Published in final edited form as:

Exp Gerontol. 2014 February ; 50: 114–121. doi:10.1016/j.exger.2013.12.001.

Analysis of 27 vascular-related proteins reveals that NT-proBNP is a potential biomarker for Alzheimer's disease and mild cognitive impairment: A pilot-study

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Abstract

Alzheimer's disease (AD) is a severe neurodegenerative disease. Cerebrovascular changes often accompany AD-related pathology. Despite a considerable progress in the diagnostic accuracy of AD, no blood biomarkers have been established so far. The aim of the present study was to search for changes in plasma levels of 27 vascular-related proteins of healthy controls, patients with mild cognitive impairment (MCI) and AD. In a sample of 80 participants we showed that out of these 27 proteins, six proteins were slightly changed (up to 1.5×) in AD (alpha2-macroglobulin, apolipoprotein-A1, plasminogen activator inhibitor, RAGE, Tissue Inhibitors of Metalloproteinases-1 and Trombospondin-2) and one marker (serum amyloid A) was enhanced up to 6× but with a very high variance. However, N-terminal pro-brain natriuretic peptide (NT-proBNP) was significantly enhanced both in MCI and AD patients (1.9×). In a second analysis of a sample of 110 subjects including younger healthy controls, we confirmed that NT-proBNP has the potential to be a stable candidate protein for both diagnosis and AD disease progression.

Keywords

Diagnosis; Plasma; Biomarker; Alzheimer; Mild cognitive impairment; Multiplex ELISA; NT-proBNP

1. Introduction

Alzheimer's disease (AD) is a severe neurodegenerative disease resulting in progressive impairment in memory and cognition. The disorder is pathologically characterized by

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Disclosure statement There are no actual or potential conflicts of interests.

extracellular beta-amyloid plaque deposition, intraneuronal tau pathology, neuronal cell death, vascular dysfunction and inflammatory processes. Since life expectancy in the general population increased within the last 100 years from about 40 to about 80 years and age is the main risk factor for AD, the number of patients suffering from AD and mixed forms of dementia will dramatically increase within the next 50 years. It is expected that there will be about 80 million AD patients worldwide in 2050. It will be essential to delay and counteract symptoms in AD and to start therapeutic treatment as early as possible. A valid and easily accessible diagnostic procedure should be the basis for an early treatment (Humpel, 2010).

A probable diagnosis of AD can be established with a confidence of N90%, based on clinical criteria, laboratory tests, neuroimaging and neuropsychological evaluation (Desai and Grossberg, 2005). Definitive diagnosis of AD requires a post mortem detection of beta-amyloid plaques and tau-pathology (Fradinger and Bitan, 2005; McKeel et al., 2004). However, an early sensitive diagnosis of AD is still difficult because early symptoms are frequently masked by a variety of disorders, including vascular changes or cognitive deficits caused by depression. Established laboratory markers of AD are limited to the analysis of 3 biomarkers in cerebrospinal fluid (CSF): beta-amyloid(1–42) and total tau and phospho-tau-181 (Blasko et al., 2006; Blennow, 2005; Hampel et al., 2010a; Zetterberg et al., 2010). However, CSF analysis is limited due to the invasive collection. Further, CSF analysis does so far not distinguish between AD and other forms of dementia, such as e.g. vascular dementia or frontotemporal lobe dementia. Thus there is a great need to search for biomarkers in blood. Several studies have been conducted in blood samples in order to establish specific changes of proteins. Despite these great efforts, no specific blood biomarker could be established as a biomarker yet (Borroni et al., 2006; Hampel et al., 2010b; Humpel, 2010; Kurz et al., 2002; Lewczuk and Wiltfang, 2008; Pelech, 2008).

AD shares many of the risk factors of cerebrovascular disease. There is increasing supportive evidence that vascular risk factors contribute to the pathogenesis of AD (De la Torre, 2002; Humpel, 2011; Iadecola, 2004; Kudo et al., 2000; Zlokovic, 2005). Mixed pathology (AD plus vascular pathology) is increasingly being identified in autopsy studies of people aged 80 or over with dementia, and not all vascular autopsy changes are well covered by MRI imaging. However, it is speculated that neurodegeneration in AD may arise from a chronic mild cerebrovascular dysregulation caused by continuous exposure to the risk factors over years (Humpel, 2011), which precedes hypoperfusion (De la Torre, 2002; Iadecola, 2004). Some supportive epidemiological studies (De la Torre, 2002; Humpel, 2010; Rocchi et al., 2009; Zlokovic, 2005) suggest that these risk factors could be old age, atherosclerosis, stroke, diabetes, hyperhomocysteinemia, hypertension, hyperlipidemia, head injury, transient silent strokes, high serum viscosity, thrombogenic factors, cardiac disease, the apolipoprotein E4 allele, smoking, alcohol consumption, high cholesterol, fat food, reduced vitamin B12 uptake, high fibrinogen levels, depression, and others. Blood plasma may be a rich source of biomarkers in AD and may reflect a systemic metabolic signature of AD or be a change in plasma secondary to a disease-specific process in the brain (Mattila and Frey, 1995).

In the present pilot study, we examine whether plasma proteins related to the vascular system are altered in MCI or AD. These proteins include markers involved in wound healing

(for example, alpha2-macroglobulin, fibronectin, thrombomodulin, keratinocyte growth factor, platelet-derived growth factor, thrombopoietin, thrombospondin, tissue plasminogen activator and inhibitor, fibrinogen) as well as markers involved in cardiovascular pathology and atherosclerosis (for example, N-terminal prohormone of brain natriuretic peptide, TNF-related apoptosis-inducing, programmed cell death protein 1, myeloperoxidase, neutrophil gelatinase-associated lipocalin, receptor for Advanced Glycation Endproducts (RAGE), serum Amyloid A, TNF-like weak inducer of apoptosis, tissue inhibitors of metalloproteinases (–1 and –2), beta-defensin-2, apolipoprotein A-1 and B-100 (ApoB-100), oxidized low density lipoprotein (oxLDL) and antibodies against oxidized lipids (oLAbs). However, these selected vascular proteins comprise only a small number of possible vascular proteins. We have selected these proteins because they represent pathophysiological processes involved in neurodegenerative diseases. Some of these proteins have been reported to be up- or down-regulated in previous reports. The availability of commercial (multiplex) ELISA kits was also a selection criterion. The present pilot study will reveal whether a single protein or a set of vascular proteins is altered in MCI and/or Alzheimer patients who don't suffer from a clinically significant vascular disease.

2. Methods

2.1. Study design

A total number of 110 participants (healthy controls, AD and MCI) was included in this study during a time period of 2004–2012. All patients were older than 60 years and were recruited from the Memory Clinics at the Department of Psychiatry in Innsbruck or the Landeskrankenhaus Hall/Tirol in Austria. Healthy subjects were also recruited at this site. They were mainly healthy caregivers and volunteers. Subjective cognitive impairment was an exclusion criterion for healthy subjects. Psychiatrists clinically examined all subjects, performed a standardized neurological examination, reviewed medical records and all subjects underwent a neuropsychological assessment. For the NT-proBNP analysis also younger healthy volunteers (mean age 30 ± 1.4 years) were included. Twenty two patients were selected based on availability to follow up during a time period of 2008–2012: seven controls converted to MCI, 7 MCI patients converted to AD, and 5 patients were stable for MCI and 3 stable for AD. The study was approved by the local ethical committee of Innsbruck Medical University and was in accordance with Helsinki Declaration and all subjects gave written informed consent.

2.2. Exclusion criteria

Particular care was taken to exclude subjects with severe medical or neurological conditions sufficient to explain the cognitive deficits. Exclusion criteria for healthy subjects and patients suffering from MCI or AD included 1) current diagnosis with a psychiatric disorder (including other types of dementia, affective disorders and schizophrenia); 2) current or long-term alcohol or drug dependence; 3) primary neurological illness, stroke, Parkinson's disease, seizure disorder, and multiple sclerosis; 4) current, clinically significant cardiovascular disease as suffering from coronary artery disease, arrhythmias or valvular disease. Persons with a systolic pressure > 160 millimeters of mercury (mm Hg) were excluded as well as patients with focal neurological signs 5) clinically significant hepatic,

renal, pulmonary, metabolic or endocrine disturbances and inflammation. The Westergren erythrocyte sedimentation rate was used as an established inflammation-sensitive biomarker with proven prognostic value (Erikssen et al., 2000). 6) Participants were excluded when they had neuroradiological features that are associated with vascular dementia: small-vessel disease with extensive leukoencephalopathy or lacunae, or multiple infarcts in large-vessel territories. However, in participants classified as healthy subjects we only had MR scans in less than 10% of the participants.

2.3. Diagnostic procedure

As part of the diagnostic process, healthy control subjects and patients with depression were assessed by the same diagnostic procedure. Psychiatrists clinically examined all subjects, performed a standardized neurological examination, reviewed medical records, and conferred with referring physicians for all patients. Assessment of somatic burden was based on the annual medical assessment performed by as part of each subject's routine care. The treating physician compiled a medical problem list on the basis of the patient's history, physical examination, and available laboratory data.

All subjects underwent a neuropsychological assessment. All subjects (>60 years) answered the 30-item Geriatric Depression Scale (GDS) (Yesavage et al., 1983) and were tested for the Minimal Mental State Examination (MMSE) (Folstein et al., 1975). They were tested on verbal memory, learning, free recall and recognition subtests of the CERAD battery (Rosen et al., 1984), figural memory (free recall, CERAD), object naming (Boston Naming Test—short version, CERAD), categorical verbal fluency (animals/min, CERAD), planning (CLOX Test part 1; (Sunderland et al., 1989)), constructive abilities (copy geometrical shapes, CERAD; CLOX Test part 2). For healthy subjects, no subtest score was below one standard deviation. In general, only patients who fulfilled diagnostic criteria for MCI and AD were included. Probable AD was diagnosed according to NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) criteria (McKhann et al., 1984). MCI patients were diagnosed according to the clinical criteria of Petersen et al. (2001) including: subjective memory complaints over the last 6 month, and impaired memory function (verbal of figural) in neuropsychological assessment > 1.5 standard deviation (SD) related to age and education. Structural magnetic resonance imaging (MRI) was performed using a 1.5 T Siemens Symphony MRI scanner for all patients. In healthy subjects, neuroimaging was performed in less than 10%.

2.4. Collection of plasma

After a patient had been assigned to a group, 10 ml EDTA blood was collected and was processed within 24 h. The samples were centrifuged on a Biocoll (Biochrom) gradient (400 ×g, 30 min) and the upper plasma phase was immediately frozen at −80 °C. To test the stability half of the blood (of a few samples) was processed after 3 h and half was stored at room temperature for 24 h and then processed.

2.5. Searchlight multiplex array ELISA

The detection of 23 biomarkers was performed using the Thermo Scientific SearchLight Protein Array Technology (Aushon, THP Medical Products, Vienna) as described (Marksteiner et al., 2011). This method is a multiplexing sandwich-ELISA system based on chemiluminescent detection of analytes whose respective capture-antibodies are spotted in arrays within each well of a 96-well microplate. Briefly, diluted plasma (1:2 for NT-proBNP, KGF, PD-1, TRAIL, Thrombomodulin, TPO, TSP-1, PDGF-AB, TWEAK; 1:10 for BD-2, PDGF-AA, RAGE, TSP-2; 1:100 for SAA; 1:2000 for MPO, TMIP-1, TIMP-2, NGAL; 1:10,000 for alpha2-Macroglobulin, fibronectin; 1:100,000 for Apo-A1, ApoB-100, Fibrinogen) or calibrated protein standards were added to the wells of the plate (50 µl), incubated for 60 min at room on a shaker, then washed 3 times, and then the biotinylated antibodies (50 µl cocktail) were added that specifically bind to the captured proteins. After incubation for 30 min at room temperature on a shaker, the wells were again washed (3×) and then incubated with streptavidin-horseradish peroxidase conjugate (50 µl), again washed (3×) and the SuperSignal ELISA Femto Chemiluminescent Substrate (50 µl) was added. After incubation for 1–5 min the entire plate was imaged using a compatible CCD imaging system to capture the chemiluminescent signal from each spot within each well. The concentration of each analyte in the array was quantified by comparing the spot intensities for each unknown sample to the corresponding standard curves calculated from the standard sample results by the SearchLight Array Analyst Software. Integrated density values were proportional to the concentrations of bound proteins. Individual analytes were identified by the position of each specific capture antibody within the well. Standard curves, raw data and final pg/ml concentrations for each analyte and each sample were reviewed in the array software and exported to Microsoft Excel Software for further statistical analysis. The detection limits for each biomarker are given in Table 2. To reduce interassay variations, all runs of the same multiplex assay were performed on the same day. Each plate contained an own standard curve. The controls and patient group samples were randomly distributed across all plates.

2.6. Single ELISAs

In the cases where Searchlight Multiplex ELISAs were not available commercial single ELISAs were used as described by the manufacturer: tissue plasminogen activator (tPA) (kitPA91, Oxford Biom,ed Res, USA), plasminogen activator inhibitor-1 (PAI-1, American Diagnostica, Ref 822), for Low density lipoprotein MDA-oxLDL (Biomedica, BI-20022) and for antibodies against oxidized lipids (oLab, Omniagnostica, FT-OLAB). Briefly, for the tPA-1 Elisa, biotinylated PAI-1 working solution was added per well, incubated 30 min, washed, then 100 µl diluted (1:2) plasma samples were added to the well, incubated 30 min, washed, then the secondary antibody was added, incubated again 30 min, washed, then the substrate added, stopped and measured at 450 nm. Briefly, for the PAI-1 Elisa 20 µl plasma was added to 50 µl buffer in wells, incubated for 60 min, then 50 µl conjugate was added and again 60 min incubated, washed, the substrate added, incubated 12 min, stopped and measured at 490 nm. Briefly, for the MDA-oxLDL Elisa, 20 µl plasma was added to 100 µl assay buffer in wells, incubated 90 min, washed, the conjugate was added, incubated 90 min, washed, substrate was added, incubated for 30 min, stopped and measured at 450 nm. Briefly, for the oLab Elisa, 10 µl plasma was added to 210 µl buffer, incubated 2 h, washed,

then 100 µl conjugate was added, incubated 40 min, washed, the substrate added, incubated 5 min, stopped and measured at 450 nm. All assays were measured in a Zenyth 3100 ELISA reader.

2.7. Statistical analysis

One-way analysis of variance was used to compare the three diagnostic groups (healthy controls, MCI, AD) with respect to the plasma levels of vascular markers. Post-hoc pairwise comparisons were performed by means of Fisher's least-significant difference (LSD) method. No further adjustment for multiple testing was required in the case of three groups, as significance in the global F-test and also in the LSD testing ($p < 0.05$) was sufficient to keep the family-wise error rate at 0.05 (Meier, 2006; Shaffer, 1986). For the stability assay a paired Student *t*-test was used. Logistic regression was applied to calculate predicted probabilities of diagnostic group membership based on NT-proBNP plasma levels. These probabilities were entered into a receiver operating characteristic (ROC) analysis to obtain estimates of the sensitivity and specificity and of the area under the ROC curve. The stability of NT-proBNP plasma levels over time (3 h vs. 24 h.) was checked by means of a paired *t*-test and the intra-class correlation coefficient (ICC).

3. Results

3.1. Selection of patients

In the first study, 80 subjects were included with the following diagnosis: 23 healthy controls (age 71 ± 1.4 years), 24 MCI patients (age 74 ± 1.6 years) and 33 AD patients (age 81 ± 1.0 years; Table 1A). In the second part of this study, we analyzed a larger sample size including 110 participants (Table 1B). A one way ANOVA did not reveal a statistical difference between the "screening" and "NT-proBNP" groups with respect to all variables. In this set of experiment, we also included a group of young healthy subjects ($n = 15$, 30 ± 1 years) (Table 1B). The mean age of the MCI group was 75 ± 1.5 years with a mean MMSE score of 27 ± 0.3 (Table 1) and the AD subjects had an average age of 81 ± 0.9 years and were thus significantly older than control subjects (Table 1). The mean MMSE of AD patients was 20 ± 0.8 which was significantly different from control subjects (Table 1A). The GDS score was not different between controls, MCI and AD (Table 1).

3.1.1. Plasma levels of vascular markers, a multimarker approach—Out of 27 tested markers (Table 2), 8 were significantly enhanced in AD patients, compared to healthy controls: alpha2-macroglobulin (1.6×), ApoA1 (1.7×), NT-proBNP (1.9×), PAI (1.3×), RAGE (1.1×), SAA (6.2×), TIMP-1 (1.5×) and TSP-2 (1.5×). However, NT-proBNP (1.9×) was increased in MCI and AD. Whereas, no other markers were significantly altered in MCI patients compared to healthy control subjects, or to AD patients. The plasma levels of NT-proBNP were further determined in a larger sample size (Fig. 1B). Again, NT-proBNP levels were significantly enhanced in MCI ($p = 0.01$) and AD patients ($p = 0.0009$). In addition, NT-proBNP levels were measured in a younger group of healthy subjects (Table 1B). Healthy young and older subjects showed comparable NT-proBNP plasma levels (Fig. 1). In order to test the stability of plasma NT-proBNP blood was processed 3 h or 24 h after blood withdrawal in a sample of 6 subjects. No significant difference was observed between

NT-proBNP plasma levels at 3 h (1254 ± 287 pg/ml, $n = 6$) and at 24 h (1156 ± 328 pg/ml, $n = 6$). Moreover, the intra-class correlation (ICC) of 0.93 indicated a high level of stability (degree of freedom = 5; t value = 0.817; t -probability = 0.450; correlation = 0.934).

3.1.2. Plasma NT-proBNP levels and course of disease—In order to study the changes of plasma levels of NT-proBNP depending on course of disease, 22 patients were followed up and evaluated every year or every second year. When controls converted to MCI the plasma NT-proBNP levels increased approx. 800 pg/ml per year (Fig. 2). When MCI converted to AD the plasma NT-proBNP levels increased approx. 700 pg/ml per year. Plasma NT-proBNP were permanently increased in AD patients over years when compared to controls. Patients which were stable in AD had an increased plasma NT-proBNP level of approx. 900 pg/ml per year (Fig. 2). Interestingly, when patients were stable in MCI, the plasma levels decreased to about 500 pg/ml per year (Fig. 2).

3.1.3. ROC analysis—Results of the ROC analyses are shown in Table 3 and in Fig. 3. Discrimination between MCI patients and controls by NT-proBNP plasma levels yielded fairly high values for the area under the ROC curve (AUC of 0.777) and the sensitivity (79.2%), whereas the specificity was markedly lower (64%). Discrimination between AD patients and controls resulted in similar levels of sensitivity (81.8%) and specificity (64%), with AUC values being slightly lower (0.732). Similar findings were also obtained when the two patient groups (MCI and AD) were pooled and contrasted with the control group.

4. Discussion

In the present study, we measured 27 vascular-related proteins which may directly or indirectly contribute to the pathology found in AD and MCI patients. We showed that 8 proteins were significantly increased in AD patients when compared to healthy subjects. However, only NT-proBNP levels were increased in both MCI and AD patients compared to healthy subjects. Thus, NT-proBNP may be a sensitive marker for MCI and AD.

4.1. Vascular plasma marker affected in AD

Out these 27 markers, we found that 8 were affected in AD: alpha2-macroglobulin, apolipoprotein A1, NT-proBNP, PAI, RAGE, SAA, TIMP-1 and TSP-2. Plasma levels of 6 proteins (alpha2-macroglobulin, ApoA1, PAI, RAGE, TIMP-1 and TSP-2) were only moderately increased with a maximum increase of about 50%. SAA was markedly enhanced (6x), but the plasma levels of AD and MCI patients had a very high variance, resulting in a poor reliability. Thus, a high sensitivity of SAA levels to different conditions limits their use as vascular markers of AD. However, further studies may determine their within- and between-individual variance.

Several studies have been performed using microarrays for soluble vascular marker measurements. Some of the findings are in agreement with the present study, whereas some are in disagreement. Factors relating to assay performance can contribute to differences in observed values for vascular markers. Such a high variance and inconsistency is also seen between different laboratories. It has e.g. been shown that thrombomodulin is enhanced in AD patients (Borroni et al., 2002), while we did not see any effect. Similarly, Laske et al.

(2011) reported that out of 24 tested markers only 3 displayed significances in serum: cortisol, oLAB and ox-LDL. However, when we tested oLAB and ox-LDL we could not show any significant changes. This discrepancy may be the consequence of the fact that both markers were tested in plasma and not serum. Ghidoni et al. (2008) reported decreased plasma levels of soluble RAGE in MCI, while RAGE was slightly enhanced in our study.

Proteomics has been established as a potent tool to screen for many proteins. Ray et al. (2007) identified for the first time a panel of 18 signaling proteins leading to an accuracy of 90% in the diagnosis of AD, however, this finding could not be reproduced by others including us (Marksteiner et al., 2011). Three other recent papers used proteomics to identify novel biomarkers in blood of AD patients. Hu et al. (2012) analyzed 190 biomarkers in 600 patients and found four interesting candidates: ApoE, BNP, C-reactive protein and pancreatic polypeptide. As we used the erythrocyte sedimentation rate as a marker for inflammation instead of C-reactive protein, we do not know whether C-reactive protein is changed in our sample. Llano et al. (2012) measured 146 biomarkers in 527 patients and reported 5 analytes of interest: ApoA-II, ApoE, serum glutamic oxaloacetic transaminase, alpha1-microglobulin and BNP. And finally, Soares et al. (2012) investigated a panel of blood markers in about 500 patients and found eotaxin-3, pancreatic polypeptide, tenascin C and NT-proBNP to be enhanced and ApoE decreased.

4.2. Plasma NT-proBNP as a diagnostic marker

Natriuretic peptides (NP) are a family of peptides including atrial NP (ANP) and brain NP (BNP), as well as the larger precursor proteins midregional pro-ANP (MR-pro ANP) and N-terminal proBNP (NT-proBNP). It is well known that NPs show significant diagnostic properties in acute myocardial infarction (Venugopal, 2001). It is also well established that NT-proBNP is a biomarker for diagnosing and monitoring congestive heart failure (Bayes-Genis, 2006).

Recently it has been reported that ANP is enhanced in mild AD/MCI (Ewers et al., 2010). In parallel plasma MR-proANP is also enhanced in AD (Buerger et al., 2011). It seems to be evident from 2 independent groups that BNP is enhanced in AD (Hu et al., 2012; Llano et al., 2012). In addition, in another study BNP plasma levels are increased in subcortical vascular dementia but not in AD (Kondziella et al., 2009). NT-proBNP levels are about 10 times higher than BNP levels and these 2 markers significantly correlate with each other (Bionda et al., 2006). Plasma NT-proBNP levels are in the range of 1000–1700 pg/ml (Nishiyama et al., 2009), which is in line with our study. BNP levels are approx. 60 pg/ml (Kubo et al., 2012). Alterations of renal function have less impact on BNP than on NT-proBNP levels (Bionda et al., 2006). There is some recent evidence that aging, vascular dementia and AD correlate with enhanced NT-proBNP plasma levels (Nilsson et al., 2006; Soares et al., 2012). Our data are in full agreement and show for the first time that NT-proBNP serves as a stable and useful blood biomarker to diagnose AD and possibly MCI.

4.3. Criteria for a good biomarker and clinical implications

In the present study we found that participants with a diagnosis of MCI and AD had increased NT-proBNP levels compared to healthy subjects. More specifically we found that

increased levels of NT-proBNP levels were the only changes of vascular proteins found both in MCI and AD patients. We took particular care to exclude subjects with medical or neurological conditions sufficient to explain the cognitive deficits. Especially, patients with clinically significant cardiovascular events were excluded. Kidney function as well as blood pressure was similar in all groups. In a study that also excluded participants with a history of cardiovascular disease increased NT-proBNP levels were detected in community-dwelling older adults with poor cognitive function (Daniels et al., 2011). Further studies are needed to elucidate the path-ophysiological role of NT-proBNP in MCI and AD patients which may reflect a combination of cardiac, neurovascular and neurodegenerative etiologies.

The most established biomarker candidates for AD include brain imaging studies using magnetic resonance imaging or positron emission tomography and proteins in CSF. Out of 27 markers tested in our laboratory, NT-proBNP was some of the characteristics to become a useful future blood biomarker. The requirements for a good biomarker for diagnosing AD or neurodegenerative processes are well defined (Humpel, 2010; Sprott, 2010). In general, a biomarker should reflect some biological processes of physiological aging and of the AD pathology. It should also correlate to any therapeutic interventions. Any results obtained by a specific biomarker must be replicable in independent groups and it should be statistically significant. And finally, a biomarker assay must be non-invasive, easy, cheap and stable (Cedazo-Minguez and Winblad, 2009; Frey et al., 2005). Thus, for NT-proBNP the first analytic steps have been successful because our results are comparable to those obtained by different other groups.

4.4. Study limitations

A main limitation of the present study is the small size of the samples. This study should therefore be followed by future large-scale multi-center studies. These future studies should also include other types of dementia, especially vascular dementia. Although we have controlled for clinically relevant cardiovascular features, the selective increase in NT-proBNP could be caused by vascular pathology rather than specific AD features. A very recent study with larger samples has found that many factors significantly affected levels of NT-pro-BNP. Dadu et al. (2013) found that age, sex, silent brain infarcts (BMI) and hypertension influenced levels in serum. In our study, however, we did not find that sex had an influence on NT-pro-BNP levels. In addition, a recent report showed that one must consider renal function, gender and left ventricular ejection fraction (LVEF) when using NT-proBNP as a cardiac biomarker (Srisawasdi et al., 2010).

Another strong limitation is that the controls were not age-matched to the AD patients. The analysis of 23 controls who were on average 10 years younger than the AD patients, and had a lower frequency of smoking, make it possible that nonspecific plasma biomarker differences could have been found. Nevertheless, we did not find a significant difference between very young controls (30 years) and old controls (72 years) for NT-proBNP. A further study with age-matched groups is necessary to make sure that age is not a confounder responsible for the differences between control subjects and AD patients.

Our study has several other additional limitations. Several reasons may account for some of the divergent results obtained in various studies: (1) a difference can be observed between

plasma and serum levels. In fact the mean levels of a single individual biomarker may vary between serum, EDTA-plasma, citrate plasma and heparin plasma. (2) Different commercial ELISA systems may provide different concentrations of the biomarkers, depending on the sensitivity and specificity of the used antibodies. An antibody may detect full length forms or spliced forms or precursor forms. (3) Some biomarkers may show enhanced binding capacities to plasma/serum proteins, and only a pre-treatment may release the soluble forms. (4) The stability of the biomarkers may vary. In our study we did not test the stability of every single marker. (5) The processing of blood may be of importance. We collected plasma after centrifugation on a density Biocoll gradient (to collect also peripheral blood mononuclear cells for another study), while others often centrifuge blood directly or collect serum without centrifugation. (6) Freezing and thawing of plasma may influence the biochemical biomarker properties. (7) Blood cells are a source of storage compartment for several of the measured proteins and incomplete purification may affect the analysis. (8) The ethnicity of patients may have an effect on protein levels as well, although in the present study we used samples from the same ethnical group. (9) The comparability of our study is also limited as we could not determine the apolipoprotein E status for all participants. However, a further replicatory study should include the ApoE status as well as C-reactive protein. Thus, in summary, controlled standardized procedures to measure biomarkers in blood must be established to compare the data in different centers.

In conclusion, our data show that out of 27 vascular-related markers 8 are affected and that one (NT-proBNP) seems to be a useful and stable marker for diagnosing AD. NT-proBNP revealed to become a candidate of interest for diagnosis AD and MCI in plasma.

Acknowledgment

The study was supported by a Translational Project of the Austrian Science Funds (L429-B05). We thank Ursula Kirzenberger-Winkler for excellent technical help.

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Fig. 1.

Plasma NT-proBNP levels of young healthy controls (yCo), old controls (oCo), patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD); see Table 1.

Statistical analysis was performed by one way ANOVA with a subsequent Fisher PLSD posthoc test. Values in parenthesis give the number of analyzed subjects.

Fig. 2.

Yearly conversion rates for plasma NT-proBNP levels from controls to mild cognitive impairment (CO to MCI) and MCI to Alzheimer's disease (MCI to AD), as well as yearly changes in stable MCI (MCI-MCI) and stable AD (AD-AD). Values are given as mean $\pm$ SEM. The number in parenthesis gives the number of follow up patients. The groups were statistically compared against CO-MCI using one way ANOVA and a Fisher LSD posthoc test (* $p < 0.05$).

Fig. 3.
 (A) ROC curve based on NT-proBNP plasma levels for MCI patients vs. controls. (B) ROC curve based on NT-proBNP plasma levels for patients with Alzheimer dementia vs. controls.

Table 1

Demographic data of the patients.

A: All subjects included in screening					
	Controls	MCI	AD		
n	23	24	33		
male	13	9	12		
Age [years]	71 ± 1.4	74 ± 1.6 ns	81 ± 1.0***		
BMI	26.2 ± 6.3	25.7 ± 4.9 ns	25.5 ± 5.9 ns		
MMSE	29 ± 0.2	27 ± 0.3 ns	20 ± 0.8***		
GDS	7.2 ± 1.0	7.4 ± 1.0 ns	6.3 ± 0.9 ns		
Education [years]	10.7 ± 2.7	9.6 ± 2.2 ns	9.5 ± 2.9 ns		
Current smoking [%]	29	36	42		
Diabetes type II [%]	14	16	19		
Systolic RR [mm Hg]	129 ± 18.4	134 ± 17.1 ns	138 ± 14.5 ns		
Creatinine [mg/100 ml]	0.94 ± 0.13	0.97 ± 0.12 ns	1.01 ± 0.36 ns		
Urea [mg/100 ml]	36 ± 12.3	48 ± 16.2 ns	46 ± 14.2 ns		
B: Subjects included in focused NT-proBNP analysis					
	n	Male	Age	MMSE	GDS
Young controls	15	3	30 ± 1.4***	N.A.	N.A.
Old controls	40	21	72 ± 1.1	29 ± 0.7	6.5 ± 0.8
MCI	27	11	75 ± 1.5 ns	27 ± 0.3 ns	6.7 ± 1.0 ns
AD	43	15	81 ± 0.9***	20 ± 0.7***	6.8 ± 0.9 ns

The table gives the demographic data of patients used in the screening approach (A) and in the extended approach to measure NT-proBNP (B). n gives the number of patients; MCI, mild cognitive impairment, AD Alzheimer's disease; BMI, body mass index; MMSE, Mini-mental State Examination; GDS, Geriatric Depression Scale; N.A. not applicable. Values are given as mean ± SEM. Statistical analysis was performed by one way ANOVA with a subsequent Fisher PLSD posthoc test (ns not significant). A one way ANOVA did not reveal a statistical difference between the "screening" (Table A) and "NT-proBNP" (Table B) groups in respect to all variables.

***
p < 0.001.

Table 2

Plasma levels of vascular markers in controls and patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD).

	Control	MCI	AD	DL (pg/ml)
α2 Macroglobulin (mg/ml)	7.47 ± 0.81	9.17 ± 0.94	11.88 ± 1.24**	927
Apolipoprotein A1 (μg/ml)	103 ± 17	89 ± 9	176 ± 18***	98
Apolipoprotein B100 (μg/ml)	1420 ± 216	1068 ± 142	1252 ± 94	117
Beta-defensin-2 (pg/ml)	369 ± 70	483 ± 144	603 ± 113	2.7
Fibrinogen (mg/ml)	17.3 ± 1.4	15.9 ± 1.1	19.4 ± 0.7	98
Fibronectin (μg/ml)	82.2 ± 16.5	55.9 ± 6.8	86.1 ± 23.6	117
KGF (pg/ml)	1.69 ± 0.6	1.01 ± 0.1	1.03 ± 0.1	0.9
Myeloperoxidase (ng/ml)	13.5 ± 0.9	25.7 ± 8.6	16.1 ± 1.5	1.7
NGAL (ng/ml)	63.0 ± 5.3	66.0 ± 6.1	74.6 ± 6.1	0.4
NT-proBNP (pg/ml)	718 ± 172	1403 ± 207*	1406 ± 184**	1.3
oLab (U/ml)	2.58 ± 0.59	1.82 ± 0.28	2.10 ± 0.42	0.5 U/ml
oxLDL (μg/ml)	12.3 ± 1.4	11.1 ± 1.0	11.5 ± 1.6	310,000
PAI-1 (ng/ml)	74.8 ± 7.16	80.1 ± 8.1	98.8 ± 8.9*	3000
PD1 (pg/ml)	330 ± 158	172 ± 30	177 ± 45	9.7
PDGF-AA (pg/ml)	405 ± 100	411 ± 82	716 ± 140	0.9
PDGF-AB (ng/ml)	5.1 ± 3.0	4.1 ± 1.2	5.5 ± 1.6	12
RAGE (pg/ml)	743 ± 92	644 ± 85	827 ± 118*	1.4
Serum Amyloid A (μg/ml)	7.7 ± 1.7	42 ± 17	48 ± 16*	1953
Thrombomodulin (pg/ml)	2238 ± 154	2310 ± 289	2408 ± 257	70
TIMP-1 (ng/ml)	104 ± 7	131 ± 9	162 ± 23*	4.8
TIMP-2 (ng/ml)	142 ± 8	137 ± 10	149 ± 8	4.8
tPA (ng/ml)	4.16 ± 0.35	4.3 ± 0.7	4.48 ± 0.21	50
Thrombopoietin (pg/ml)	256 ± 43	250 ± 40	338 ± 46	5.8
TRAIL (pg/ml)	67.8 ± 5	66.4 ± 5	58.0 ± 4	1.5
Trombospondin-1 (ng/ml)	441 ± 28	463 ± 38	512 ± 30	293
Trombospondin-2 (ng/ml)	24 ± 1.7	25 ± 2	36 ± 4**	29
TWEAK (pg/ml)	487 ± 27	416 ± 43	484 ± 27	7.1

Plasma was isolated from patients (control, n = 23; MCI, n = 24; AD, n = 33; see Table 1) and analyzed as given in Methods. Values are expressed as mean ± SEM. Statistical analysis was performed by one way ANOVA with a subsequent Fisher PLSD posthoc test. The detection limit (DL) is given for each assay.

Abbreviations: KGF, Keratinocyte growth factor; NGAL, neutrophil gelatinase-associated lipocalin; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; oLab, Antibodies against oxidized lipids; oxLDL, Oxidized Low density lipoprotein; PAI, Plasminogen activator inhibitor; PD-1, Programmed cell death protein-1; PDGF, Platelet-derived growth factor; RAGE, receptor for advanced glycosylation end products; TIMP, Tissue Inhibitors of Metalloproteinases; tPA, Tissue plasminogen activator; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis.

* p < 0.05.

** p < 0.01.

*** p < 0.001.

Table 3

Results of logistic regression and ROC analyses based on NT-proBNP plasma levels.

Prediction	Logistic regression				ROC analysis ^b		
	OR ^a (95% CI)	χ^2	df	p-Value	AUC (95% CI)	Sensitivity	Specificity
MCI vs. control	2.36 (1.20–4.62)	6.26	1	.012	0.777 (0.648–0.905)	79.2%	64.0%
AD vs. control	2.35 (1.28–4.33)	7.61	1	.006	0.732 (0.603–0.862)	81.8%	64.0%
MCI/AD vs. control	2.40 (1.33–4.31)	8.52	1	.004	0.751 (0.638–0.863)	80.7%	64.0%

OR = odds ratio; CI = confidence interval; df = degrees of freedom; AUC = area under the ROC curve.

^aThe odds ratio was calculated per 1000 points (pg/ml) increase in NT-proBNP plasma level

^bThe cut-off value was chosen to maximize the sum of sensitivity and specificity.