AD, while haplogroup J overrepresented [74]. Van der Walt et al. [75] showed that males classified as haplogroup U had a significant increase in risk of AD, while females demonstrated a significant decrease in risk with the same U haplogroup. To assess the relationship between mtDNA haplogroup and AD in an Iranian population, Fesahat sequenced the two mtDNA hypervariable segments in 30 AD patients and 100 control subjects. They found that haplogroups H and U are significantly more abundant in AD patients, assuming that these two haplogroups might act synergistically to increase the penetrance of AD disease [76]. By studying an Italian sample of subjects, Carrieri et al. [77] hypothesized that K and U haplogroups may act by neutralizing the effect of the major AD risk factor ApoE $\varepsilon 4$ allele. However, this association was not confirmed recently by a collaborative study performed by Elson et al. [78], in which the authors analyzed the complete mtDNA coding region sequences from more than 270 AD patients and normal controls. The authors described no statistically significant association between haplogroup and disease status. They also observed that for both synonymous and nonsilent changes, the overall numbers of nucleotide substitutions were the same for the AD and control sequences.

Regarding our experience, in our laboratory we did not find any evidence for an etiological role of haplogroups in AD risk [56]. We studied the frequency of the European mtDNA haplogroups in a clinically well-defined group of 209 unrelated patients and 191 controls, both with clear Tuscan origin (in order to minimize the risk of false associations between gene markers and disease). The frequency of haplogroups was not significantly different between the two groups. Furthermore, there was no significant difference between genders as far as mtDNA haplogroups distribution in both AD patients and control groups is concerned. Takasaki, studying a Japanese population of 96 AD patients, described an association between AD and the haplogroups G2a, B4c1, and N9b1. In addition, to compare mitochondrial haplogroups of the AD patients with those of other classes of Japanese people, the relationships between four classes of Japanese people (96 Japanese centenarians, 96 Parkinson's disease (PD) patients, 96 type 2 diabetic (T2D) patients, and 96 nonobese young males) and their haplogroups are also described. The four classes of people are associated with a set of haplogroups, therefore different from those of the AD Japanese patients [79]. A study conducted on a Polish population found that HV cluster is significantly associated with the risk of AD, regardless of the APOE4 status, reporting no evidence for the involvement of haplogroup U, K, J, or T in AD risk [80].

The most recent report about a possible link between AD and mtDNA genotypes shows evidence for subhaplogroup H5 as a risk factor for late onset AD [81]. Santoro et al. analyzed 936 AD patients and 776 controls comparable for age and ethnicity from the central-northern regions of Italy. Haplogroups H3, H4, H5, and H6 [82] are associated with an increased production of ROS than those with haplogroup T. Accordingly, it was hypothesized that subjects with haplogroup H could be more prone to oxidative stress

than those with other haplogroups [18], and consequently more susceptible to neurodegenerative diseases. They found that subhaplogroup H5 appears to be associated with a higher risk of AD in both the total sample and the female group. They also found that subhaplogroup H5 interacts with age in modifying AD risk; in fact H5 subjects younger than 75 years old had a higher AD risk than non-H5 subjects. Age is a strong risk factor for AD [83] and the authors hypothesized that age 75 could be considered as a threshold, under which risk factors such as subhaplogroup H5 and APOE could independently exert their major effect on the development of the disease, while over this age, other risk factors, such as ageing itself, would largely prevail. Tanaka and colleagues also agree that inherited mtDNA common polymorphisms could not be the single major causes of AD but that some rare variants in the protein-coding-region may have protective effects for high-risk populations with the APOE $\varepsilon 4$ allele. The authors however indicate that the np956-965 poly-c insertion and 856A>G variant might be a risk factor for AD [84].

6. Conclusions

In the past 15 years, research has been directed at clarifying the involvement of mitochondria and defects in mitochondrial oxidative phosphorylation in several disorders. It has been speculated that mtDNA mutations that accumulate with age might lead to impaired energy generation and to increased amount of ROS, both resulting in cell damage. Polymorphisms in mtDNA may cause subtle differences in mitochondrial respiratory chain activity and free radical overproduction. This could predispose an individual, or a population sharing the same mtDNA genotype, to an earlier onset of apoptotic processes, such as accumulation of somatic mtDNA mutations and mitochondrial impairment. The opposite could be true for different polymorphism(s), which could be beneficial increasing mitochondrial respiration and/or reducing ROS production [57, 85]. A critical role for mitochondrial dysfunction and oxidative damage in neurodegenerative diseases has been greatly strengthened by recent findings. However, despite the morphological and biochemical evidence of mitochondrial dysfunctions in various tissues of patients with neurodegenerative disorders, the role of the mitochondrial genome and of its haplogroups as a risk factor is still debated. MtDNA haplogroups have been associated with a number of different neurodegenerative diseases, but to date, the only disease consistently associated with a different mtDNA haplogroup frequency is PD. Although it has been suggested that inherited haplogroups K and U may influence AD risk in Caucasians, this is still an unresolved question. To date, mtDNA haplogroups do not seem to play a major role in AD. The mtDNA alterations that cybrid models induce to hypothesize might be due to somatic factors. APP, Aβ-induced mitochondrial toxicity, oxidative stress, somatic mtDNA damage, mitochondrial dysfunction and apoptosis seem to be interconnected in vicious manner that reinforces this dysfunction by causing mtDNA damage, impairment of the mitochondrial respiration and oxidative stress leading to neurodegeneration and dementia. Researches performed in order to allow the identification of AD risk factor also in preclinical stages might be useful in the development of more effective therapeutic interventions at a potentially reversible stage.

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Research Article

Assessing the Sociocultural Impacts of Emerging Molecular Technologies for the Early Diagnosis of Alzheimer's Disease

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Novel technologies for early diagnosis of Alzheimer's disease (AD) will impact the way society views and deals with AD and ageing. However, such "sociocultural" impacts are hardly acknowledged in standard approaches of technology assessment. In this paper, we outline three steps to assess such broader impacts. First, conceptual analysis of the ideas underlying technological developments shows how these technologies redraw the boundary between Alzheimer's disease and normal ageing and between biological and social approaches of ageing. Second, imaginative scenarios are designed depicting different possible futures of AD diagnosis and societal ways to deal with ageing and the aged. Third, such scenarios enable deliberation on the sociocultural impact of AD diagnostic technologies among a broad set of stakeholders. An early, broad, and democratic assessment of innovations in diagnostics of AD is a valuable addition to established forms of technology assessment.

1. Introduction

The early detection of Alzheimer's disease is a widely shared goal in current biomedical research. At many labs and in many hospitals around the world, scientists are working hard to develop knowledge and technologies to enable such early detection. Most attempts are focused on the identification of biomarkers that might indicate early stage Alzheimer's or even predict the disease. Recent recommendations and proposals to adapt the definition of and the guidelines for diagnosing Alzheimer's disease reflect these endeavors. The new guidelines by the American National Institute on Aging and the Alzheimer's Association conceive AD as a three-staged disease process, including a preclinical stage, a stage of mild cognitive impairment, and the final stage of Alzheimer's dementia. The authors of these guidelines suggest that biomarkers can play a role in delineating the preclinical as well as the mild cognitive impairment stage and might be used to increase the certainty that AD pathophysiology is the basis of the clinical syndrome [1, 2]. In the proposals for the DSM V, Alzheimer's Disease is included as a subtype of major as well as minor neurocognitive disorders. In the case of minor neurocognitive disorders, the category most relevant to early detection, the proposal requires not only evidence of memory complaints, but also "supporting evidence for the Alzheimer etiology (e.g., a positive test for a known mutation in an Alzheimer's disease associated gene) or with evolving research, documentation based on biomarkers or imaging" [3]. In both the NIA/AA guidelines and the DMS V proposals, then, molecular biomarkers are expected to play an important role in delineating and diagnosing stages preceding full-blown AD, even though their precise contribution is still unclear.

This does not mean, however, that early detection of Alzheimer's Disease is uncontroversial. Researchers working on such early detection may be confronted with questions about, for example, the value of early detection when a cure for Alzheimer's is still lacking, about the potential medicalization of the ageing process, about the right of potential Alzheimer patients/families to codecide on a technology that might have a great impact on their lives, and about the distribution of liabilities. These are difficult questions to deal

with, and scientists may not be in the best position to answer them. From a societal perspective, however, it is important that such considerations are taken into account before early detection for Alzheimer's disease is introduced at a wide scale.

Health technology assessment (or medical technology assessment) is often used by policy makers to judge the desirability of novel technologies before they are introduced. However, most forms of HTA are limited in scope. They focus on the clinical efficacy of the novel diagnostic or therapeutic tool, its potential risks or side effects, and its efficiency. The fact that those technologies might also have an impact on the organization of medical practice and on society and culture at large, is neglected. Such sociocultural impacts are, of course, difficult to foresee, let alone to quantify. This does not make them less relevant, however. If we want to include such broader impacts, a different type of assessment is needed in addition to the quantitative tools of HTA.

In this paper, we describe how sociocultural impacts (in particular impacts on views on and practices of ageing) could be included in the assessment of emerging diagnostic technologies for Alzheimer's disease. After explaining the practical and theoretical background of the research from which this paper resulted, we will outline the three steps of our approach. First, a conceptual analysis of the ideas underlying technological developments can show how a new type of diagnosis might redraw the boundary between Alzheimer's disease and normal ageing. Second, we investigate how possible futures can be represented to stakeholders. A promising route is to produce imaginative sociotechnical scenarios depicting different possible future ways to deal with AD, ageing, and the aged. Third, we review the possibilities for deliberation on the potential sociocultural impacts. Scenarios like the ones outlined here can be presented to a broad set of stakeholders (including patients, families, medical professionals, medical industry, policy, and insurance companies), which allows an interactive assessment of sociocultural impacts. We conclude that the potential of current innovations in diagnostics of AD merits a democratic assessment, including an early and broad assessment of sociocultural impacts.

2. Practical and Theoretical Background

The work described here originated in the context of a multidisciplinary research project, in which philosophers, ethicists, and social scientists cooperate with a Dutch biomedical research consortium developing molecular tools for early detection of Alzheimer's. The biomedical research project is called the "Leiden Alzheimer Research Nederland" or LeARN-project. Partners in the consortium are Leiden University Medical Centre, VU University Medical Centre, University Medical Centre St. Radboud, Maastricht University Medical Centre, Philips Electronics, Schering Plough (part of MSD), BAC, Cyclotron and Virtual Proteins. The LeARN-project is funded by the Centre for Translational Molecular Medicine. (For more information on the CTMM and the LeARN-project, http://www.ctmm.nl/.) The multidisciplinary project from which this paper originates uses the LeARN-project as a case study, but is independently funded by the Dutch Organization for Scientific Research (NWO). The LeARN consortium aims to develop tools for early diagnosis of AD. Three types of medical technology are used to achieve this goal: PET scans, MRI scans, and CSF analysis. The biomedical project investigates which (combination of) molecular biomarkers are able to detect AD in a sufficiently reliable way. The aim of our multidisciplinary project is to contribute to responsible innovation by anticipating the potential social and ethical aspects of early (molecular) diagnosis of AD as developed within the LeARN project. Our reflections on the potential impact of this type of emerging technologies, however, are relevant to any attempt to find molecular biomarkers for early diagnosis of AD.

One of the starting points of our project is the observation that new biomedical technologies may have a broad set of impacts on medical practice, society, and culture, but that society hardly takes these into account when assessing the desirability of a novel technology. This is unfortunate, both from an ethical as well as a practical point of view. In an ethical sense, responsible innovation means that the innovation at hand should be acceptable to all those for whom something is at stake in the innovation process. And this, in turn, presupposes that these stakeholders have the opportunity to reflect on all types of considerations on the desirability of an innovation [4, 5]. A more democratic innovation process should address this wider set of considerations. On a practical note, anticipating the potential broader impact of a technology helps to prevent unpleasant surprises, or even a backlash, later on [6–8]. As, for example, scientists working on the genetic modification of crops learnt to their dismay, not all innovation that seems useful to science is embraced by a lay public. Many medical innovations as well never make it to daily use in health care because they do not fit the needs and values of their targeted users. Anticipating the impact of emerging technologies in society may contribute to more robust and useful technologies.

This is, however, hard to realize. After all, emerging technologies do not exist yet and are characterized by many uncertainties. It is not clear yet what they will look like, how they will perform, and how they will be used, and this makes it difficult to determine how desirable they might be for future users. It may even be difficult to identify who these users will be. In the case of early detection for AD, for example, it is far from clear which (set of) molecular biomarkers will prove to be sufficiently reliable (if any), how useful they will be for prognosis, who would use this type of diagnostics, and in what way. This might be an argument to first wait and see and not to speculate on things to come before the technology has evolved to the stage of a prototype or can be experimented with. However, in social studies of science and technologies, it is well known that at such a later stage it is much more difficult to steer technology development in a different direction. So, we may be caught in what Collingridge [9] called the "dilemma of control": at an early stage of technology development, it is difficult to foresee what the technology will be like and what it will do, but at a later stage it is too late to effectively steer the development.

One approach developed in science and technology studies to circumvent this problem is "constructive technology assessment" (CTA) [6, 10]. Starting from the assumption that early anticipation of future developments is necessary to enable any attempt at socially orienting technology development, this approach aims to engage stakeholders early on. In such engagement activities, ideals and expectations guiding the work of technology developers can be scrutinized and opened up for broader deliberation, ultimately leading to a broadening of the criteria used to design a socially and ethically desirable technology.

One of the tools used in engagement activities are "sociotechnical scenarios" that tease out the different ways in which technology, society, and culture (including morality) might interact with each other [11, 12]. The use of scenarios has its origin in business strategy and later on also in policy studies [13]. In particular, the famous Shell group developed a methodology to produce and use scenarios [14, 15]. Scenarios are neither predictive, nor completely fictitious. In general, they are used to test whether strategies are robust under unexpected future circumstances. The plural character of scenarios enables business leaders or policy makers to prepare for the future, whatever in the end will be realized. Since they are grounded in both historical analysis and an exploration of how different stakeholders respond to the developments at hand, the narratives can be characterized as products of "controlled speculation" [16]. Using such scenarios in focus groups or interactive workshops with stakeholders enables sensible deliberation on emerging technologies, while at the same time acknowledging the uncertainties involved [10].

The project described here also attempts to create space for stakeholder engagement early in the process of technology development and to provide input for such engagement in the form of controlled speculation on the future. More than other CTA projects, however, it builds on a conceptual analysis of the mutual interaction between concepts of disease (i.e., AD) and ageing on the one hand, and technology development on the other. As argued by Boenink [17], the analysis of emerging shifts in the concepts of disease and health helps to anticipate ethical issues related to emerging biomedical technologies. Whereas the particular direction and performance of an emerging technology may still be uncertain, the underlying framings of disease and health, or normality and abnormality, are often visible at an early stage already. Such conceptual shifts help us to anticipate subsequent shifts in the organization of health care and nonmedical practices, thus opening them up for ethical and social debate. This is particularly true for technologies related to Alzheimer 's disease, since the history of Alzheimer's disease shows how novel diagnostic and therapeutic technologies for AD framed the disease and, by implication, also views of ageing in new and unexpected ways [18, 19]. Moreover, as Joyce and Loe [20] argue, technology in general is an important element in the imagery, expression, and performance of modern ageing. The first step of our approach, therefore, aims to reconstruct the history of Alzheimer's disease as a concept more or less distinct from other diseases as well as "normal ageing," and to investigate how the existing boundaries between AD and ageing might be redrawn by research into the biology of AD.

3. Technology, Alzheimer's Disease, and Ageing

Several authors have reconstructed and analyzed the history of Alzheimer' disease [18, 19, 21]. They all share the observation that the label "Alzheimer's disease" has been interpreted and used in various ways since Kraepelin included it in his nosology of psychiatric disease in 1910. Both scientific and technological developments and developments in society have influenced the way the disease was framed. The evolving definitions, in turn, have significantly determined how society deals with elderly people displaying complaints and signs that tend to be associated with dementia. Definitions of Alzheimer's disease have coevolved with scientific and technological as well as social developments. We will highlight here a few excerpts of this history of interacting technological and sociocultural developments, as a starting point to explore how it might further evolve in the future.

The case Alzheimer himself described in 1907, which would later become the basis for Kraepelin's definition of AD, was about a 51-year-old woman, Auguste D., who displayed clinical features of what was then called "senile dementia." What initially distinguished her case from other cases of senile dementia was her relatively young age. At autopsy, however, Alzheimer observed another difference. The brain tissue displayed not only the plaques often associated with senile dementia. By using a newly developed silver staining technique, Alzheimer also made visible specific neurofibrillary tangles [22]. It soon became clear that these tangles were present in many other cases of senile dementia as well, including those of elderly patients. When Kraepelin defined "Alzheimer's disease" a few years later, he therefore focused on the *clinical* criterion of age; according to his definition, AD was a form of *pre*-senile dementia. This immediately engendered a discussion on the question whether the age difference is sufficient to identify AD as a disease sui generis (Alzheimer himself was among those who thought it was not) and how both presenile and senile dementia relate to ageing processes in general [18]. The identification of AD as distinct from senile dementia seemed to suggest that, whereas the AD was clearly distinct from the normal ageing process, senile dementia might not be so different from normal ageing after all [23].

Notwithstanding such debates, the age-based criterion continued to be used for diagnosing AD till the 1970s. As a result, the prevalence of the disease was not very high. Only after the age criterion was dropped in the 1970s, the number of people diagnosed with AD rose steeply [18]. This evolution from "presenile" and "senile dementia" to "dementia (including the Alzheimer type)" can be understood as the result of converging social and technological developments that had been going on in the 1960s. On the one hand, the position of elderly people in society had changed. With the growing number of individuals living well beyond 65, the "third age" started to be regarded as an attractive period of life. Ageing need not to be feared,

and if it was accompanied by marginalization and stigma, this was due to social processes that should be changed. The concept of "ageism" was coined in 1968 by gerontologist Robert Butler to denounce all attempts to stereotype and discriminate people just because they are old. From this perspective, labeling elderly people as "senile" often was an easy way not to take them seriously. It became politically relevant to distinguish the broad, pejorative use of the term senility from a careful biomedical diagnosis of dementia. Such a diagnosis would at the same time help to protect the golden shine of the third age against the gloomy shadow of deterioration during the fourth age.

On the other hand, several developments in science and technology intensified the search for a correlation between the clinical symptoms of senility and pathological signs in the brain [24]. The invention of novel counting methods for the number of plaques and tangles in brain tissue, and the emergence of electron microscopy, enabled novel takes on the pathology related to AD and senile dementia. The counting methods were thought to show that the density of plaques relates to the severity of the clinical phenomena and thus to make a case for a clear biological substrate for these symptoms. Electron microscopic investigations of plaques and tangles revealed that these had different morphological features. The plaques were identified in 1964 as consisting of amyloid; the substance of the tangles would remain obscure for some more time. The findings raised all kinds of novel hypotheses with regard to the biological process underlying the formation of plaques and tangles and their role in causing the clinical symptoms. Although the causal process itself was not clear at all, the idea gained ground that dementia and Alzheimer's disease were essentially biological processes and should be diagnosed and treated on that level [23]. As a result, the relative importance of clinical symptoms and biological signs was reversed. The presence of brain pathology became a prerequisite for diagnosing dementia in general (including Alzheimer's) [18].

Another significant shift in the framing of Alzheimer's disease and ageing occurred in the 1970s and 1980s, when neurotransmitters became an important focus of interest, resulting in a strong focus on memory complaints. The interest in neurotransmitters was due to developments in therapeutic technology, which started with the observation that drugs blocking cholinergic activity in the brain (prescribed to women in child birth) produce dementia like memory disruptions. This was further developed into the hypothesis that a lack of acetylcholine in the brain led to neuronal death and subsequently to problems with short term memory—the so-called "cholinergic hypothesis." This hypothesis led to several attempts to develop drugs countering this cholinergic deficit, resulting in cholinesterase inhibitors like tacrine. The effects of these drugs were limited, because they only slow down the degradation of acetylcholine. When a substantial number of neurons have died, the effect of the drugs will decrease. Accordingly, cholinesterase inhibitors can only be effective in the early stages of dementia.

What is striking in this episode from the history of Alzheimer's disease is that the complex phenomenology of the disease was narrowed down to memory loss. Whereas the definition of AD from the start had included a broad set of symptoms, including other cognitive problems and personality problems, scientific and clinical attention now focused on the loss of memory functions. Since deteriorating memory is associated with ageing anyway, this narrow focus invited a reconnection of AD and ageing. This connection is still visible today, in the often heard half cunning, half anxious comment that "this shows I'm off on the Alzheimer track" when a (in all likelihood minor) slip of memory has occurred. The promises of drugs slowing down the process of memory loss thus lead to an increased social anxiety about forgetfulness, and a concurrent neglect of the other symptoms earlier is thought to be correlated with AD and dementia.

The most recent episode highlighted in the history of AD is the flurry of genetic and subsequent molecular research that started in the 1990s. With the invention of transgenic mouse models and the rise of genetic research in humans, the hunt was open for "the" gene causing AD. As with most other diseases, results were more modest than hoped for. To date, four genes have been identified that are related to AD. Three of them (called APP, presenilin-1, and presenilin-2) are linked to cases of AD beginning at an early age, and they are usually transmitted in an autosomal dominant way. These monogenetic variants of AD account for less than 1% of all AD cases. A fourth gene (APOE) is associated with an increased risk of AD in the general population (usually also associated with early onset of the disease) and is thus one cause among others in a multifactorial variant of AD [25]. Here, as before, the novel technologies engendered a shift in the conceptualization of AD, now producing a distinction between genetic (or familial) and sporadic AD, which seemed to be associated with, but not identical to, a distinction between early and late onset AD. The identification of specific genetic risk groups has driven attempts to actively screen individuals from such families with offers of genetic testing; however, most countries do not offer population screening for the common late-onset form of AD [26].

Even though the genetic variants of AD do not seem to be very prevalent, molecular research into the function of the APP gene (and later also into the presenilin genes) led to an influential hypothesis about the causal pathway underlying AD: the amyloid cascade. The idea that AD is a disease with progressive stages had been around for decennia [23]. The amyloid cascade hypothesis gave it a clear pathological foundation, by tracing the presence of amyloid plaques and the subsequent neuronal death back to genetic alterations causing excessive production of Aβ42 protein [27, 28]. The hypothesis, although not without critics and having evolved substantially since it was first proposed in 1991, is quite influential in current AD research. It has strongly reinforced thinking of AD as a gradual process, in which biological changes precede clinical manifestations. This helped to make sense of the long standing finding that some individuals who during life did not display any clinical symptoms, at autopsy do show the plaques and tangles characteristic for AD. The conceptualisation of "a-symptomatic AD" in turn stimulated proposals to develop molecular tools for early diagnosis of AD.

Despite the recent emphasis on the biological processes of AD, several authors stressed the importance of clinical manifestations as well. Petersen et al. (1999) showed that there is a group of people experiencing cognitive decline, who do not satisfy current diagnostic criteria for AD. Analogous to the presupposition of a gradually developing process on the molecular level, they proposed to use the label of "Mild Cognitive Impairment" for this group and suggested that it may be a transitional stage between normal functioning and dementia in general or AD in particular [29]. The value of this concept is currently highly debated, as it is unclear how it might relate to biological processes [30]. It is clear, however, that current thinking on AD both on the molecular and the clinical level tends towards conceptualising the disease in terms of a gradual process with signs and symptoms that become more manifest as time progresses.

4. The Future of AD and Aging: Some Scenarios

Given these ongoing changes in the clinical practice and understanding of AD, the question is how the envisioned early diagnosis will affect the social and cultural meaning of the disease and of aging in general. To assess such broader impacts, we developed "possible futures" or scenarios serving two goals. First, they invite reflection on the broad range of impacts technological development in the field of AD diagnostics may have. In addition, they enable early and public deliberation on the desirability of such impacts, as well as identification of the conditions for creating a desirable future. When subjected to debate among a broad set of stakeholders, such scenarios can help to democratise technological development and steer it in a socially and ethically desirable direction, thus contributing to a responsible innovation process.

The scenarios presented here share a general starting point: they presuppose that attempts to develop biomedical technologies for early detection of AD, in one form or another, will not suddenly disappear. Their form and societal impact will depend, however, on the actual path of development and their success, as well as their embedding, all of which are unknown right now. These uncertainties can be reduced to some extent, however, by three observations based on the history of AD and ageing presented above. First, promises and expectations with regard to explaining and curing AD, even when widely shared in the research community, often do not materialize. Second, up to now, it has been difficult to explain AD in terms of a single, linear causal pathway. And finally, opening up bodily processes that were formerly invisible often produces a fragmentation of what counts as "normal bodily functioning."

Starting from these general observations and inspired by the specific conceptual and historical developments sketched in Section 2, we developed three scenarios. In the first, we explore what might happen if current attempts to identify molecular biomarkers for AD are successful. The second scenario, in contrast, outlines a future in which it proves to be difficult to associate AD unequivocally with underlying biological processes. The third scenario depicts a future (further away in time than the first two) in which initial failures to identify informative biomarkers lead to novel technological developments.

4.1. Scenario 1: Forgetfulness as a Biological Problem. This scenario starts from the assumption that the promise of molecular research is indeed realized: (a set of) biomarkers is/are identified that neatly distinguishes a specific group of people from others, in terms of their chance to develop clinically manifest AD. Not only a substantial part of those patients diagnosed with AD score positive on the biomarker, but also many of those with MCI. This greatly reinforces the legitimacy of the MCI label, which until recently was still contested as a pseudodisease. In due time, research shows that those MCI patients scoring positive on the biomarker test are significantly more likely to develop full-blown AD in the years to come. This motivates several memory clinics to experimentally introduce the biomarker test in their diagnostic workup. There is some professional and societal debate, however, whether it is ethically sound to offer this test in the absence of a cure for AD. What use is it to know that you are off on a prospect of gradual decline if nothing can be done about it? Some neurologists argue that the test may offer reassurance to those who test negative. Psychiatrists and gerontologists point out that although a cure is lacking, therapeutic options and different care arrangements are available. Knowing your biomarker status may help to make informed decisions with regard to the future. In The Netherlands, some voices claim that a positive biomarker test might enable people to lay down clear advanced directives at what stage they consider their life to have become worthless. This might help to solve the difficult situation AD patients applying for legal euthanasia. Currently, such requests are usually rejected, either because the patient's situation is not yet clearly hopeless, or because his/her mental capacities have deteriorated to such an extent that the request is not considered autonomous anymore. Others respond, however, that this is exactly the reason to abstain from biomarker testing. In the end, the novel professional guidelines for AD diagnostics advise to perform a biomarker test, but only when the patient is well informed about the implications, and when post test counselling is provided in case of a positive test result.

As memory problems become clearly linked with biological functioning, subjective experience of one's cognitive functioning is more and more distrusted. Physicians are confronted with an increase of middle aged individuals wondering whether their cognitive functioning is deteriorating. Such worries are reinforced by self-tests for regular cognitive checkups available on the internet, aiming to sell training programs which promise to improve cognitive functioning. Some employers are quick to offer cognitive check-ups as a service to their employees, stating that testing is not meant to demote anybody, but might help to adjust the working environment to one's evolving capabilities. In general, however, there is a poor relation between the results of such tests and the biomarker test. Physicians, therefore,

denounce these tests and advise to rely on medical diagnosis only.

In due time, the drugs with a modest effect only on AD patients are found to effectively prevent memory complaints in asymptomatic individuals who score positive on the biomarker test. This reinforces the demand for testing. It also leads to a medicalization of memory loss and an increase in social norms for cognitive functioning. Those who can no longer live up to these demands are even slightly blamed, because they could have opted for biomarker testing and drug treatment earlier. The government and employers start campaigns promoting "healthy ageing," in which forgetfulness is presented as one of those annoying phenomena from the past. Ultimately, almost everyone above age fifty, only a few stubborn people excepted, starts using the biomarker test as a regular checkup. The test now actually serves as the new golden standard for diagnosing AD. Neuropsychological tests for diagnosing AD consequently almost go out of use.

Only after a substantial number of years, psychiatrists observe that the ratio between patients with dementia and those with other diseases in mental hospitals and nursing homes has changed. The huge number of AD patients has decreased because people seek help earlier and memory complaints can be treated quite effectively. The number of patients displaying behavioural and personality disorders has increased, however, partly because these phenomena are now no longer associated with AD. The success of the biomarker tests, moreover, has decreased the funding as well as the attention for these other complaints, resulting in a nursing home population that is difficult to deal with. Physicians and nurses willing to work at such institutions are scarce; in particular the young ones shy away from a confrontation with a part of humankind that so obviously does not satisfy what one would expect humans to be.

4.2. Scenario 2: Biomarkers as an Add-On to the Diagnostic Toolbox. Despite the amount of time, work, and money spent on the identification of biomarkers for AD, the high expectations of AD research at the beginning of the 21st century do not materialize. MRI techniques seem to be able to single out some groups of patients highly likely to develop full-blown AD, but this targets only a very small subset of the whole AD population. Moreover, quite a few patients are excluded from having a 7T MRI because they have a pacemaker or other metal implants. Insurance companies are not willing to pay for MRI scans with an apparently low added value, thus in fact blocking the inclusion of this technology in the diagnostic workup. Biomarkers identifiable in CSF do slightly better: a CSF test combining several biomarkers does identify a large subset of MCI patients likely to develop AD. However, whereas it predicts quite well the onset of memory complaints, there is no clear correlation with other symptoms of AD. At consensus meetings to prepare novel professional guidelines for AD diagnostics, there is huge controversy whether or not to include the CSF test. Ultimately, most countries include the test in the guidelines, but only in conjunction with other laboratory and clinical tests aiming to identify the full spectrum of potential AD symptoms.

With the increase of potential tests included in the diagnostic workup, however, the chances of test results contradicting each other increase as well, complicating the diagnostic process. Some medical professionals start talking in terms of "proven" and "suspected" cases of AD—with proven referring to the presence of amyloid that is made visible in vivo for the first time in those that test positive on the CSF test. Others argue that it would be more precise to distinguish "amyloid deposit disease" (ADD) as distinct from AD and MCI. Pharmaceutical companies that have invested a lot of money in research how to counteract the amyloid cascade organize clinical symposia focusing at the novel ADD phenomenon, but they cannot hide that the results of their research efforts are as yet very limited.

On a less grand scale, research into the role of lifestyle and environmental factors in causing AD continues. The lack of clear categories and associated therapies does engender a lot of confusion and anxiety among elderly. Patient organizations for AD start campaigns to promote regular cognitive check-ups for elderly (using the slogan "Don't forget to check your memory!") and make such cognitive self tests available on their website. If the result is below average, people are advised to see a physician and ask for AD diagnostics including the CSF test. This medical orientation is contested by some physicians, elderly, and family members of AD patients, leading to a split in the patient organization. The mission of the novel patient organization is to help people live well with dementia. This organization supports projects designing new forms of housing or implementing new ways of working with elderly in organizations, in due time claiming some successes in improving quality of life for both patients and their social environment. Most elderly experiencing cognitive or behavioural problems, however, are disappointed if they do not test positive on the CSF test but receive the label MCI or "suspected" AD instead. They feel they are sentenced to a vague but deadly disease, without any prospect of a future cure. Moreover, both their informal caregivers and the biomedical professionals start treating them as if they were completely helpless already. "It's really annoying," a lady from the patient organization says. "Most of my friends have given a key of their house to the neighbours, in case they might forget their key. But if I stand some time in front of my door to find my key, my neighbour comes out and says: Did you forget your key again? Let me open the door for you!"

4.3. Scenario 3: The Normal Becomes Personal. Ongoing AD biomarker research definitely produces a huge increase in data on individual bodily functioning, but it is difficult to identify biomarkers that are sufficiently informative about chances of developing AD. One of the problems is that many biomarkers characterise very small groups only. Moreover, those biomarkers that do identify substantial groups at risk of AD often lead to conflicting results, which makes them difficult to interpret. This lack of clear progress makes funding organizations wary to finance more biomarker research, and for some time developments in this domain

come to a halt. Developments in the technology platforms enabling biomarker tests do continue, however.

An important step is made when blood serum proves to be as reliable as source of biomarkers as CSF. In addition, lab-on-a-chip technology or even ingested sensors that communicate measuring results to a computer system become available, making it possible to regularly perform biomarker tests in a way that is hardly burdensome to the test subjects. This motivates some researchers to start monitoring a set of biomarkers in healthy individuals and MCI and AD patients. When this ambitious and time-consuming study is completed, huge variations within these groups (instead of between them) come to the fore. Subsequent prospective research, with a larger set of healthy individuals, points out that what is normal for one may be quite abnormal for another person. On the long term, however, deviations from one's personal pattern prove to be informative with regard to a person's chances to develop complaints related to AD.

After many years, then, the procedure for diagnosing AD is radically transformed. Instead of having a one-time set of tests in the memory clinic, people are sent home with a monitoring device. What is more, physicians and patient organizations alike start urging middle-aged people to have the personal pattern of their AD biomarkers established before decline sets in, because only this will provide a good reference point for later diagnosis. The roles and responsibilities of clinicians and patients are radically redistributed, with the latter ones taking an active part in diagnosis. Some patients (or rather clients, since they are healthy individuals) become quite good at interpreting their test results in view of their own functioning, deciding for themselves which deviations are significant and which ones are not. When changes are deemed significant, a set of therapeutic options is advised. Next to the prescription of drugs (which still have a limited effectiveness), clients are urged to continue working and be productive as long as possible, exercise regularly, and eat healthy.

In general, people become more aware of their own ageing process. Since they are regularly confronted with changes in bodily parameters, they become also more keen on identifying changes in their physical, cognitive, and behavioural performance. Clinicians have a hard time, though, first of all because the role and influence of medical professionals is on the wane. Large groups of people self-confidently assert that they are very well capable of managing their own health. Commercial companies are all too happy to offer assistance: the market for web-based health monitoring tools, personal health advice, and healthy ageing coaches is booming. But even if people do seek medical advice, it is difficult to offer it in an evidence-based way. Deciding whether, when, and how to intervene becomes much more complex, since scientific research is hardly able to keep up with the data produced by ubiquitous and permanent monitoring. Although, as some reflexive clinicians note, this might be more like an illusion lost than a real change. With the benefit of hindsight, they state, past medical interventions now can be seen for what they always were: experiments lacking a sound scientific basis.

5. Deliberating Sociocultural Impacts

As said above, scenarios like the three presented here can be used to broaden the imagination as well as the deliberation of those who are involved or may be involved in the future. The scenarios also enable discussion of what is actually problematic in the current situation: what are the problems and needs surrounding AD and how might the attempt to detect AD early affect those problems and needs? Such exercises can be organised with stakeholders in various ways [31, 32]. Generally defined, stakeholders are (groups of) actors who are invested in a particular outcome of the envisioned technological developments, that is, they have something to gain or to lose from it [33, 34]. This is no doubt the case for patients (in particular future ones), clinicians (physicians, nurses, and all kinds of paramedics) involved in the diagnosis and care for AD patients, family members and other informal care givers, the technology developers (scientists, industry), clinical guideline developers, and insurance companies. But as the scenarios imply, impacts may be felt as well by the elderly in general, those caring for the elderly, employers, policy makers, and in the Dutch case even the committees assessing euthanasia cases. When inviting stakeholders for engagement activities, choices will have to be made; a careful balancing of the number of participants and their respective backgrounds is necessary to ensure an even handed deliberation process. What is feasible depends, of course, on the design of the deliberation (discussed below). In addition to the question which groups to include, the question how to find representatives of the groups to be included deserves attention. After all, not all individuals may be representative of the group they are supposed to speak for. In the case of AD, in particular, the inclusion of patients requires careful consideration. If it is feasible to find patients diagnosed with AD willing to participate, these will usually be at an early stage of the disease. To make sure that the voice of those familiar with later stages is also heard, inclusion of family members, nurses, or patient organizations should be considered.

Once stakeholders have been identified, the question is how to elicit their responses [32]. One method for doing this is to convene so-called "focus groups": homogeneous groups of a particular type of stakeholders. In this case, several meetings with different groups are held, to probe the considerations of, say, patients and elderly, clinicians, and informal care givers, respectively. Having separate deliberations with these groups has several advantages. First, it enables the inclusion of a larger number of representatives of a specific group, thus possibly disclosing a variety of viewpoints among this group. In addition, it will be easier to discern to what extent specific considerations are widely shared within a specific group of stakeholders. Finally, in this setting, it will often be easier to create an atmosphere of trust, stimulating participants to voice all their considerations without worry about being overwhelmed by others. This may hold particularly for patients and caregivers. In general, then, focus groups enable the disclosure of a rich variety of considerations and viewpoints. Moreover, they lend themselves very well to a phased setup, in which first the problems with,

as well as the good qualities of the present situation can be explored, before responses to ongoing developments are probed by presenting scenarios like the ones outlined above. The ultimate goal of any focus group is to list the conditions future developments should satisfy for a specific group of stakeholders to accept these developments. The conditions identified can be technological, social, organizational, financial, or cultural. As a result, who will be the addressees of the focus group's conclusion is an open question.

Another method for probing stakeholders' responses, which may be combined with the focus group method, is to bring different stakeholders together in a larger interactive setting. Since different groups of stakeholders may radically differ from, or even contradict, each other, it can be difficult to weigh or prioritize outcomes of separate focus groups. Moreover, it is unclear who is in the best position to perform this weighing. In addition, the moral legitimacy of all stakeholders' considerations can increase when these are checked and judged to be legitimate by a larger set of stakeholders. In an interactive, multistakeholder setting, chances will increase that the ultimate conclusions not just seek to further the interests of a specific group, but are based on widely shared values. Interactive deliberation enables the production of truly ethical judgments. Finally, the opportunities for creative thinking on how to address the legitimate concerns of specific stakeholders may increase, thus contributing not only to a well-considered problem analysis, but also to possible solutions or improvements. The actual process of deliberating the different scenarios will not differ very much from the one used in focus groups, although in an interactive setting the person chairing the session will have to be even more diligent in explicitly connecting participants to further listening and a serious consideration of all that is brought to the fore.

If feasible, then, having several focus groups first before bringing stakeholders together in an interactive workshop might combine the best of both worlds. In addition, a thorough exploration of stakeholders' views on the present situation of AD diagnostics (in a descriptive and an evaluative way) will help to keep the discussion focused on what needs to be done here and now, as well as to improve the understanding of participants' responses to the scenarios in the subsequent phase. By explicitly returning to the diagnosis of the present after having deliberated the imagined futures, these deliberations are most likely to result in conclusions as to what needs to be done, by whom, here and now to ensure responsible innovation.

6. Conclusion

In view of the current investments pursuing molecular technologies for early diagnosis of Alzheimer's disease, as well as the recent proposals for expanding diagnostic categories in guidelines for diagnosing this disease, it is important to evaluate the broader impacts these developments may have (and to some extent already have) on society and culture. We have argued that such an assessment might enhance the democratic character of technology development and lead to more robust technologies. We also indicated that, to be effective, such an assessment should take place at a relatively early stage of technology development. This paper outlines how one might proceed in three steps, focusing in particular on impacts on the way society views and deals with ageing. First, a conceptual analysis of the history of AD and aging provides valuable insights in the broader impact of AD diagnosis. Secondly, these insights can be used to create imaginative, plausible scenarios about the future, which might then, thirdly, be used as input for deliberation among stakeholders via focus groups and broad interactive workshops. Ultimately, such a process will help to identify the conditions for responsible innovation of AD diagnostics, by enabling innovators and societal stakeholders to become mutually responsive to each other's needs and values.

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Research Article

Genomic Copy Number Analysis in Alzheimer's Disease and Mild Cognitive Impairment: An ADNI Study

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Copy number variants (CNVs) are DNA sequence alterations, resulting in gains (duplications) and losses (deletions) of genomic segments. They often overlap genes and may play important roles in disease. Only one published study has examined CNVs in late-onset Alzheimer's disease (AD), and none have examined mild cognitive impairment (MCI). CNV calls were generated in 288 AD, 183 MCI, and 184 healthy control (HC) non-Hispanic Caucasian Alzheimer's Disease Neuroimaging Initiative participants. After quality control, 222 AD, 136 MCI, and 143 HC participants were entered into case/control association analyses, including candidate gene and whole genome approaches. Although no excess CNV burden was observed in cases (AD and/or MCI) relative to controls (HC), gene-based analyses revealed CNVs overlapping the candidate gene *CHRFAM7A*, as well as *CSMD1*, *SLC35F2*, *HNRNPCL1*, *NRXN1,* and *ERBB4* regions, only in cases. Replication in larger samples is important, after which regions detected here may be promising targets for resequencing.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia and accounts for 50–80% of dementia cases. Currently, an estimated 5.3 million Americans have AD, the seventh leading cause of death in the United States. The hallmark abnormalities of AD are deposits of the protein fragment amyloid β (plaques) and twisted strands of the protein tau (tangles). Amnestic mild cognitive impairment (MCI) is a clinical condition in which a person has problems with memory, with or without other cognitive deficits, that are noticeable to others and show up on psychometric testing but are not severe enough to interfere significantly with daily functioning. About 14–18% of individuals aged 70 years and older have MCI, and these individuals are likely to progress to dementia, particularly AD, with an annual conversion rate of 10–15% [1].

Genetic factors play a key role in the development and progression of AD. AD has a high heritability, with 58–79% of phenotypic variation estimated to be caused by genetic factors [2]. Early-onset AD (onset < 65 years) accounts for a small percentage (<5%) of cases and is primarily caused by mutations in three genes that affect the cerebral levels

of amyloid β peptide: *AβPP* (amyloid-β protein precursor) on chromosome 21, *PSEN1* (presenilin 1) on chromosome 14, and *PSEN2* (presenilin 2) on chromosome 1 [3]. Late-onset AD (LOAD) accounts for the majority of AD cases, but only the e4 allele of the *APOE* (apolipoprotein E) gene on chromosome 19 has been consistently replicated across studies. Recently, three large genome-wide association studies (GWASs) identified five additional loci: *CLU* (clusterin), *CR1* (complement component (3b/4b) receptor 1), *PICALM* (phosphatidylinositol binding clathrin assembly protein), *BIN1* (bridging integrator 1), and *EXOC3L2* (exocyst complex component 3-like 2) to be strongly associated with AD [4–6]. These loci also showed strong association in replication studies [7], further supporting a role in AD susceptibility.

Copy number variants (CNVs) are segments of DNA, ranging from 1 kilobase (kb) to several megabases (Mb), for which differences in the number of copies have been revealed by comparison of two or more genomes. These differences can be copy number gains (duplications or insertional transpositions), losses (deletions), gains or losses of the same locus, or multiallelic or complex rearrangements. CNVs have been implicated in various neuropsychiatric disorders such as autism and schizophrenia [8]. To date, the role of CNVs in LOAD has only been examined in one study [9]. These authors performed a genome-wide scan of AD in 331 dementia cases (in which >80% of patients had a clinical diagnosis of AD) and 368 controls. Although no CNVs, which are typically rare occurrences, were significant at genome-wide threshold, Heinzen et al. were able to identify a duplication in the *CHRNA7* gene warranting further investigation. To date, no study has looked at the role of CNVs in MCI.

In the present report, we conducted a preliminary CNV analysis using genotype data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort to examine the role of CNVs in susceptibility to MCI and LOAD. ADNI is an ongoing multiyear public-private partnership to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), genetic factors such as single nucleotide polymorphisms (SNPs) and CNVs, other biological markers, and clinical and neuropsychological assessments can be combined to improve early diagnosis and predict progression of MCI and early AD. Here, we used the genome-wide array data acquired on the ADNI cohort to determine whether AD and MCI participants (cases) showed an excess burden of CNVs relative to controls and to characterize any genomic regions where CNVs were detected in cases but not controls.

2. Materials and Methods

2.1. Alzheimer's Disease Neuroimaging Initiative. The ADNI was launched in 2004 by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private pharmaceutical companies, and nonprofit organizations, as a $60 million, multiyear public-private partnership. The Principal Investigator of this initiative is Michael W. Weiner, M.D., VA Medical Center and University of California—San Francisco. ADNI is the result of efforts of many coinvestigators from a broad range of academic institutions and private corporations. Presently, more than 800 participants, aged 55 to 90, have been recruited from over 50 sites across the US and Canada, including approximately 200 cognitively normal older individuals (i.e., healthy controls or HCs) to be followed for 3 years, 400 patients diagnosed with MCI to be followed for 3 years, and 200 patients diagnosed with early AD to be followed for 2 years [10]. Longitudinal imaging, including structural 1.5T MRI scans collected on the full sample and [^{11}C]PiB- and [^{18}F]FDG-PET imaging on a subset, and performance on neuropsychological and clinical assessments were collected at baseline and at followup visits in 6–12-month intervals. Other biomarkers are also available including *APOE* and whole genome genotyping on the full ADNI sample, and longitudinal cerebrospinal fluid (CSF) markers on a subset of the sample. Written informed consent was obtained from all participants, and the study was conducted with prior institutional review boards approval. Further information about ADNI can be found in [11] and at http://www.adni-info.org.

2.2. Participants. Participants in the present report included 655 non-Hispanic Caucasian individuals from the ADNI cohort who had DNA samples extracted from peripheral blood. Those with DNA samples derived from cell lines were excluded from the present analysis because cell line transformation might influence CNV results [12, 13]. Current diagnoses were downloaded from the ADNI database as of 04/29/2010 (AD = 288, MCI = 183, HC = 184). In addition to AD participants who had a baseline and current diagnosis of AD, we included MCI participants who had converted from a baseline diagnosis of MCI to a current diagnosis of AD (MCI Converters) as well as one participant who had converted from a baseline diagnosis of HC to a current diagnosis of AD in the AD group. Similarly, in addition to MCI participants who had a baseline and current diagnosis of MCI, we included seven HC participants who had converted from a baseline diagnosis of HC to a current diagnosis of MCI in the MCI group. Data used in preparation of this report is publicly available on the ADNI web site (http://loni.ucla.edu/ADNI/). The focus of ADNI is on incident LOAD. To our knowledge, no participants in the present study carry a known causal mutation [14].

2.3. Genotyping. Blood samples from each participant were obtained and sent to Pfizer for DNA extraction and were also banked at The National Cell Repository for Alzheimer's Disease (NCRAD; http://ncrad.iu.edu/). Genotyping was performed by the Translational Genomics Research Institute (TGen) (Phoenix, AZ) using the Illumina Human610-Quad BeadChip as previously described [15, 16]. As indicated by the manufacturer's documentation, the Human610-Quad BeadChip contains 620,901 markers. This array provides

dense genomic coverage (89%) in the CEU (Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain (CEPH) collection) population analyzed here with a median marker spacing of 2.7 kb. In addition, 27,635 markers are included in "unSNPable" regions likely to contain CNVs that are not easily assessed by SNPs. Coverage is provided for 3,938 CNV regions (184,064 markers) reported in the Toronto Database of Genomic Variants (http://projects.tcag.ca/variation/) at an average of 37.7 markers per region. Markers have an average of 15–18-fold redundancy to improve signal quality for detection of CNVs (mean Log R Ratio SD < 0.2, see below).

Normalized bead intensity data for each sample was loaded into GenomeStudioV2009.1 software (Illumina, Inc., CA) along with the manufacturer's cluster file to generate SNP genotypes. The Log R Ratio (LRR) and B Allele Frequency (BAF) values computed from the signal intensity files by GenomeStudio for each sample were exported and used for the generation of CNV calls. Initial genotyping was performed by TGen using BeadStudio software (Illumina, Inc., CA). In January 2010, we reprocessed the array data using GenomeStudioV2009.1, and this data set will be made available on the ADNI website in a followup data release.

2.4. Inference of LRR and BAF. The two alleles of an SNP are designated as allele A and allele B. GenomeStudio software uses a five-step six-degree of freedom affine transformation to normalize signal intensity values of the A and B alleles (referred to as X and Y). The normalized values are then transformed to a polar coordinate plot of normalized intensity $R = X_{norm} + Y_{norm}$ and composition (copy angle) $\theta = (2/pi) * \arctan(Y_{norm}/X_{norm})$, where X_{norm} and Y_{norm} represent transformed normalized signals from alleles A and B for a particular locus (Illumina's genotyping data normalization methods white paper). The LRR value for a sample is calculated as follows:
LRR = $\log_2$(normalized R value/expected R value) for the SNP.

Linear interpolation of the R value at the SNP's θ value for a sample, relative to the R values of the surrounding clusters, is used to compute the expected R value.

The BAF for a sample shows the θ value for an SNP, corrected for cluster positions, which were generated from a large set of previously studied normal individuals. BAF is described by the following equation:

$$\begin{aligned} \text{BAF} &= 0 \quad \text{if } \theta < \theta_{AA} \\ &= 0.5 * \frac{(\theta - \theta_{AA})}{(\theta_{AB} - \theta_{AA})} \quad \text{if } \theta < \theta_{AB} \\ &= 0.5 + 0.5 * \frac{(\theta - \theta_{AB})}{(\theta_{BB} - \theta_{AB})} \quad \text{if } \theta < \theta_{BB} \\ &= 1 \quad \text{if } \theta \geq \theta_{BB}, \end{aligned} \tag{1}$$

where θ_{AA} = mean θ value of all genotypes in AA cluster plotted in polar normalized coordinates, θ_{AB} = mean θ value of all genotypes in AB cluster plotted in polar normalized coordinates, and θ_{BB} = mean θ value of all genotypes in BB cluster plotted in polar normalized coordinates (GenomeStudio Genotyping Module v1.0 User Guide).

2.5. Generation of CNV Calls and Quality Control. CNV calls were generated for the 655 non-Hispanic Caucasian participants whose DNA was derived from periph-eral blood. PennCNV software (2009Aug27 version) (http://www.openbioinformatics.org/penncnv/), which implements a hidden Markov model (HMM) [17], was used to generate the CNV calls. The hg18 "all" PennCNV hidden Markov model (hmm), population frequency of B allele (pfb), and gcmodel files were used to ensure that CNV-specific markers were included. All samples were subjected to extensive quality control (QC). Since samples that have below optimal genomic wave QC values can be considered unreliable [18], we applied the GC-model wave adjustment procedure, using PennCNV's gcmodel file. A frequency distribution plot of the number of CNV calls for all samples was made, and samples were excluded if the number of CNV calls made for that individual was greater than the 90th percentile of the frequency distribution. One sample was observed to have multiple deletions and duplications on chromosome 18 (Supplementary Figure 1 in Supplementary Materials available Online at doi:10.4061/2011/729478) and was excluded from further analysis as it may be a mosaic sample [19]. Samples were also excluded if they met the following criteria: LRR SD > 0.35, BAF Drift > 0.002, or Waviness Factor (WF) >0.04. The LRR SD is a measure of signal-to-noise ratio. Sometimes, when a sample has genotyping failure, many SNP markers will have abnormal BAF patterns (i.e., they do not cluster to 0, 0.5, or 1), yet their LRR looks normal. The BAF Drift takes into account these abnormal BAF patterns. The WF measures the waviness of the signal curves, as artificial gains and losses in the genome can be created by peaks and troughs of the wave.

Analyses were also restricted to autosomes due to the complications of hemizygosity in males and X-chromosome inactivation in females. Finally, to ensure only high-confidence CNVs were included in the analysis, CNVs for which the difference of the log likelihood of the most likely copy number state and less likely copy number state was less than 10 (generated using the confidence function in PennCNV), CNVs that were called based on data from fewer than 10 SNPs, and CNVs that had >50% overlap with centromeric, telomeric, and immunoglobulin regions as defined in Need et al. [20] were excluded. 501 participants (AD = 222, MCI = 136, HC = 143) passed all QC checks and were included in further CNV analyses.

2.6. Case/Control Association Analyses. Case/control association analyses using CNV calls generated for the AD, MCI, and HC participants were performed using PLINK v1.07 [21] (http://pngu.mgh.harvard.edu/purcell/plink/) to investigate any differences in CNV calls between cases and controls (AD versus HC; MCI versus HC). Two approaches were used: (1) a candidate gene approach using AD genes, identified from the AlzGene database [22] (http://www.alzgene.org/)

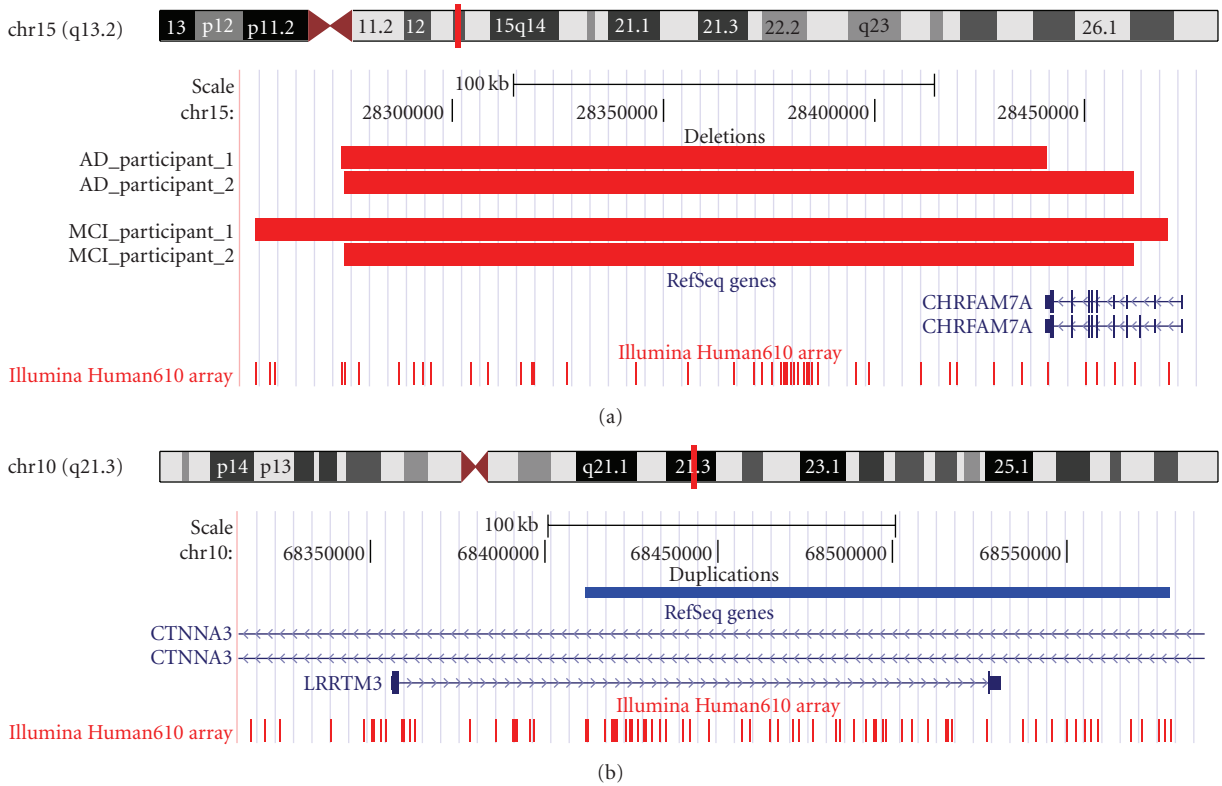

FIGURE 1: Examples of candidate genes (a) *CHRFAM7A* and (b) *LRRTM3*, overlapped by CNV calls from at least one Alzheimer's disease and/or one mild cognitive impairment participant, but no healthy controls. The red rectangles represent deletions, and the blue rectangles represent duplications (March 2006 (NCBI36/hg18) assembly).

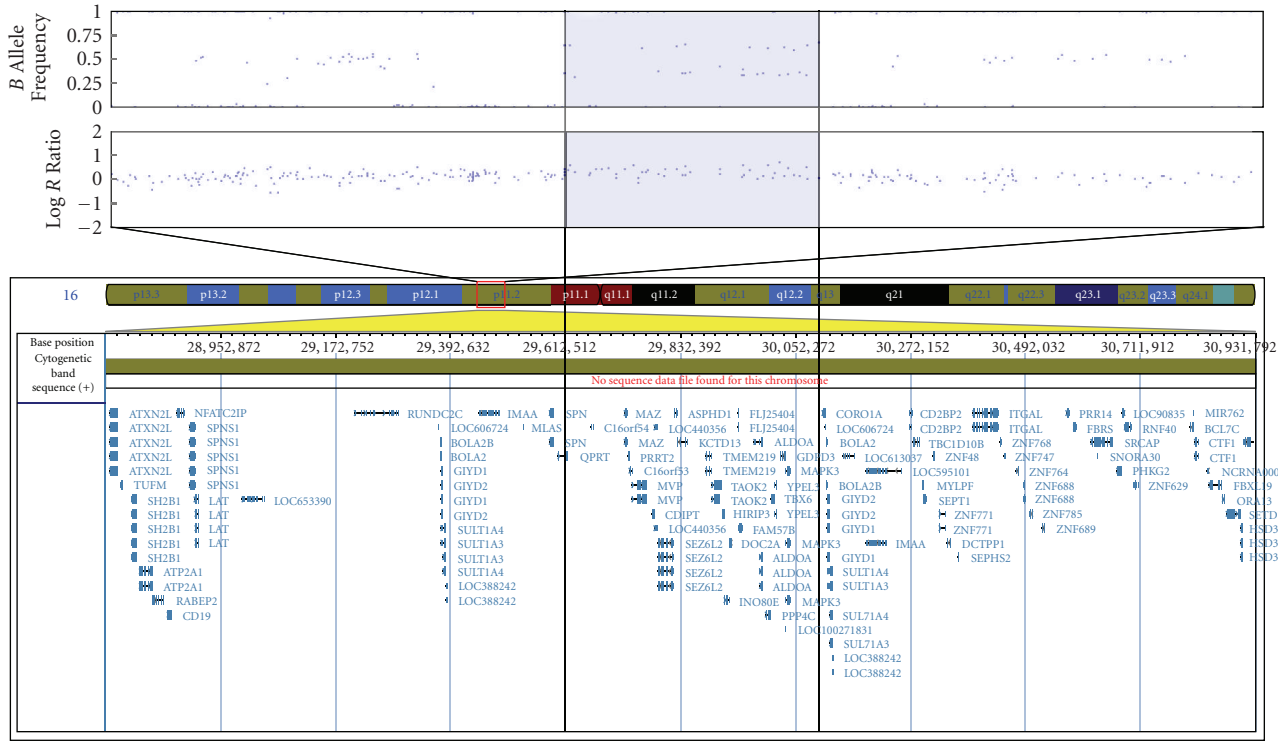

FIGURE 2: Representative image of B Allele Frequency and Log R Ratio of the participant who had a duplication at 16p11.2. The purple-shaded portion indicates the duplicated region (Human Genome Build 36.1).

as having a positive association with AD in at least one study, consisting of 294 genes as of 04/22/2010, and (2) a whole genome approach using PLINK's entire gene list (hg18 coordinates), consisting of 17,938 genes. The AlzGene database provides a comprehensive and regularly updated synopsis of genetic studies in AD. In both approaches, CNV segments either partially or completely overlapping gene regions were analyzed. Both deletions and duplications were included in the analyses.

Representative plots of CNV calls (Figure 1) were generated in UCSC Genome Browser (http://genome.ucsc.edu/) [23] (March 2006 (NCBI36/hg18) assembly). Plots were produced using the genome browser track for the Illumina Human-610 array obtained from the PennCNV website. Representative plots of LRR and BAF values for samples (Figure 2, Supplementary Figures 1 to 3) were generated using the Illumina Genome Viewer plugin within GenomeStudio (Human Genome Build 36.1).

3. Results

3.1. Description of CNV Calls by Current Diagnostic Group. The sample demographics and CNV call characteristics of the 501 participants who passed all QC checks are shown in Tables 1 and 2. A total of 6,737 CNV calls (4,746 deletions and 1,991 duplications) were observed in these participants. The average number of SNPs per CNV call was 25 and the average length of a CNV call was 105.93 kb. A higher CNV call rate and a lower average CNV call size were observed in deletions compared to duplications. On comparing the three diagnostic groups, AD and MCI participants appeared to have a higher CNV call rate for deletions and a lower CNV call rate for duplications, but these were not statistically significant ($P < .05$) when evaluated by permutation. We also evaluated whether CNV burden was higher in cases than controls in the *APOE* e4 negative participants. There was a similar trend toward a higher CNV call rate for deletions and lower CNV call rate for duplications in AD and MCI participants, but these were not statistically significant ($P < .05$; data not shown). A large proportion of deletions and duplications were found in the 0.1–0.5 Mb size range (Table 3). Two AD participants were found to have very large CNV calls (>2 Mb) (Supplementary Figures 2 and 3). One AD participant had a deletion on chromosome 4 (Supplementary Figure 2), which includes the following genes: *NDST4* (N-deacetylase/N-sulfotransferase 4), *TRAM1L1* (translocation-associated membrane protein 1-like 1), and *MIR1973* (microRNA 1973). The other AD participant had a duplication on chromosome 11 (Supplementary Figure 3), which includes the gene *LUZP2* (leucine zipper protein 2).

3.2. Case/Control Association Analyses

3.2.1. Candidate Gene Approach. We identified regions overlapping 294 AD candidate genes with CNV calls from at least one case (AD and/or MCI) but no controls (HC). As expected, cell sizes were very small in each group leading to low power. Resulting CNV calls along with *APOE* genotype and age at onset (for the AD at baseline group) are presented in Table 4 for reference although these did not meet conventional significance ($P < .05$). A number of genes, such as *CHRFAM7A* (*CHRNA7* (cholinergic receptor, nicotinic, alpha 7, exons 5–10) and *FAM7A* (family with sequence similarity 7A, exons A–E) fusion), had CNV calls from only AD or MCI participants partially overlapping them. Figure 1 shows representative plots of two of these genes (*CHRFAM7A* and *LRRTM3*).

3.2.2. Whole Genome Approach. We also identified CNV calls present in cases (AD and/or MCI) but not controls (HC) within regions overlapping 17,938 genes. There was no significant ($P < .05$) gene after correction for multiple testing. We, therefore, focused on genes that had an uncorrected $P < .05$. The genes identified included *CSMD1* (CUB and Sushi multiple domains 1), *HNRNPCL1* (heterogeneous nuclear ribonucleoprotein C-like 1), and *SLC35F2* (solute carrier family 35, member F2) (Table 5). We also observed CNVs overlapping two genes associated with neuropsychiatric disorders: *NRXN1* (neurexin 1) [24, 25] and *ERBB4* (v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)) [26], but these did not reach significance ($P < .05$). An MCI participant, who subsequently converted to clinical AD, was also observed to have a duplication comprising 23 genes in the 16p11.2 region (Figure 2).

4. Discussion

The present report represents an initial analysis of CNVs in the ADNI dataset and is the first CNV analysis of patients with MCI. After extensive QC, we analyzed CNV calls generated in cases (AD and MCI) compared to controls (HC), using whole genome and candidate gene association approaches.

Comparison of the CNV calls between the three diagnostic groups showed no excess CNV burden (rate of calls) in AD and MCI participants compared to controls. This is consistent with previously published results [9]. Two AD participants were found to have CNV calls >2 Mb. One AD participant had a duplication on chromosome 11 (Supplementary Figure 3) which includes the gene *LUZP2* (leucine zipper protein 2). This gene has been shown to be expressed only in the brain and spinal cord in adult mouse tissues [27]. The authors of the study also found this gene to be deleted in some patients with Wilms tumor-aniridia-genitourinary anomalies-mental retardation (WAGR) syndrome. Another AD participant had a deletion on chromosome 4 (Supplementary Figure 2), which includes the following genes: *NDST4* (N-deacetylase/N-sulfotransferase 4), *TRAM1L1* (translocation-associated membrane protein 1-like 1), and *MIR1973* (microRNA 1973). None of these genes have been previously associated with AD susceptibility. Further investigation by either cytogenetic techniques such as fluorescence in situ hybridization (FISH) or molecular biology techniques such as real-time quantitative polymerase

Table 1: Sample demographics.

Current diagnosis	Alzheimer's disease	Mild cognitive impairment	Healthy controls	*P* value
Number of participants	222	136	143	—
Gender (Males/Females)	133/89	87/49	82/61	not significant
Baseline age (Mean ± SD)	75.10 ± 7.27	75.88 ± 7.17	75.83 ± 5.32	not significant
Years of education (Mean ± SD)	15.30 ± 3.05	15.85 ± 3.01	16.24 ± 2.62	0.009
APOE group (e4 negative/e4 positive)	73/149	70/66	108/35	<0.001
Age of onset (Mean ± SD)	74.08 ± 7.73	—	—	—

Table 2: Characteristics of CNV calls in the three diagnostic groups.

	Alzheimer's disease ($n = 222$)	Mild cognitive impairment ($n = 136$)	Healthy controls ($n = 143$)
Deletions:			
Number of CNVs	2128	1340	1278
Rate per participant	9.59	9.85	8.94
Average size (kb)	73.24	76.32	79.38
Duplications:			
Number of CNVs	886	498	607
Rate per participant	3.99	3.66	4.24
Average size (kb)	157.24	154.06	170.30

chain reaction (PCR) and deep resequencing is required to determine the clinical relevance of these regions.

A case/control association analysis was then performed using a candidate gene approach and a whole genome approach to determine if there was an excess of CNV calls partially overlapping genes in AD or MCI participants relative to controls, suggesting potential involvement of these genes in AD or MCI susceptibility.

The candidate gene approach revealed several interesting genes (Table 4 and Figure 1). The *CHRFAM7A* gene had CNV calls in cases (two AD and two MCI) but not in controls. *CHRFAM7A*, located on chromosome 15, consists of a partial duplication of the *CHRNA7* (cholinergic receptor, nicotinic, alpha 7) gene (exons 5-10) fused to a copy of the *FAM7A* (family with sequence similarity 7A) gene (exons A-E) [28]. The *CHRFAM7A* gene contains a polymorphism consisting of a 2-base pair deletion (−2 bp) at position 497-498 of exon 6. This −2 bp polymorphism has been associated with schizophrenia [29]. The *CHRFAM7A* genotype without the −2 bp allele has also been shown to be significantly overrepresented in AD ($P = .011$), dementia with Lewy bodies ($P = .001$), and Pick's disease ($P < .0001$) participants [30]. Heinzen et al. found a duplication in six out of 276 dementia cases (2%) and one out of 322 controls (0.3%) within the schizophrenia and epilepsy-associated risk region at 15q13.3, affecting the *CHRNA7* gene [9]. In the present study, we found a deletion in one out of 222 AD participants (0.45%) and one out of 136 MCI participants (0.74%), as well as a duplication in two out of 143 HC participants (1.40%). This gene codes for one of several neuronal cholinergic nicotinic receptors. Genetic variants in *CHRNA7* and other cholinergic receptor genes have been implicated in AD susceptibility [9], and further investigation of this gene family is warranted. The number of CNV calls overlapping the identified genes is small, as we had a small sample size ($n = 501$) after QC for analysis limiting power. Nevertheless, all identified genes have been previously investigated in AD studies and thus represent potential candidate genes. Replication studies with larger sample sizes as well as laboratory validation are required to confirm the role of these genes in AD susceptibility.

The whole genome approach revealed three genes at uncorrected $P < .05$, as shown in Table 5. *CSMD1* (CUB and Sushi multiple domains 1) has been shown to be primarily synthesized in the developing central nervous system (CNS) and epithelial tissues [31]. It is enriched in the nerve growth cone, suggesting that it may be an important regulator of complement activation and inflammation in the developing CNS. *HNRNPCL1* (heterogeneous nuclear ribonucleoprotein C-like 1) is predicted to play a role in nucleosome assembly by neutralizing basic proteins such as A and B core hnRNPs (Uniprot: http://www.uniprot.org/). *SLC35F2* (solute carrier family 35, member F2), also known as lung squamous cell cancer-related protein LSCC-3, is integral to membrane and transport (Gene Ontology: http://www.geneontology.org/).

We also identified CNVs overlapping two candidate genes associated with neuropsychiatric disorders: *NRXN1* and *ERBB4,* from the whole genome approach in cases, but not in controls. Deletions in the *NRXN1* (neurexin 1) gene were observed in four AD participants and three MCI participants; deletions in the *ERBB4* (v-erb-a erythroblastic leukemia viral oncogene homolog 4) gene were observed in four AD participants and one MCI participant, respectively. *NRXN1*, a member of the neurexin family on chromosome 2, is a cell surface receptor that binds neuroligins. The Ca^{2+}-dependent neurexin-neuroligin complex

Table 3: Participants grouped by CNV call size.

Call size	Alzheimer's disease ($n = 222$)		Mild cognitive impairment ($n = 136$)		Healthy controls ($n = 143$)	
	Deletions n (%)	Duplications n (%)	Deletions n (%)	Duplications n (%)	Deletions n (%)	Duplications n (%)
0.1–0.5 Mb	174 (78.38)	183 (82.43)	104 (76.47)	100 (73.53)	114 (79.72)	120 (83.92)
0.5–1.0 Mb	6 (2.70)	27 (12.16)	8 (5.88)	18 (13.24)	8 (5.94)	27 (18.88)
1.0–1.5 Mb	0 (0.00)	8 (3.60)	0 (0.00)	4 (2.94)	2 (1.40)	8 (5.59)
1.5–2.0 Mb	0 (0.00)	2 (0.90)	0 (0.00)	1 (0.74)	1 (0.70)	0 (0.00)
>2.0 Mb	1 (0.45)	1 (0.45)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

Table 4: Genes that have CNV calls from at least one Alzheimer's disease (AD) and/or one mild cognitive impairment (MCI) participant and no healthy controls using the candidate gene approach.

Chromosome	Region	Start (bp)	End (bp)	Number of AD participants	*APOE* genotype	Age at onset[a]	Number of MCI participants	*APOE* genotype
5	*PPP2R2B*	145949260	146441226	1	e3/e3	N/A	0	—
6	*ATXN1*	16407321	16869700	1	e3/e4	83 years	0	—
7	*MAGI2*	77484309	78920826	1	e2/e4	N/A	0	—
7	*RELN*	102899472	103417198	0	—	—	1	e4/e4
9	*GRIN3A*	103371455	103540683	1	e3/e3	74 years	0	—
10	*LRRTM3*	68355797	68530873	1	e3/e3	55 years	0	—
10	*LIPA*	90963305	91001640	0	—	—	1[b]	e3/e3
12	*PPM1H*	61324030	61614932	1	e2/e3	N/A	1	e2/e3
15	*CHRFAM7A*	28440734	28473156	2	e3/e3	N/A	2	e3/e3
					e3/e4	N/A		e3/e4
15	*ADAM10*	56675801	56829469	1	e3/e3	N/A	1[b]	e3/e3
21	*DNAJC28*	33782107	33785893	1	e3/e4	74 years	0	—
21	*DOPEY2*	36458708	36588442	0	—	—	1	e3/e4
22	*GSTT1*	22706138	22714284	1	e3/e3	59 years	0	—

[a] Age at onset of AD symptoms, available only for participants with a baseline diagnosis of AD; N/A: Not available.
[b] The same participant had CNV calls overlapping the two genes.

is present in the CNS at synapses and is required for efficient neurotransmission and formation of synaptic contacts [32]. This gene has been found to have reduced expression with AD severity [33], and its disruption has been shown to be associated with schizophrenia [20, 25, 34] and autism [24, 35]. Deletions in this gene have also been shown to predispose to a variety of developmental disorders including autism spectrum disorders, language delays, and mental retardation [36]. Interestingly, an SNP (rs6463843) flanking the *NXPH1* (neurexophilin 1) gene was identified by our group in a GWAS of neuroimaging phenotypes in the ADNI cohort [37]. The *NXPH1* gene, a member of the neurexophilin family, forms a tight complex with alpha neurexins, and the SNP was found to be associated with reduced global and regional grey matter density. The *ERBB4* gene, also on chromosome 2, is a member of the type I receptor kinase subfamily, that encodes a receptor for neuregulin 1 (*NRG1)*. The neuregulin-ErbB receptor signaling pathway plays a role in development, synaptic function, and neural network activity and has been implicated in schizophrenia [38]. One AD participant had a large duplication that included 23 genes in the 16p11.2 region (Figure 2). CNVs in this region have previously been associated with autism [39–41], schizophrenia [42], cognitive impairment and speech/language delay [43], and obesity [44, 45], but not AD or MCI. Because the ADNI employed a case/control design, DNA from family members was not available for linkage analysis. This limitation precluded determination as to whether CNVs were *de novo* or inherited.

The ADNI cohort provides a unique opportunity for discovery analyses such as this initial CNV analysis. With multiple types of potential biomarkers, including structural and molecular imaging, blood and CSF markers, genetic information, and behavioral data, analysis of the ADNI data has the potential to enhance knowledge of the underlying mechanisms leading to MCI and to AD.

The present study has several limitations related to participant inclusion and exclusion and the software and algorithms used in the analyses. CNV calls in the present report were generated from DNA samples derived only from peripheral blood-78 participants whose DNAs were derived

Table 5: Significant (uncorrected $P < .05$, relative to healthy controls) genes present in either Alzheimer's disease (AD) and/or mild cognitive impairment (MCI) participants, but not healthy controls using the whole genome approach.

Chromosome	Region	Start (bp)	End (bp)	Number of AD calls	*P* value for AD calls	Number of MCI calls	*P* value for MCI calls
8	*CSMD1*	2780281	4839736	9	0.0114	4	0.0556
1	*HNRNPCL1*	12829847	12831165	6	0.0493	4	0.0549
11	*SLC35F2*	107166926	107234864	5	0.0820	6	0.0120

from lymphoblastoid cell lines (LCLs) were excluded. LCLs are generated by transforming peripheral B lymphocytes by the Epstein-Barr virus (EBV). EBV-transformed cells are shown to have significant telomerase activity and develop aneuploidy, along with other cellular changes such as gene mutations and reprogramming in the postimmortal cellular stage of transformation [13]. Thus, to avoid CNV call discrepancies that may arise between the different DNA sources, we chose to include only those participants whose DNA was derived from peripheral blood. Additional QC was also performed, resulting in only 501 samples that passed all QC checks. To date, no definitive QC criterion has been established to ensure only high-quality samples are included in CNV analyses. Therefore, the QC criterion applied in the present study may have been too stringent leading to the exclusion of samples which otherwise may have had informative CNV data. In future studies, we propose to analyze multiple QC thresholds to determine the optimum QC criteria.

Another limitation is that the CNV calls analyzed in the current study were generated using only one software program (PennCNV). Several detection algorithms including HMMs, segmentation algorithms, *t*-tests, and standard deviations of the LRR are available for identifying CNVs from genome-wide SNP array data. A comparison of these methods has been performed by Dellinger et al. Even though the PennCNV program was found to have moderate power in detecting CNVs, it also had a low false positive call rate. The program was found to detect less CNV calls in comparison to other methods and did not accurately detect small CNVs (3-4 SNP CNVs) [46]. However, in our analyses, we have included CNV calls that had at least 10 SNPs. Obtaining the same CNV calls from another algorithm would help further reduce false positive CNV calls.

The heterogeneity of the MCI group of participants also represents a possible limitation of the present study. Although biomarkers such as CSF and PiB-PET can help differentiate MCI participants who have an AD-like profile from those who have a normal profile, this data was only available for a small number of ADNI-1 participants which would have limited power to detect differences in CNVs. In the next phases of the project (ADNI-GO and ADNI-2), all subjects will have CSF and amyloid PET data, enabling further examination of this issue.

5. Conclusion

In sum, we have conducted an initial CNV analysis in the ADNI cohort dataset. Although no excess CNV burden was found in cases relative to controls, a number of interesting candidate genes and regions were identified. Replication in larger samples will be critical to confirm these findings. Additional region-based analyses may help elucidate the role of these CNVs, and deep resequencing studies may be warranted for some of these regions if they replicate in other cohorts.

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The Alzheimer's Disease Neuroimaging Initiative (ADNI)

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Research Article

Association Study of Genetic Variants in *CDKN2A/CDKN2B* Genes/Loci with Late-Onset Alzheimer's Disease

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Alzheimer's disease (AD) is the most common form of dementia clinically characterized by progressive impairment of memory and other cognitive functions. Many genetic researches in AD identified one common genetic variant ($\varepsilon 4$) in Apolipoprotein E (*APOE*) gene as a risk factor for the disease. Two independent genome-wide studies demonstrated a new locus on chromosome 9p21.3 implicated in Late-Onset Alzheimer's Disease (LOAD) susceptibility in Caucasians. In the present study, we investigated the role of three SNP's in the *CDKN2A* gene (rs15515, rs3731246, and rs3731211) and one in the *CDKN2B* gene (rs598664) located in 9p21.3 using an association case-control study carried out in a group of Caucasian subjects including 238 LOAD cases and 250 controls. The role of *CDKN2A* and *CDKN2B* genetic variants in AD is not confirmed in our LOAD patients, and further studies are needed to elucidate the role of these genes in the susceptibility of AD.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia clinically characterized by insidious onset and progressive impairment of memory and other cognitive functions [1], ultimately resulting in complete dependency and death of the patient.

The key features of AD brains are neuronal and synapse loss, extracellular plaques composed of amyloid-β (Aβ) peptides and intraneuronal neurofibrillary tangles consisting of hyperphosphorylated tau protein.

Although most patients develop AD at later age, with a prevalence estimates ranging from 4.4% in persons aged 65 years to 22% at ages 90 and older [2], it is mainly the research performed on the rare autosomal dominant early-onset form of AD that provided valuable insights into disease pathogenesis.

Several penetrant autosomal dominant mutations have been identified (http://www.molgen.ua.ac.be/ADMutations/), leading to early-onset familial AD within three genes: presenilin 1 (*PSEN1*) [3], presenilin 2 (*PSEN2*) [4], and amyloid precursor protein (*APP*) genes [5].

The Apolipoprotein E gene (*APOE*) was identified as a major risk factor contributing to the pathogenesis of late-onset Alzheimer disease's (LOAD) [6]. However, the *APOE* e4 allele is neither necessary nor sufficient for the occurrence of the disease.

Many SNPs have been analyzed to identify new susceptibility candidate genes for the LOAD [7].

Recently, two independent genome-wide studies have demonstrated a new locus on chromosome 9p21.3 implicated in LOAD susceptibility in Caucasians [8, 9].

Züchner and colleagues in their work individuated several SNPs in three different genes: the cyclin-dependent kinase inhibitors (*CDKN2A, CDKN2B*) and methylthioadenosine phosphorylase (*MTAP*), and they conducted an allelic association test to evaluate the genetic effect of these genes [9].

In light of these results, we investigated the role of three SNP's in the *CDKN2A* gene (rs11515, rs3731246, and rs3731211) and one in the *CDKN2B* gene (rs598664) using an association study carried out in a group of Caucasian LOAD patients.

2. Materials and Methods

2.1. Patients. Our study group included 488 Caucasian subjects: 238 LOAD patients (63.9% females, age at onset 72.9 ± 5.6 years, mean ± SD, Mini Mental State Examination score 20.2 ± 5.4 points) and 250 nondemented controls (62.4% females, mean age 71.5 ± 5.7 years; SD) (Table 1).

All subjects were enrolled in the study at the Neurology Unit of the Department of Neurological and Psychiatric Sciences of the University of Florence and at Paul Flechsig Institute for Brain Research, University of Leipzig, Germany.

Patients (19.7% autopsy proven) were clinically evaluated according to published guidelines, and the AD diagnosis fulfilled the Diagnostic and Statistical Manual of Mental Disorders criteria (DSM-IV) [10, 11]. Presence of a family history of dementia was considered an exclusion criterion.

All controls were carefully assessed using a rigorous clinical history evaluation and a general/neurological examination, in order to exclude the presence of any neurological disorder.

The study protocol was approved by the local ethics committee and conducted in accordance with the provisions of the Helsinki Declaration; informed consent for genetic screening was obtained from the study participants or, where appropriate, a relative or legal representative.

2.2. Genotyping. DNA was extracted from white blood cells using the phenol-chloroform procedure and all of the genotyping was performed by KBioscience (http://www.kbioscience.co.uk/). KBiosciences uses a novel form of competitive allele specific PCR system (KASPar) that is a proprietary KBioscience invented genotyping chemistry. The accuracy greater than 99% is achieved and a routine quality control measures is performed on all genotyping.

APOE genotypes were determined in all subjects using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods, as previously described [12].

2.3. Statistics. Differences in distribution between cases and controls were tested using chi-square test (EpiInfo software v. 3.3.2 available free at http://www.cdc.gov/EpiInfo/).

In order to evaluate the association between genotypes and alleles and AD, odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated using logistic regression models (SPSS software ver. 18.0 for Windows (Chicago, ILL, USA)). Further analysis were performed by using logistic regression models adjusted for gender, age (in continuous), and presence of *APOE* e4.

A P-value of <.05 was considered statistically significant.

A power analysis was performed using COMPARE2 software (available free at http://www.brixtonhealth.com/).

Table 1: Main characteristics of all studied subjects.

Characteristics	LOAD[a]	Controls
Total	238	250
Gender, no. (%)		
Female	152 (63.9)	156 (62.4)
Male	86 (36.1)	94 (38.6)
Recruitment site, no. (%)		
Italy	218 (91.6)	230 (92)
Germany	20 (8.4)	20 (8)
Age at onset[b], mean (±SD)	72.9 (±5.6)	71.5 (±5.7)
MMSE points, mean (±SD)	20.2 (±5.4)	28.2 (±1.4)
APOE ε4, no. (%)		
No	133 (55.9)	221 (88.4)
Yes	105 (44.1)	29 (11.6)

[a] LOAD cases were defined by the occurrence of Alzheimer's disease with an onset age ≥65 years.
[b] At examination tests for controls.

Linkage disequilibrium (LD) between the SNPs was analyzed using PowerMarker software, version 3.25) (http://statgen.ncsu.edu/powermarker/) [13].

3. Results

Table 1 gives the distribution of 238 LOAD cases and 250 controls according to gender, recruitment site, age, and other selected characteristics.

Cases and controls had similar recruitment site and age distribution. The mean of the Mini Mental State Examination score (MMSE) points was higher in controls than cases. Conversely, cases reported more frequently the presence of E4 compared to controls (44.1% versus 11.6%).

The frequencies of all SNPs were in Hardy-Weinberg equilibrium in AD patients and controls.

Moreover, no correlation between mean age at onset and all SNPs genotypes was observed and no interaction between *APOE* gene was found (data not shown).

We found no differences in the allelic or genotypic frequencies between AD patients and controls for any of the SNPs (Table 2). We found no association between AD and rs11515 genotype ($\chi^2 = 3.47$; $P = .17$) and allele ($\chi^2 = 3.2$; $P = .07$), rs3731246 genotype ($\chi^2 = 1.13$; $P = .56$) and allele ($\chi^2 = 1.17$; $P = .27$), rs3731211 genotype ($\chi^2 = 0.57$; $P = .75$) and allele ($\chi^2 = 0.0$; $P = .96$), rs598664 genotype ($\chi^2 = 1.11$; $P = .57$), and allele ($\chi^2 = 1.15$; $P = .28$). None of the unadjusted ORs were statistically significant, and when we adjusted for gender, age (in continuous), and *APOE* e4 these results did not substantially change (data not shown).

Moreover, as reported by Züchner and colleagues, we found that rs11515 SNP was in Linkage Disequilibrium (LD) with rs37361246 ($r^2 = 0.64$), rs3731211 ($r^2 = 0.33$) and rs598664 ($r^2 = 0.55$).

4. Discussion

Recent studies reported informative results regarding genomewide association and linkage studies on chromosome 9

Table 2: *CDKN2A/B* polymorphisms: genotype, allele frequencies, and unadjusted odds ratios (OR) and corresponding 95% confidence intervals (CI) for 238 Late-Onset Alzheimer's Disease (LOAD) cases and 250 controls.

	LOAD[a] no. (%)	Controls no. (%)	χ^2 (df, *P*-value)	unadjusted OR (95% CI)
Total	238	250		
CDKN2A				
Rs 11515				
Genotypes			3.47 (2, .17)	
CC	169 (71)	163 (65.2)		1[b]
CG	62 (26)	72 (28.8)		0.83 (0.54–1.27)
GG	7 (3)	15 (6)		0.45 (0.16–1.21)
Alleles			3.20 (1, .07)	
C	400 (84)	398 (79)		1[b]
G	76 (16)	102 (21)		0.74 (0.53–1.04)
Rs 3731246				
Genotypes			1.13 (2, .56)	
GG	183 (76.9)	182 (72.8)		1[b]
GC	50 (21)	61 (24.4)		0.82 (0.52–1.28)
CC	5 (2.1)	7 (2.8)		0.71 (0.19–2.55)
Alleles			1.17 (1, .27)	
G	416 (87.4)	425 (85)		1[b]
C	60 (12.6)	75 (15)		0.82 (0.56–1.2)
Rs 3731211				
Genotypes			0.57 (2, .75)	
TT	105 (44.1)	106 (42.4)		1[b]
AT	99 (41.6)	112 (44.8)		0.89 (0.60–1.33)
AA	34 (14.3)	32 (12.8)		1.07 (0.59–1.93)
Alleles			0.00 (1, .96)	
T	309 (64.9)	324 (64.8)		1[b]
A	167 (35.1)	176 (35.2)		0.99 (0.76–1.31)
CDKN2B				
Rs 598664				
Genotypes			1.11 (2, .57)	
AA	182 (74.5)	181 (72.4)		1[b]
AG	51 (21.4)	62 (24.8)		0.82 (0.52–1.28)
GG	5 (2.1)	7 (2.8)		0.71 (0.19–2.55)
Alleles			1.15 (1, .28)	
A	415 (87.2)	424 (84.8)		1[b]
G	61 (12.8)	76 (15.2)		1.35 (0.89–2.07)

[a] LOAD cases were defined by the occurrence of Alzheimer's disease with an onset age ≥65 years.
[b] Reference category.

as candidate region for LOAD. In particular, Pericak-Vance and colleagues identified the LOAD locus on 9p21.3 in a genomewide microsatellite-based linkage screen on 466 AD families [14] and confirmed in a genetic study of a consanguineous Israeli-Arab community [15]. In 2007 Hamshere and colleagues analyzing 723 affected relative pairs with genomewide linkage analysis showed evidence for disease locus for LOAD on chromosome 9p [8]. In 2008 Züchner identified a chromosomal area under the linkage peak containing several potential AD candidate genes and analyzed *CDKN2A* and *CDKN2B* genes. Among the different SNPs identified, the rs11515 localized in the 3′-UTR of *CDKN2A* was the most significant even if no transcription-factor-binding site, micro-RNA target site, or conserved regulatory potential has been detected; other SNPs are all in intron regions.

Many studies have been carried out on numerous putative gene polymorphisms candidate in AD pathogenesis and, with the exception of *APOE*, that is, the only confirmed genetic risk factor, results are still contrasting.

In our case-control association study we analyzed four SNPs, three in the *CDKN2A* gene (rs15515, rs3731246, and rs3731211) and one in *CDKN2B* (rs598664) in a sample of 488 Caucasian subjects including 238 LOAD and 250 controls.

Our data set of 238 LOAD patients was evaluated as having a power to detect an OR of 2.0 at a 5% significance rate between 86.7 and 96.6 for all SNPs. However, to detect an OR at 1.5—the usual genetic main-effect for complex disorder—our data has a power between 40.4 and 59 for all SNPs. Thus, the study is not sufficiently powered and this is one of the limitations of our study. We did not find any association between these SNPs and AD risk in contrast to Züchner's results: this may be due to the different study design. In fact, Züchner and colleagues reported a linkage and association study in 674 AD families, whilst we conducted a case control association study. Moreover, only in 47 patients (19.7%) of AD cases the autopsy confirmed the diagnosis.

Furthermore no correlation between mean age at onset and all SNPs genotypes, and no interaction between *APOE* gene was observed, suggesting that the genes do not act synergistically.

5. Conclusion

In conclusion, our results did not confirm the hypothesis, suggested by Züchner and colleagues that *CDKN2A* and *CDKN2B* at 9p21 are implicated in the susceptibility in the LOAD.

In fact, our data show no evidence of an association between all the studied SNPs and disease risks, thus a possible role of *CDKN2A* and *CDKN2B* as a genetic risk factor implicated in the susceptibility to AD was not confirmed. In lights of these contrasting results further studies are needed to elucidate the role of these genes in the susceptibility of AD.

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Research Article

Specific Silencing of L392V *PSEN1* Mutant Allele by RNA Interference

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RNA interference (RNAi) technology provides a powerful molecular tool to reduce an expression of selected genes in eukaryotic cells. Short interfering RNAs (siRNAs) are the effector molecules that trigger RNAi. Here, we describe siRNAs that discriminate between the wild type and mutant (1174 C → G) alleles of human Presenilin1 gene (*PSEN1*). This mutation, resulting in L392V PSEN1 variant, contributes to early onset familial Alzheimer's disease. Using the dual fluorescence assay, flow cytometry and fluorescent microscopy we identified positions 8th–11th, within the central part of the antisense strand, as the most sensitive to mismatches. 2-Thiouridine chemical modification introduced at the 3′-end of the antisense strand improved the allele discrimination, but wobble base pairing adjacent to the mutation site abolished the siRNA activity. Our data indicate that siRNAs can be designed to discriminate between the wild type and mutant alleles of genes that differ by just a single nucleotide.

1. Introduction

Alzheimer's disease (AD) is the most common form of a progressive dementia in humans. The most frequently occurring variant is a "late-onset" (LOAD), sporadic form of the disease developing usually after age 65. The LOAD appears as a result of a complex interaction among environmental factors and individual predisposing genetic traits. Almost sixty years ago, Sjögren et al. demonstrated that some patients with AD had an autosomal dominant Mendelian pattern of disease inheritance [1]. Early onset familial cases of Alzheimer's disease (FAD) are rather rare and account for only a few percent of the total population of patients but in this form disease symptoms appear at an unusually early age, between 30–50 year of life. Mutations in the three genes coding Amyloid Precursor Protein (*APP*), Presenilin 1 (*PSEN1*), and Presenilin 2 (*PSEN2*) were identified as responsible for development of the dementia in almost 50% of patients with FAD. Identification of the amyloid-beta (Aβ) peptides isolated from brains of patients with AD and trisomy 21 (Down syndrome) suggested localization of the parent *APP* gene on chromosome 21 [2–5]. Linkage to disease mapped in FAD families led to the discovery of the first autosomal dominant missense mutations in *APP* segregating with disease risk [6]. This was followed by identifications of autosomal dominant FAD mutations in the *PSEN1* [7] and *PSEN2* [8, 9] genes, on chromosomes 14 and 1, respectively. The summary of FAD mutations is maintained at the Alzheimer's Disease and Frontotemporal Dementia Mutation Database [10]. Accordingly, there are currently described totally 227 sequence defects in the human FAD genes: 32 mutations in *APP* gene, 181 mutations in *PSEN1* gene, and 14 mutations in *PSEN2* gene, identified, respectively, in 89, 399, and 23 families.

Human Presenilin 1 (PSEN1, called alternatively as PS1, AD3, FAD, S182) is a 467-residues, ~50 kDa protein

with 9-TMD (transmembrane domain) topology [11, 12]. PSEN1 together with nicastrin (Nct), anterior pharynx-defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) constitutes a multisubunit membrane-bound protease complex of γ-secretase and is essential for its activity, stability and interaction between all γ-secretase components [13–15]. In the γ-secretase complex, Presenilin 1 is cleaved, in intramolecular autocatalytic event, into N- and C-terminal fragments (~30 kDa NTF and 20 kDa CTF), which form an active heterodimer. Several mutations in *PSEN1* gene, detected in FAD patients, can inhibit processing of polypeptide chain. The most well-studied PSEN1 function in γ-secretase complex is catalysis of the intramembranous cleavage of a number of proteins, for example, Notch, APP, N-, and E-cadherins. Due to the fact that only a fraction of cellular protein actually forms the γ-secretase complex [16], it was postulated that Presenilin 1 may have functions beyond those of the γ-secretase complex. For example it is involved in neuronal Ca^{2+} signaling and homeostasis [17], and it also interacts with and stabilizes β-catenin [18, 19]. The main role of the γ-secretase is participation in the APP processing. The CTF 83 and CTF 99 fragments of APP, formed after its proteolysis by α- or β-secretases, respectively, are cleaved within their transmembrane regions to release the C-terminal domain (AICD) and generate Aβ peptides. Nearly 90% of the secreted amyloid peptides are Aβ40 or shorter forms, while the remaining 10% are Aβ42 or longer peptides. AD-linked mutations in *PSENs* genes influence the γ-secretase cleavage-site specificity, favouring cleavage at the position 42 relative to the position 40, thus increasing the Aβ42/Aβ40 ratio. Research from the 1990s indicates that mutations in the *PSEN1* gene may be responsible for 30–60% of early onset Alzheimer's cases. The hydrophilic loop (amino acids 263–407) of PSEN1, in which many pathogenic mutations are localized, appears to be crucial for the protein function, since it includes the binding domains to different PSEN1 partners [20].

Dominantly inherited disorders constitute particularly attractive targets for allele-specific gene silencing by short interfering RNAs due to the fact that patients with these disorders carry both the wild type gene and mutant allele causing the disease. Specific silencing of a mutant allele that expresses the toxic form of the protein, without reducing the level of the wild type allele, might constitute a promising approach for the cure or prevention of such disorders. Most of the disease-associated genes differ by a single point mutation, making them targets of choice for allele-specific silencing. The list of trials on silencing mutant alleles associated with neurodegenerative disorders includes Huntington disease (HD) [21–27], Parkinson disease (PD) [28], amyotrophic lateral sclerosis (ALS) [29–33], spinocerebellar ataxia (SCA) Type 1 (SCA1) [34], and Type 3 (SCA3) causing Machado-Joseph disease [35, 36], frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [35], slow channel congenital myasthenic syndrome (SCCMS) [37], and prion protein-induced disease [38]. Although several studies have used RNAi to investigate the effect of silencing of *PSEN1* and its homolog *PSEN2* on γ-secretase processing and cell metabolism [39, 40], to our best knowledge no studies have been reported on the allele-specific silencing of any *PSEN1* mutant allele. Mutation L392V in PSEN1 carries the single nucleotide substitution (C → G) at position 1174 of coding sequence in exon 11, followed by amino acid substitution (Leu → Val) at the position 392 of polipeptide chain in TMD 7. Mutant form of PSEN1 is involved in the production of longer forms of the Aβ peptide resulting in its accumulation in the brain, and, in consequence, generation of early-onset Alzheimer's disease. Mean age onset of the disease in patients with L392V PSEN1 mutation was determined as 42.5 years and mean age of death was determined as 52.6 years [10]. According to data from Alzheimer's Disease and Frontotemporal Dementia Mutation Database, this mutation has been already found in 50 patients with FAD in European (Italy, France) and Asian (Japan) population [10, 41–46]. We selected the L392V mutant and wild type alleles of Presenilin 1 as a model system for the evaluation of allele-specificity of siRNA duplexes. A series of siRNA molecules fully complementary to the mutant gene were screened first by a dual fluorescence assay, then by FACS flow cytometry and their activity was confirmed by fluorescence microscopy and observation of reduced level of Aβ42 determined by ELISA. Our data suggest that siRNAs can be designed to discriminate between the wild type and the mutant alleles that differ by just a single nucleotide.

2. Materials and Methods

2.1. Synthesis and Purification of RNA Oligonucleotides. The oligoribonucleotides were synthesized according to the routine phosphoramidite approach [47], using LCA CPG glass support and commercially available nucleoside phosphoramidites (ChemGenes). Synthesis was performed on the Gene World DNA synthesizer under the conditions recommended by the manufacturer. Oligonucleotides were cleaved from the solid support as 5′-DMT-derivatives, then deprotected and purified according to the described procedure [48]. Support-bound oligonucleotides were treated with 33% ethanolic methylamine (Sigma-Aldrich) and DMSO 1 : 1 (v : v) mixture at 65°C for 15 min and then with triethylamine-trihydrofluoride (Sigma-Aldrich) at 65°C for 15 min. The reaction mixture was frozen at −20°C for 30 min, quenched with cold 1.5 M NH_4HCO_3 and poured into a conditioned SepPak cartridge (Waters). Shorter oligomers were eluted with 14% CH_3CN in 50 mM CH_3COONa and 50 mM NaCl. The remaining oligomer was treated with 2% aqueous TFA (trifluoroacetic acid) for 15 min at room temperature and washed with water, 1 M NaCl, and water. The product was eluted from the cartridge with 30% CH_3CN. The structure and purity of oligomers were confirmed by MALDI-TOF mass spectroscopy (Table 1) and by 20% polyacrylamide/7 M urea gel electrophoresis (data not shown).

2.2. Assembly of siRNA. siRNA duplexes were assembled in phosphate saline buffer (PBS, without Ca^{2+} and Mg^{2+}) by mixing equimolar amounts of complementary oligonucleotides, heating at 95°C for 2 min, and slow cooling down

Table 1: Sequences and MALDI-TOF MS data of oligoribonucleotides used for the preparation of siRNAs. The position of mismatch for pairing with the wild type gene is indicated in bold and underlined.

siRNA	Strand	Sequence	MW (calculated)	MALDI-TOF MS *m/z*
P1	S:	5′-**G**UGGUUGGUAAAGCCUCAGTT-3′	6724.1	6768.3
	As:	3′-TT**C**ACCAACCAUUUCGGAGUC-5′	6587.2	6592.1
P2	S:	5′-U**G**UGGUUGGUAAAGCCUCATT-3′	6685.1	6681.9
	As:	3′-TTA**C**ACCAACCAUUUCGGAGU-5′	6611.2	6608.2
P3	S:	5′-UU**G**UGGUUGGUAAAGCCUCTT-3′	6662.1	6660.3
	As:	3′-TTAA**C**ACCAACCAUUUCGGAG-5′	6634.2	6632.1
P4	S:	5′-GUU**G**UGGUUGGUAAAGCCUTT-3′	6702.1	6697.1
	As:	3′-TTCAA**C**ACCAACCAUUUCGGA-5′	6594.2	6592.6
P5	S:	5′-UGUU**G**UGGUUGGUAAAGCCTT-3′	6702.1	6699.6
	As:	3′-TTACAA**C**ACCAACCAUUUCGG-5′	6594.2	6590.5
P6	S:	5′-GUGUU**G**UGGUUGGUAAAGCTT-3′	6742.0	6736.5
	As:	3′-TTCACAA**C**ACCAACCAUUUCG-5′	6554.3	6552.9
P7	S:	5′-AGUGUU **C** UGGUUGGUAAAGTT-3′	6766.0	6764.0
	As:	3′-TTUCACAA**C**ACCAACCAUUUC-5′	6515.3	6514.1
P8	S:	5′-CAGUGUU**G**UGGUUGGUAAATT-3′	6726.1	6723.5
	As:	3′-TTGUCACAA**C**ACCAACCAUUU-5′	6555.3	6552.7
P9	S:	5′-ACAGUGUU**G**UGGUUGGUAATT-3′	6726.1	6725.1
	As:	3′-TTUGUCACAA**C**ACCAACCAUU-5′	6555.3	6553.2
P10	S:	5′-UACAGUGUU**G**UGGUUGGUATT-3′	6703.0	6705.5
	As:	3′-TTAUGUCACAA**C**ACCAACCAU-5′	6578.3	6578.6
P11	S:	5′-CUACAGUGUU**G**UGGUUGGUTT-3′	6679.0	6720.2
	As:	3′-TTGAUGUCACAA**C**ACCAACCA-5′	6617.2	6615.5
P12	S:	5′-UCUACAGUGUU**G**UGGUUGGTT-3′	6679.0	6679.4
	As:	3′-TTAGAUGUCACAA**C**ACCAACC-5′	6617.2	6617.1
P13	S:	5′-UUCUACAGUGUU**G**UGGUUGTT-3′	6640.1	6640.2
	As:	3′-TTAAGAUGUCACAA**C**ACCAAC-5′	6641.3	6664.1
P14	S:	5′-UUUCUACAGUGUU**G**UGGUUTT-3′	6601.1	6601.1
	As:	3′-TTAAAGAUGUCACAA**C**ACCAA-5′	6400.0	6701.1
P15	S:	5′-UUUUCUACAGUGUU**G**UGGUTT-3′	6601.1	6620.4
	As:	3′-TTAAAAGAUGUCACAA**C**ACCA-5′	6665.3	6686.7
P16	S:	5′-AUUUUCUACAGUGUU**G**UGGTT-3′	6624.1	6669.3
	As:	3′-TTUAAAAGAUGUCACAA**C**ACC-5′	6642.3	6640.1
P17	S:	5′-CAUUUUCUACAGUGUU**G**UGTT-3′	6584.1	6583.3
	As:	3′-TTGUAAAAGAUGUCACAA**C**AC-5′	6682.2	6679.5
P18	S:	5′-UCAUUUUCUACAGUGUU**G**UTT-3′	6545.2	6583.8
	As:	3′-TTAGUAAAAGAUGUCACAA**C**A-5′	6706.2	6705.0
P19	S:	5′-UUCAUUUUCUACAGUGUU**G**TT-3′	6545.2	6543.6
	As:	3′-TTAAGUAAAAGAUGUCACAA**C**-5′	6706.2	6704.4

to room temperature (~2 hours). Formation of the resulting duplexes was confirmed by 4% agarose gel electrophoresis (data not shown).

2.3. Construction of Plasmids Coding EGFP-PSEN1 Fusion Gene. Human Presenilin 1 (*PSEN1*, GenBank NM_000021) coding sequence containing 1404 base pair (bp) was isolated from a total RNA, extracted from human cells (SH-SY5Y, human neuroblastoma) by reverse transcription (RT) and polymerase chain reaction (PCR) using One Step RT-PCR Kit (Qiagen). Reactions were performed in the following conditions—RT: 45°C 30 min, 95°C 15 min, and PCR: amplification 40 cycles (95°C 30 s; 55°C 30 s; 68°C 2 min) and termination at 72°C for 10 min. Sequences of primers were as follow: (Fow1) 5′-AAAAAAGAATTCAGATCTATGACAGAGTTACCTGCAC-3′ and (Rev1) 5′-AAAAAAGGATCCCTAGATATAAAA-TTGATGGAATGC-3′. Primers were designed to introduce EcoR I, Bgl II restriction sites to the 5′ end and BamH I restriction site to the 3′ end of *PSEN1* gene, appropriate for cloning into pUC18 (Invitrogen) and pEGFP-C1 (BD Biosciences) plasmids. The cloning process was carried out using *E. coli* competent cells, strain TOP10 (Invitrogen). Site-directed mutagenesis for introducing a point mutation in the *PSEN1* gene (C → G at the position 1174) was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) in the conditions recommended by the manufacturer. Sequences of the mutagenic primers were as follow: 5′-CATTTTCTACAGTGTTGTGGTTGGTAAAGCCTCAGC-3′ and 5′-GCTGAGGCTTTACCAACCACAACACTGTA-GAAAATG-3′. Correctness of inserts sequences (wild type, Wt-PSEN1, and mutated Mut-PSEN1) was confirmed by the sequencing reaction (IBB, Warsaw). Inserts coding Wt-PSEN1 or Mut-PSEN1 were introduced at the 3′ end of *EGFP* (Enhanced Green Fluorescent Protein) gene in the pEGFP-C1 expression plasmid. Plasmids pEGFP-PSEN1(1400) and pEGFP-Mut-PSEN1(1400) were used to study *PSEN1* gene expression changes of in eukaryotic cells. Due to the low level of the EGFP-PSEN1 (EGFP with full length PSEN1) fusion protein expression in HeLa cells, a new fusion genes coding *EGFP* with shorter fragment of Wt-PSEN1 or Mut-PSEN1 were constructed. To create appropriate plasmids (pEGFP-Wt-PSEN1(400) and pEGFP-Mut-PSEN1(400)) the fragments of *Wt-PSEN1* or *Mut-PSEN1* coding region (1061–1404 nt position) were amplified by polymerase chain reaction (PCR) from the pEGFP-Wt-PSEN1(1400) or pEGFP-Mut-PSEN1(1400) plasmids, using the following primers: (Fow2) 5′-AAAAAAGTCGACTAGTAACACCTGAGTCAC-GAGCTGC-3′ and (Rev1) 5′-AAAAAAGGATCCCTA-GATATAAAATTGATGGAATGC-3′. PCR products were digested by Sal I and BamH I restriction enzymes and 344 bp inserts were cloned into pEGFP-C1 plasmid. The accuracy of sequences of both inserts was confirmed by the sequencing reaction (IBB, Warsaw).

2.4. Cell Culture and Transfection Conditions. HeLa (human cervical carcinoma) cells were cultured in RPMI (GIBCO, BRL, Paisley) supplemented with 10% FBS (GIBCO, BRL, Paisley) and antibiotics (penicillin 100 units/mL, streptomycin 100 mg/mL, Polfa) at 37°C and 5% CO_2. Twenty-four hours before the experiment, cells were plated in 96-well plate, (plates with black walls and transparent bottom, Perkin-Elmer) at the density of 15×10^3 cells per well. Directly before the transfection, the cell medium containing antibiotics was replaced with the new one, free of antibiotics. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) at a ratio 1.5 : 1 (1.5 μL of Lipofectamine 2000 per 1 μg of nucleic acid) according to the manufacturer's protocol. For dual fluorescence assay (DFA), HeLa cells were cotransfected with DNA plasmids: reporter plasmid pDsRed-N1 (BD Biosciences) (15 ng/well) and pEGFP-Wt-PSEN1(400) (100 ng/well) or pEGFP-Mut-PSEN1(400) (100 ng/well) and siRNAs (1 nM) dissolved in OPTI-MEM medium (GIBCO, BRL, Paisley). After 5 hours of incubation, transfection mixture was replaced with fresh medium with antibiotics. After next 48 hours of incubation at 37°C in 5% atmosphere of CO_2, the cells were washed three times with PBS buffer (without Ca^{2+} and Mg^{2+}) and lysed with NP-40 buffer (150 mM NaCl, 1% IGEPAL, 50 mM Tris-HCl pH 7.0, 1 mM PMSF) overnight at 37°C. Prepared cell lysates were used for fluorescence determination.

Two cell lines of human fibroblasts (i) expressing wild type PSEN1 (CELMA) and (ii) L382V PSEN1 mutant (NOV4), were cultured in Dulbecco's MEM (Sigma-Aldrich Co., Saint Louis, MO), supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) in 24-well plate at 37°C and 5% CO_2. Before transfection, the culture medium was replaced with fresh medium, free of antibiotics. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) and appropriate siRNAs at the ratio 2 : 1. To test the effect of siRNA on Aβ level, the cells were transfected with siRNAs (150 or 300 nM). After transfection, cells were incubated for 5-6 hours and then medium with transfection mixture was replaced with the fresh, culturing medium with antibiotics and 5% FBS. After 72-hour incubation at 37°C and 5% CO_2, cells were washed three times with PBS buffer (without Ca^{2+} and Mg^{2+}), the fresh medium with 0.2% FBS was added and cells were incubated for the next 24 hours. Then the culturing medium was collected with 1 mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, serine protease inhibitor, Sigma) for determination of Aβ42 level by ELISA (Enzyme-linked immunosorbent assay).

2.5. Dual Fluorescence Assay. Fluorescence values of EGFP and RFP (red fluorescent protein) were determined using a Synergy HT reader (BIO-TEK). Quantification of data was done with KC4 software. Excitation and emission wavelengths for each protein were as follows—EGFP: λ_{Ex} = 485/20 nm and λ_{Em} = 528/20 nm and RFP: λ_{Ex} = 530/25 nm and λ_{Em} = 590/30 nm. The activity of siRNA duplexes was calculated as the ratio of EGFP to RFP fluorescence values according to the following equation. Activity of siRNA (%) = 100%− (sample EGFP-X/RFP : control EGFP-X/RFP) ×100% were (EGFP-X means the fluorescence value of EGFP-X fusion protein, X = Wt-PSEN1 or Mut-PSEN1).

Every time an average value of fluorescence was mean of eight repeats calculated after eliminating extreme values. Each siRNA activity value given on the plots is the average of mean values from three independent experiments. The level of fluorescence (EGFP/RFP) in control cells (transfected with pDsRed-N1 and pEGFP-Wt PSEN1(400) or pEGFP-Mut-PSEN1(400) plasmids and control nonsilencing siRNA duplex) was taken as a reference (100%).

2.6. Determination of Aβ42 Level. Chromogenic sandwich ELISA kit (Signet Laboratory, Denham, MA) was used according to the manufacturer's procedure. To analyze the level of Aβ42 in conditioned medium (after transfection of siRNAs) collected as described in p.2.4., the 100 μL samples of medium were placed in a 96-well ELISA plate, precoated with a capture antibody (specific to the N-terminus of human Aβ42 peptide) and incubated at 4°C for 16 hours. After washing, a reporting antibody, that binds to the C-terminus of Aβ42, was added. After incubation for the next 2 hours and extensive washing, a horseradish peroxidase conjugated secondary antibody was added. Color developed with substrate o-phenylenediamine was measured at λ_{492} using a Synergy HT reader, and received data were quantified using KC4 software. The siRNA activity was calculated as the ratio of Aβ42 in experimental and control sample (cells treated with transfection reagent only). Results showed at the graph (Figure 6) are the average of two independent experiments.

2.7. FACS Flow Cytometry. For flow cytometry assay, HeLa cells were seeded on the 6-well plate (3×10^5 cells per well) and cotransfected with appropriate plasmids: pDsRed-N1 (0.5 μg/well) and pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400) (1.5 μg/well) and siRNAs: control nonsilencing siRNA and duplexes targeting the mutated form of PSEN1 transcript such as siRNA no 2, 7, 10, 11, 12 and 16 (denoted as P2, P7, P10, P11, P12, and P16, resp.) (5 nM) using Lipofectamine 2000, according to the manufacturer's protocol. After 48 hour incubation at 37°C and 5% CO_2, cells were washed three times with ice cold PBS buffer (without Ca^{2+} and Mg^{2+}) and collected for flow cytometry assay. Flow cytometry was performed on BD FACS Calibur Flow Cytometry System (Becton Dickinson) using Ar-ion laser (488 nm). Fluorescence dot plots and histograms were generated using Cell Quest software.

2.8. Statistical Analysis. The values of EGFP-PSEN1 fluorescence were normalized to the values of RFP fluorescence for each studied sample. All results are given as a percent of the fluorescence of sample toward fluorescence of control cells (transfected with Lipofectamine 2000 only or with control, nonsilencing siRNA) with standard deviation of mean. The Shapiro-Wilks' W test was used to analyze the normality of distribution of the resulted data. Statistical analysis of differences between two groups of data (Wt-PSEN1 versus Mut-PSEN1) were calculated by the use the Student's t-test (for data with normal distribution) or by the nonparametric Mann-Whitney's U-test. The differences with $P < .05$ were considered as statistically significant.

3. Results and Discussion

3.1. Construction of the Expression Plasmids Carrying the Wild Type and Mutant Alleles of PSEN1 Gene. The wild type and 1174 C → G, L392V mutant of *PSEN1* gene were selected as a model system to assess the allele-specificity of the siRNAs and to select the duplexes, that discriminate between two target RNAs differing by the single nucleotide. Plasmids coding the wild type and mutant type genes were prepared as described in Section 2. At first, the full length (1404 bp) wild type and mutant *PSEN1* genes were cloned in ORF (open reading frame) at the 3′-UTR of *EGFP* gene coded in the pEGFP-C1 plasmid. The expression products were designed to contain the EGFP protein fused by five amino acids linker (-Ser-Gly-Leu-Arg-Ser-) with intact Wt-PSEN1 or Mut-PSEN1 protein. The resulting plasmids pEGFP-Wt-PSEN1(1400) or pEGFP-Mut-PSEN1(1400) were cotransfected with the reporter plasmid pDsRed-N1 coding RFP, into HeLa cells. After 48 hour incubation, only very low levels of the fluorescence of EGFP fusion proteins were observed. Probably, attaching the 473-aa long polypeptide chain at the C-termini of EGFP resulted in abnormal folding of the fusion protein and, in consequence, in the low level of the fluorescence. Therefore these plasmids were excluded from further experiments and new, shorten fusion genes were prepared, containing a 344 bp 3′-terminal fragment of *PSEN1* gene, in the wild-type and mutated versions, attached to the 3′-UTR of *EGFP* gene in the pEGFP-C1 expression plasmid. Additionally, the translation stop codons TAG and TAA were introduced into *EGFP* upstream of the coding sequence of fragment of the *PSEN1* gene. The length of the obtained plasmids was confirmed by 0.5% agarose gel electrophoresis (Figure 1). We expected the high value of the fluorescence of EGFP in the case of intact fusion transcript *EGFP-PSEN1* and low or no fluorescence if the fusion transcript is degraded by RNAi. Results of the dual fluorescence assay for all reporter plasmids (pEGFP-Wt-PSEN1(1400); pEGFP-Mut-PSEN1(1400); pEGFP-Wt-PSEN1(400) and pEGFP-Mut-PSEN1(400)) are shown in Figure 2. Plasmids pEGFP-Wt-PSEN1(400) and pEGFPMut-PSEN1(400) were used in further studies of the siRNA activity toward the *Wt-* and *Mut-PSEN1* genes both expressed in HeLa cells.

3.2. Design, Synthesis, and Evaluation of the Activity of siRNAs Targeting Mutant Allele of PSEN1

3.2.1. siRNA Molecules. We synthesized a set of 19 siRNAs, designed across the L392V point mutation in the human *PSEN1* gene. The L392V mutant contains a guanosine at the position 1174 of mRNA, whereas the wild-type mRNA bears a cytidine at that position. Each siRNA antisense strand was fully complementary to the mutant PSEN1 mRNA, but in relation to the wild type mRNA contained a C:C mismatch (Figure 3). The structure of all tested oligonucleotides was confirmed by mass spectrometry (MALDI-TOF MS, Table 1), and their purity was assessed on 20%

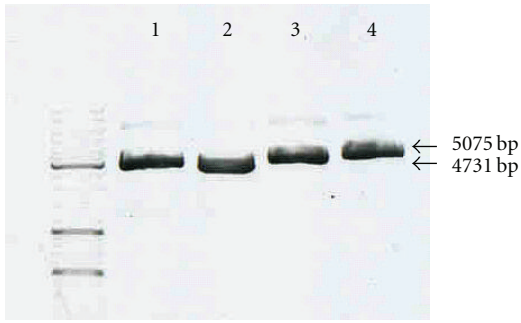


Figure 1: Electrophoretic analysis of the plasmids carrying the wild type (Wt) and mutant (Mut) alleles of the *PSEN-1* gene and original, commercially available plasmids: (1) pEGFP-C1 (4731 bp), (2) pDsRed-N1 (4689 bp) (3) pEGFP-Wt-PSEN1(400) (5075 bp), (4) pEGFP-Mut-PSEN1(400) (5075 bp) (0.5% agarose gel).

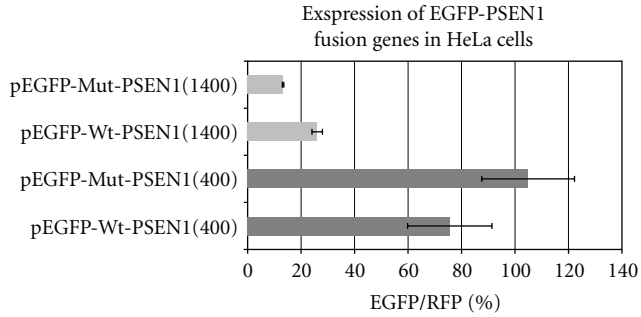


Figure 2: Comparison of the fluorescence values of EGFP-PSEN1 fusion proteins expressed from the *EGFP-PSEN1* gene coding plasmids. Plasmids pEGFP-Wt PSEN1(1400) and pEGFP-Mut PSEN1(1400) contain full length (1404 bp) wild type (Wt) and mutein (Mut) (C → G 1174) *PSEN1* gene introduced at the 3′-UTR of *EGFP* gene; plasmids pEGFP-Wt-PSEN1(400) and pEGFP-Mut-PSEN1(400) contain insert with shorter version (344 bp) of *PSEN1* (Wt or Mut) introduced at the 3′-UTR of the *EGFP* gene.

denaturing PAGE (data not shown). All siRNA duplexes used in these experiments had the typical structure of 19 bp fully complementary duplex with 2-nt overhangs at each 3′-end with typically used two thymidine units (TT). This approach was intended for the proper interaction of RNA duplex with proteins of RNAi machinery [49]. Ago2 protein, the main catalytic core of the RISC (RNA-induced silencing complex) binds the guide strand, which leads the complex to the complementary mRNA strand. Ago2-mediated RNA cleavage requires perfect Watson-Crick base pairing between the guide strand of siRNA and the target mRNA, spanning both, the seed region (positions from 2nd to 8th nucleotide) and the cleavage site (phosphate group opposite to the internucleotide linkage located between 10th and 11th nucleotide, counted from the 5′-end of the guide strand [50].

3.2.2. Silencing Activity Measured in the Dual Fluorescence Assay. In order to evaluate the silencing activity of siRNAs, we used the already described dual fluorescence reporter system [51–55]. This assay is based on the measurement of the relative fluorescence intensity of EGFP and RFP proteins, expressed from exogenously delivered plasmids cotransfected together with siRNA. In our case, the HeLa cells were transfected with pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400) and pDsRed-N1 as well as with P1–P19 siRNAs listed in Table 1 and Figure 3. The results of the fluorescence measurements are shown in Figure 4. The P8–P11 siRNAs, with the perfect complementarity to the mutant allele, but in the same time with one mismatch (cytidine:cytidine, C:C) to the wild type gene placed at the central positions of the duplex, efficiently silenced the mutant gene (up to 90%, $P < .0001$), while only slightly reducing the wild type gene expression (silencing ca. 10–30%, $P < .0001$). Interestingly, duplexes P1 and P2 showed a very strong silencing property towards both—the wild type and the mutated genes. This high silencing activity of the latter duplexes may be related to the high thermodynamic asymmetry of the duplex ends (discussed in Section 3.4) in comparison to other siRNA duplexes or to the better access of siRNA to the target region of the mRNA. The remaining P3, P5, P6, P7, and P12–P19 duplexes were almost inactive in relation to both the wild type as well as the mutant gene.

3.2.3. Silencing Activity Measured by Flow Cytometry. HeLa cells cotransfected with pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400), pDsRed-N1 plasmids, and with selected duplexes P2, P7, P8, P9, P10, P11, P12, and P16 siRNAs were analyzed by FACS flow cytometry. The amount of cells containing the EGFP and RFP fluorescence in the tested samples (cells transfected with siRNAs) was determined and compared with the amount of cells in control samples (cells treated with transfection reagent only or treated with nonsilencing, control siRNA). The duplexes perfectly complementary to the L392V PSEN1 mRNA but with one mismatch (C:C) towards the wild type gene, lockated at the central position of the antisense strand (as P8 and P9), exhibited the highest allele-specificity and silencing efficiency exclusively towards the mutant gene (Figure 5). Thus, the data obtained in this analysis confirmed the above described results.

3.2.4. Evaluation of the Level of Aβ42 by ELISA. The activity of siRNAs towards endogenous target genes was evaluated by the measurement of the level of extracellular Aβ42 released from the transfected cells. For this purpose human fibroblasts derived from the patients with FAD, carrying L392V mutantion in PSEN1 (NOV4), were used [56, 57]. Human fibroblasts, carrying the wild type of PSEN1 (CELMA), derived from healthy individuals were used as a control [56, 57]. The amount of the Aβ42 peptide was determined by the sandwich ELISA test. The normalized results obtained for this assay for both types of cells transfected with control, nonsilencing, and P10 siRNA are shown in Figure 6. The amount of the Aβ42 peptide released from NOV4 cells ($NOV4_{A\beta 42}$) transfected with P10 siRNA was reduced by ca. 40% ($P = .000021$) compared to fibroblasts NOV4 treated only with Lipofectamine 2000 or transfected with control nonsilencing siRNA. Application of siRNAs directed

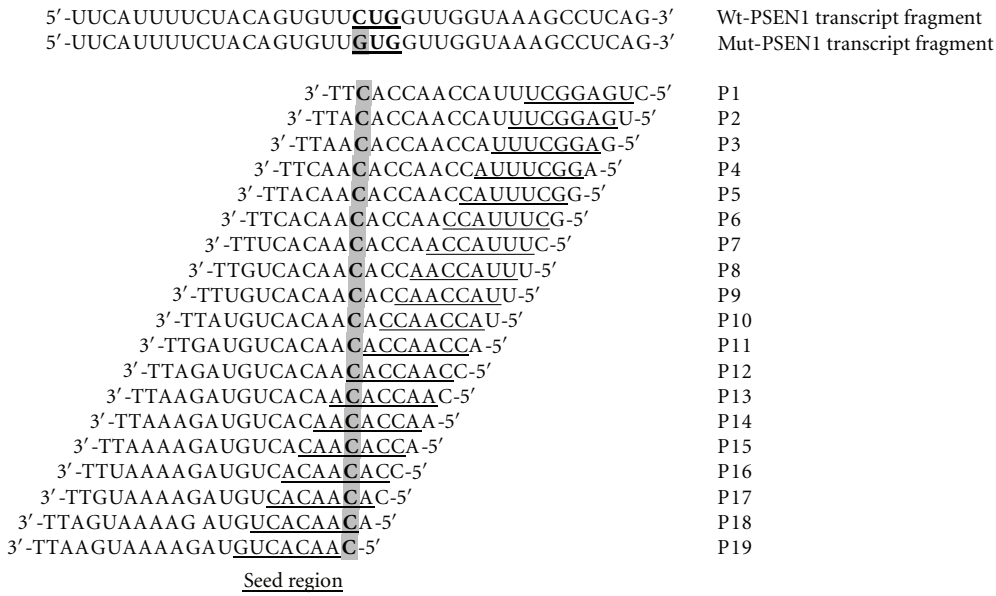


Figure 3: The sequences of the antisense strands of siRNAs used in studies. The guide strand of each siRNA is fully complementary to the mutant (C → G) PSEN1 transcript and it has one mismatch (C:C) in respect to the wild type gene. The region between 2nd to 8th nucleotide, counted from the 5′-end of the antisense strand of siRNA is known as the seed region.

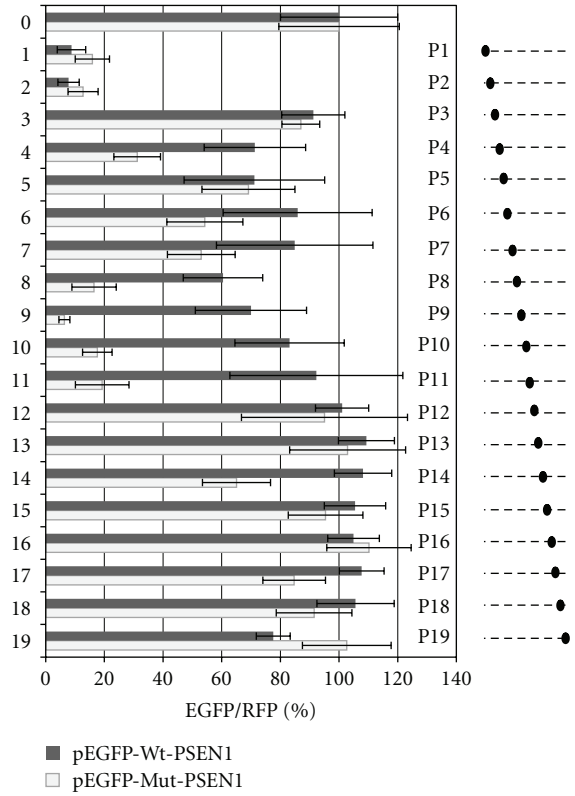
towards the mutated *PSEN1* gene did not influence the level of Aβ42 released from control fibroblasts ($CELMA_{A\beta42}$), which was much lower than in NOV4 cells (both $NOV4_{A\beta42}$ and $CELMA_{A\beta42}$ levels are showed at the graph as 100%). The results obtained may constitute a proof-of-concept that silencing of the mutant allele of *PSEN1*, exerted by fully complementary siRNA, led to the reduced expression of the mutant Presenilin1, which in consequence gave less of the toxic Aβ42 product.

3.3. Modulation of Allele-Specific Activity of siRNAs by Chemical Modifications

3.3.1. Modification of siRNA with 2-Thiouridine. The allele-specific activity was evaluated for siRNA modified with naturally occurring 2-thiouridine (s^2U) nucleoside. As it has been already demonstrated, s^2U, due to preferred C3′-*endo* conformation of the ribose ring, improves the thermodynamic stability of s^2U-containing double stranded RNA helices [52, 58, 59]. Additionally, s^2U unit, when present in the anticodon region of transfer RNA (tRNA), enhances its specificity of Watson-Crick base pairing with A and restricts wobble base pairing with G [58, 59]. We choose P10 duplex, which previously has shown satisfactory discrimination between wild type and mutant alleles, and introduced the s^2U unit at the position 18 of the antisense strand (P10-thio) (Figure 7(b)). The s^2U modification at this position of the RNA duplex (the 3′-end of duplex) improves its thermodynamic asymmetry and facilitates incorporation of the antisense strand into the RISC complex [52]. Moreover, s^2U introduction can impact positively on the uptake of siRNA into cells, due to the presence of sulfur moiety, which increases the hydrophobic properties of the molecule. Results of the dual fluorescence assay performed for HeLa cells transfected with P10-thio siRNA are presented in Figure 7(c). It is clearly shown that introduction of the s^2U unit at the 3′-end of the antisense strand of P10 siRNA further improved allele discrimination (~10%) due to the increased thermodynamic asymmetry of the duplex.

3.3.2. Silencing Activity of Sulfur-Containing siRNA Evaluated by Fluorescence Microscopy. The activity of the selected siRNAs was also observed by the fluorescence microscopy. Figure 8 shows fluorescence images of the fixed samples obtained from HeLa cells transfected with P10, P10-thio, and P11 siRNA duplexes. Here again, the cells carrying the wild type gene (*EGFP-Wt-PSEN1*(400)) expressed green fluorescent protein at the high level and independently of the siRNA sequence. In contrast, slides containing cells expressing the mutant gene (*EGFP-Mut-PSEN1*(400)) have shown less fluorescently labeled cells, and the most pronounced result was obtained for cells transfected with the P10-thio siRNA duplex, confirming high discrimination ratio of siRNA against the wild type and mutant alleles of the *PSEN1* gene.

3.3.3. Modification of siRNA with Wobble Base Pair. The region near the central positions at the antisense strand of siRNA (10th-11th) is interesting, primarily from the perspective of a nucleolytic reaction catalyzed by Ago2 [49, 50]. To investigate the influence of correct base pairing in this region on allele-specificity and the overall activity of RNA duplex, the wobble type modification was introduced at the two positions of the antisense strand, namely position 9 or 11 counting from the 5′end, adjacent to the mutation site (Figure 9(a)). According to our earlier assumption, introduc-



(a)

siRNA (Wt-PS1 versus MutPS1)	Test name	*P* value	siRNA (Wt-PS1 versus MutPS1)	Test name	*P* value
P1	Student's *t*-test	.000946	P11	Mann-Whitney's *U*-test	.000002
P2	Student's *t*-test	.023819	P12	Mann-Whitney's *U*-test	.593489
P3	Mann-Whitney's *U*-test	.386028	P13	Student's *t*-test	.000001
P4	Mann-Whitney's *U*-test	.000004	P14	Mann-Whitney's *U*-test	.0000595
P5	Mann-Whitney's *U*-test	.546494	P15	Mann-Whitney's *U*-test	.429196
P6	Mann-Whitney's *U*-test	.000107	P16	Student's *t*-test	.005251
P7	Mann-Whitney's *U*-test	.000173	P17	Mann-Whitney's *U*-test	.220432
P8	Mann-Whitney's *U*-test	.000025	P18	Student's *t*-test	.000603
P9	Mann-Whitney's *U*-test	.000089	P19	Student's *t*-test	.000019
P10	Mann-Whitney's *U*-test	.000002			

(b)

Figure 4: (a) Comparison of the fluorescence level obtained from analysis of HeLa cells cotransfected with pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400), pDsRRed-N1 and indicated siRNAs (0—control nonsilencing siRNA and siRNAs targeting the mutated form of PSEN1 transcript (denoted as P1–P19) (1 nM). Dotted lines at the right panel represent the antisense strands of the used siRNAs. Positions of the C:C mismatch between the guide strand of siRNA and the wild-type PSEN1 mRNA are indicated by the black dots. The level of the relative EGFP/RFP fluorescence of the cells transfected with control non-silencig siRNA was used as 100%. The results are mean values from three independent experiments. (b) Statistical analysis of differences between two groups of data (Wt-PSEN1 versus Mut-PSEN1) calculated by the use the Student's *t*-test (for data with normal distribution) or by the nonparametric Mann-Whitney's *U*-test. The differences with $P < .05$ were considered as statistically significant.

tion of such structural disorder into RNA duplex reduces the thermodynamic stability of the duplex and weakens the interaction between guide strand and the target mRNA. We supposed that the wobble modification could increase the allele-specificity of siRNAs targeted towards the mutant gene. Instead, we observed significant deterioration of the silencing activity of both modified siRNAs (P10W9 and P10W11) (Figure 9(b)). Most probably, introduced disorder in the base pairing like wobble G:U considerably disturbs the recognition of the double stranded RNA helix (containing mRNA and the antisense strand of siRNA) by Ago2, what results in inhibition of the silencing process.

3.4. Thermodynamic Properties of siRNA Duplexes (P1–P19) versus Their Silencing Activity. Thermodynamic stability of RNA duplexes and secondary structure of RNA are often predicted by using free-energy parameters from a near-neighbor model [60]. To assess the relative thermodynamic stability

Table 2: Differences between the free Gibbs energy of 5′-end and 3′-end of the duplex (resp. to the polarity of the antisense strand), calculated independently for three and for four consecutive base pairs shown for each duplex ($\Delta\Delta G_{37^\circ C}$ = $\Delta G_{37^\circ C}$ (5′-end) − $\Delta G_{37^\circ C}$ (3′-end)). The values of $\Delta G_{37^\circ C}$ were calculated according to [60].

siRNA	Sequence	$\Delta\Delta G_{37^\circ C}$ (kcal/mol) (for 3 bp)	$\Delta\Delta G_{37^\circ C}$ (kcal/mol) (for 4 bp)
P1	5′-GUGG—UCAGTT-3′ 3′-TTCACC—AGUC-5′	1	1.6
P2	5′-UGUG—CUCATT-3′ 3′-TTACAC—GAGU-5′	−0.2	−0.1
P3	5′-UUGU—CCUCTT-3′ 3′-TTAACA—GGAG-5′	−1.9	−2.7
P4	5′-GUUG—GCCUTT-3′ 3′-TTCAAC—CGGA-5′	−0.5	−1.8
P5	5′-UGUU—AGCCTT-3′ 3′-TTACAA—UCGG-5′	−3	−3.8
P6	5′-GUGU—AAGCTT-3′ 3′-TTCACA—UUCG-5′	−1.2	0
P7	5′-AGUG—AAAGTT-3′ 3′-TTUCAC—UUUC-5′	0.7	1.6
P8	5′-CAGU—UAAATT-3′ 3′-TTGUCA—AUUU-5′	1.7	2.7
P9	5′-ACAG—GUAATT-3′ 3′-TTUGUC—CAUU-5′	1.4	1
P10	5′-UACA—GGUATT-3′ 3′-TTAUGU—CCAU-5′	0	−1.1
P11	5′-CUAC—UGGUTT-3′ 3′-TTGAUG—ACCA-5′	−1.7	−1.4
P12	5′-UCUA—UUGGTT-3′ 3′-TTAGAU—AACC-5′	−0.7	−0.5
P13	5′-UUCU—GUUGTT-3′ 3′-TTAAGA—CAAC-5′	0.5	0.1
P14	5′-UUUC—GGUUTT-3′ 3′-TTAAAG—CCAA-5′	−0.7	−1.3
P15	5′-UUUU—UGGUTT-3′ 3′-TTAAAA—ACCA-5′	−2.7	−3.6
P16	5′-AUUU—GUGGTT-3′ 3′-TTUAAA—CACC-5′	−3.4	−4.6
P17	5′-CAUU—UGUGTT-3′ 3′-TTGUAA—ACAC-5′	−1.2	−2.1
P18	5′-UCAU—UUGUTT-3′ 3′-TTAGUA—AACA-5′	0.7	0.7
P19	5′-UUCA—GUUGTT-3′ 3′-TTAAGU—CAAC-5′	0.5	0.2

of the duplex ends, we counted up the theoretical values of Gibbs free energy, $\Delta G_{37^\circ C}$, of three and of four base pairs at the 5′-end and the 3′-end of siRNA (numbering according to the guide strand polarity) for each duplex used in our studies, by the use of the method recommended by Freier et al. [60]. The standard-state free energy of helix formation was counted as the sum of the following terms: (i) a free-energy change for helix initiation associated with forming the first base pair in the duplex, (ii) the sum of propagation free energies for forming each subsequent base pair, and (iii) free-energy increments for unpaired terminal nucleotides. Then, differences between the free Gibbs energy of the 5′-end and the 3′-end for each duplex were calculated independently for three and four consecutive base pairs according to the following equation: $\Delta\Delta G_{37^\circ C}$ = $\Delta G_{37^\circ C}$ (5′-end) − $\Delta G_{37^\circ C}$ (3′-end). The results of this analysis are shown in Table 2

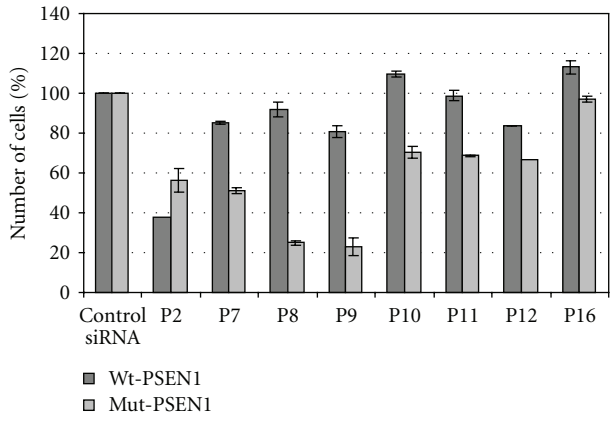
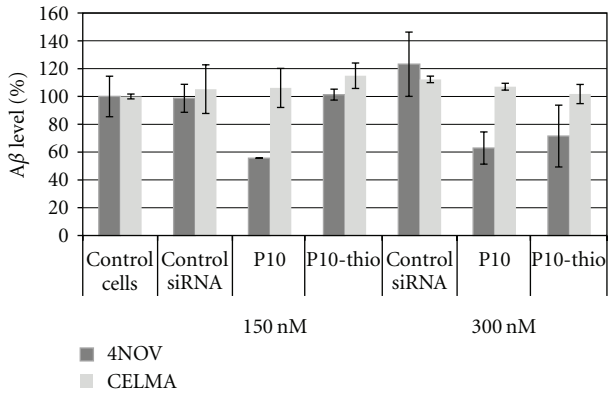
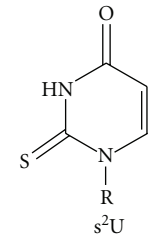


Figure 5: Results of flow cytometry assay. HeLa cells were cotransfected by pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400), pDsRed-N1, and indicated siRNAs, using Lipofectamine 2000 as a transfection reagent (details described in Section 2). HeLa cells containing both, the EGFP and RFP fluorescence, were counted in the BD FACS Calibur Flow Cytometry System (Becton Dickinson) using Ar-ion laser (488 nm). Fluorescence dot plots were generated using Cell Quest software.



siRNA (Wt-PS1 versus MutPS1)	Test name	*P* value
P10 (150 nM)	Student's *t*-test	.0010787
P10 (300 nM)	Student's *t*-test	.000021
P10-thio (300 nM)	Student's *t*-test	.000234

Figure 6: The normalized values of the extracellular Aβ42 levels in medium of cells transfected with the siRNA duplexes. Human fibroblasts expressing endogenously the wild type *PSEN1* gene (CELMA) and L392V mutant (NOV4) were cultured in the presence of P10, P10-thio, and control nonsilencing siRNA duplexes (150 and 300 nM). After 48-hour incubation, the culturing medium was collected with 1 mM AEBSF, and the level of Aβ42 was measured by sandwich ELISA test. Data are presented as percentage values to control cells treated with lipofectamine only. The results are mean values from two independent experiments. Statistical analysis of differences between two groups of data (Wt-PSEN1 versus Mut-PSEN1) were calculated by the use of Student's *t*-test (data with normal distribution). All differences were considered as statistically significant.

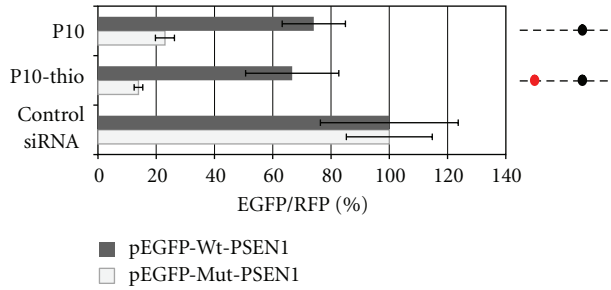


(a)

P10 5′-UACAGUGUU**G**UGGUUGGUATT-3′
3′-TTAUGUCACAA**C**ACCAACCAU-5′

P10-thio 5′-UACAGUGUU**G**UGGUUGGUATT-3′
3′-TTA**s²U**GUCACAA**C**ACCAACCAU-5′

(b)



siRNA (Wt-PS1 versus MutPS1)	Test name	*P* value
P10	Student's *t*-test	.000000
P10-thio	Mann-Whitney's *U*-test	.001194

(c)

Figure 7: (a) 2-Thiouridine (s^2U) structure, R = β-D-ribofuranoside residue. (b) Sequences of the P10 and P10-thio modified siRNAs. (c) Comparison of the relative (EGFP/RFP) fluorescence in HeLa cells transfected with reporter plasmids: pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400), pDsRed-N1 and siRNA: P10 and P10-thio (1 nM). Dotted lines represent the antisense strands of the used siRNAs. Positions of the C-C mismatch between the guide strand of siRNA and the wild-type PSEN1 mRNA are indicated by the black dots. Red dot represents the site of the s^2U modification at the antisense strand. The level of the relative EGFP/RFP fluorescence in cells transfected with control nonsilencing siRNA was used as 100%. Statistical analysis of differences between two groups of data (Wt-PSEN1 versus Mut-PSEN1) were calculated by the use the Student's *t*-test (for data with normal distribution) or by the nonparametric Mann-Whitney's *U*-test. The differences with $P < .05$ were considered as statistically significant.

and in Figure 10. The positive $\Delta\Delta G_{37^\circ C}$ values indicate the higher thermodynamic stability of the 3′-end of the duplex, thus the antisense strand should be selected as the guide strand and the siRNA duplex should be more active. The negative values of $\Delta\Delta G_{37^\circ C}$ mean the higher thermodynamic stability of the 5′-end of the duplex, thus the antisense strand is not preferred to be selected as a guide strand and, in consequence, the siRNA duplex would be expected to be less active towards the target gene. Comparison of the two graphs

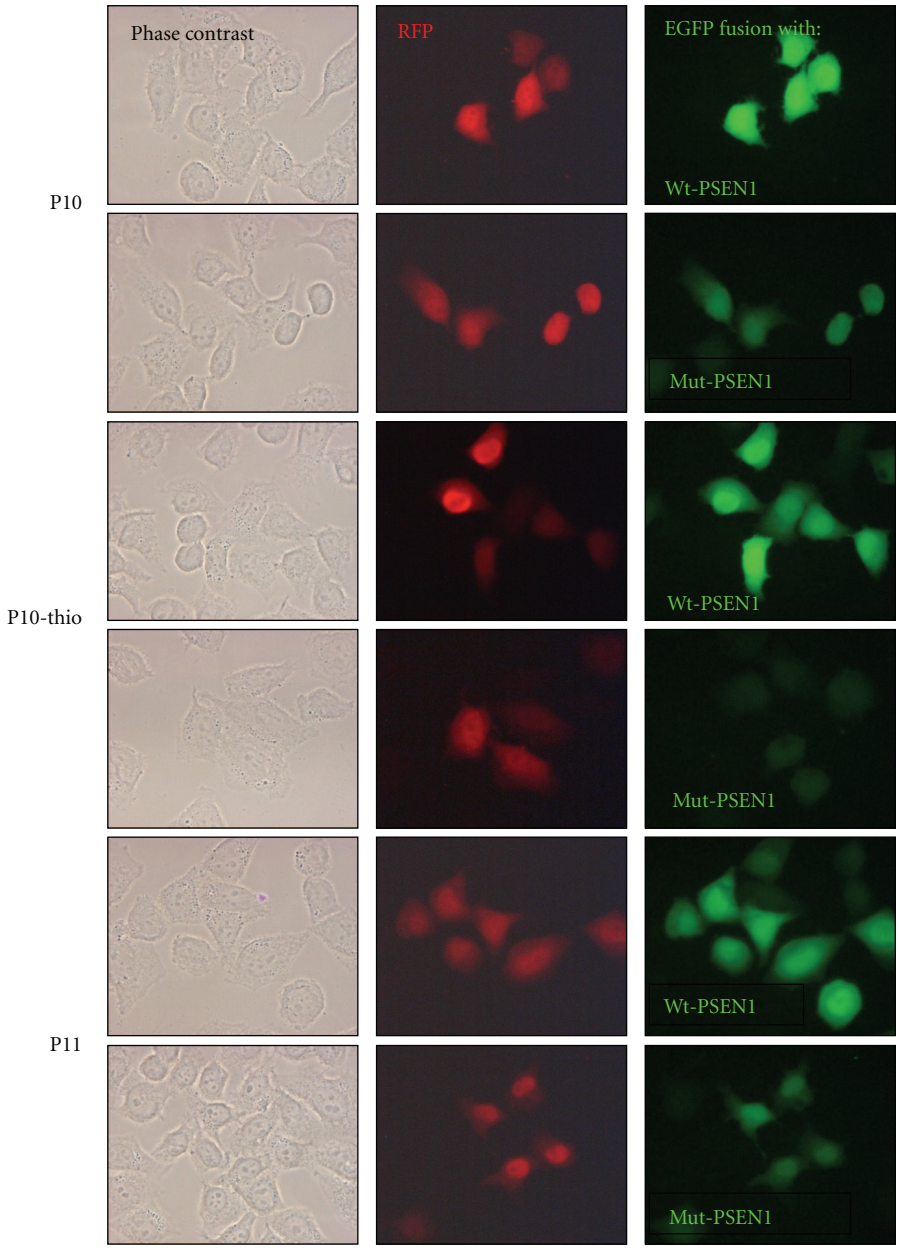
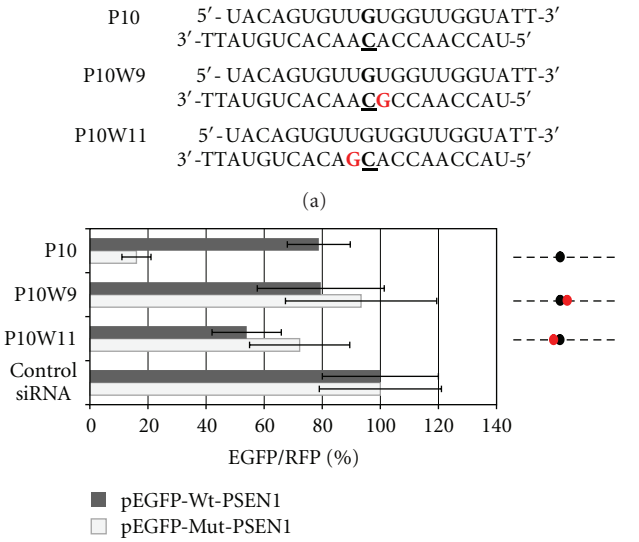


Figure 8: Silencing effects of siRNAs directed towards the *Mut-PSEN1* (C → G1174). Fluorescence microscopy image of cells expressing the wild type and mutant fusion protein in the presence of siRNA: P10, P10-thio, and P11.

representing the silencing activity (Figure 10(a)) and the thermodynamic features of the duplex ends (Figure 10(b)) can explain observed differences in the activity of the used siRNAs, for example, duplexes P3–P6 and P14–P17 are characterized by the negative values of Gibbs free energy and are neglectably active. On the contrary, duplexes P1, P2, and P7–P10 showing positive or close to zero values of Gibbs free energy are more active as RNAi triggers. In some cases, silencing efficacy of siRNA duplexes depends on other factors as the secondary structure of target mRNA or availability of target sequence for RNAi proteins [61]. Also the other, not identified factors may govern the silencing process of given mRNA targets with short interfering RNAs.

4. Conclusions

Dominantly inherited disorders, caused by the presence of the toxic mutant allele besides the wild type gene, constitute attractive targets for allele-specific gene silencing. Gene therapy aimed at the elimination of the mutant allele causing the disease can be achieved by an RNA interference technology. Clinical success of such therapies depends mostly on the ability of RNAi to discriminate between the wild type and mutant alleles. In this paper, we demonstrate the allele-specific silencing activity of the short interfering RNAs directed towards mutant allele of *PSEN1* gene. L392V *PSEN1* mutant gene contains 1174 C → G mutation, which results in



siRNA (Wt-PS1 versus MutPS1)	Test name	*P* value
P10W9	Mann-Whitney's *U*-test	.084761
P10W11	Mann-Whitney's *U*-test	.004816
P10	Mann-Whitney's *U*-test	.000000

(b)

Figure 9: (a) Sequences of the P10W9 and P10W11 siRNAs modified in the central domain at the position 9 or 11; A is replaced by G (in red) to generate the wobble base pair with complementary mRNA strand. (b) Comparison of the florescence levels obtained after co-transfection of HeLa cells with pEGFP-Wt-PSEN1(400) or pEGFP-Mut-PSEN1(400), pDsRed-N1 and indicated siRNAs (P10, P10W9, or P10W11). Dotted lines represent the antisense strands of the used siRNAs. Positions of the C-C mismatch between the guide strand of siRNA and the wild-type PSEN1 mRNA are indicated by the black dots. Red dots represent the sites of the wobble base pairs between the guide strand of siRNA and the wild- or mutated-type of PSEN1 mRNA. The level of the relative EGFP/RFP fluorescence in cells transfected with control nonsilencing siRNA was used as 100%. The results are mean values from three independent experiments. Statistical analysis of differences between two groups of data (Wt-PSEN1 versus Mut-PSEN1) were calculated by the use by the nonparametric Mann-Whitney's *U*-test. The differences with $P < .05$ were considered as statistically significant.

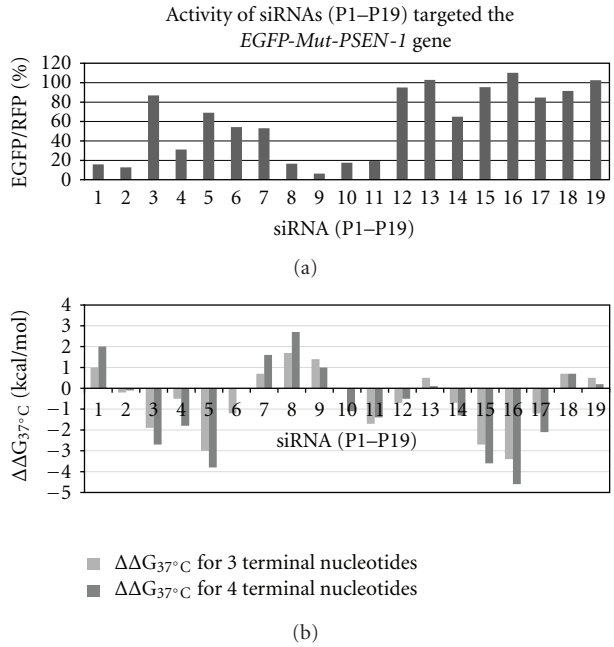


Figure 10: Comparison of the activity of siRNAs targeted *Mut-PSEN1* gene (a) with their thermodynamic data (b). siRNAs were fully complementary to the *Mut-PSEN1* transcript. Differences between the free Gibbs energy of 5′-end and 3′-end of the duplex (resp. to the polarity of the antisense strand) were calculated for three and for four consecutive base pairs: ($\Delta\Delta G_{37°C}$ = $\Delta G_{37°C}$ (5′-end) − $\Delta G_{37°C}$ (3′-end)) (compare with Table 2).

amino acid substitution, Leu to Val, at the position 392 of polypeptide chain of the protein. This mutation was found in patients with early onset FAD. At first, we synthesized a set of siRNAs, designed across the point mutation and determined their activity towards the mutant and wild type genes by a dual fluorescence assay, flow cytometry assay, and fluorescence microscopy images. The most active and allele-specific were the siRNA duplexes fully complementary to the target gene, which paired with the wild type allele with the C:C mismatch located in the central part of the mRNA-siRNA duplex (P8–P11). These duplexes silenced the mutant gene up to 90% and only slightly reduced the wild type gene, up to 10–30%. Introduction of the 2-thiouridine nucleoside into the 3′-end of the siRNA duplex (in respect to the polarity of the antisense strand) increased silencing activity of the duplex and discrimination ratio between the wild type and mutant allele (P10-thio). The wobble base pair modification introduced at the 9 or 11 positions of the antisense strand (P10W9 and P10W11), adjacent to the mutation site, reduced the thermodynamic stability of the duplex. We observed significant deterioration of the silencing activity of both modified siRNAs. Most probably, introduced disorder in the base pairing (G:U) considerably disturbed the recognition of the double stranded RNA helix (containing mRNA and the antisense strand of siRNA) by Ago2, what resulted in inhibition of the silencing process. The activity of the screened siRNAs was also evaluated by measurement of the level of extracellular Aβ42 released from the primary fibroblasts, carrying the (L392V) PSEN1 mutant (NOV4) in comparison to control fibroblasts (CELMA), carrying the wild type PSEN1, determined by ELISA. The normalized results obtained for NOV4 cells transfected with P10 and P10-thio siRNA showed reduced amount of Aβ42 by ca. 40% compared to the control fibroblasts treated only with transfection reagent. This result is a proof-of-concept that silencing of the mutant allele of *PSEN1*, exerted by fully complementary siRNA, led to reduced expression of the mutant Presenilin 1, which in consequence gave less of the toxic Aβ42 product.

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