Published in final edited form as:
J Neuroimmunol. 2009 November 30; 216(1-2): 92–97. doi:10.1016/j.jneuroim.2009.08.013.

TNF-α and antibodies to periodontal bacteria discriminate between Alzheimer's disease patients and normal subjects

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Abstract

The associations of inflammation/immune responses with clinical presentations of Alzheimer's disease (AD) remain unclear. We hypothesized that TNF-α and elevated antibodies to periodontal bacteria would be greater in AD compared to normal controls (NL) and their combination would aid clinical diagnosis of AD. Plasma TNF-α and antibodies against periodontal bacteria were elevated in AD patients compared with NL and independently associated with AD. The number of positive IgG to periodontal bacteria incremented the TNF-α classification of clinical AD and NL. This study shows that TNF-α and elevated numbers of antibodies against periodontal bacteria associate with AD and contribute to the AD diagnosis.

Keywords

periodontal antibodies; Alzheimer's disease; TNF-α; inflammation; diagnosis; biomarkers

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease afflicting the elderly. In the United States, 4.5 million people have been diagnosed with AD and this number will undoubtedly increase as the population ages and the life-span increases. The specific factors involved in the etiology and pathogenesis of AD have not been completely characterized although

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inflammation is thought to play a significant role. Increased brain inflammatory molecules such as interleukin-1β (Il-1β), interleukin-6 (Il-6) and tumor necrosis factor-α (TNF-α) participate in activating and perpetuating molecular pathways that may contribute to neurodegeneration (Akiyama et al., 2000; McGeer et al., 2006). The significance of this "inflammatory hypothesis" is that factors capable of altering the levels of inflammatory molecules may alter the expression and progression of AD (Kamer et al., 2008b). In addition, inflammatory molecules involved in the pathogenesis of AD may constitute biological predictors of the disease.

Periodontitis (PD) is a peripheral, chronic, infectious disease. Expression of PD is a function of specific periodontal bacteria and the host immune response. The interaction between periodontopathic bacteria and the host response may result in significant systemic inflammation characterized by production of inflammatory molecules including IL-1β, IL-6, and TNF-α (D'Aiuto et al., 2004). Therefore, periodontal bacteria and systemic inflammatory molecules may contribute to brain inflammation that characterizes AD and affects its expression (Kamer et al., 2008a). Support for this hypothesis comes from earlier studies that used dark field microscopy, molecular and immunological techniques to identify spirochetes including *Treponema* species (periodontal pathogens) with higher frequency in the brains of AD patients compared to non-AD subjects (Miklossy, 1993; Riviere et al., 2002). Clinical studies have reported tooth loss to be a significant risk factor for AD and/or dementia with odds ratios of 5.5 and 6.4, respectively (Gatz M, 2006; Stein et al., 2007). Although tooth loss may have multiple causes ((Al-Shammari et al., 2005; Jovino-Silveira et al., 2005), in adults one of its major causes is PD. Further support also comes from the Third National Health and Nutrition Examination Survey (NHANES III) showing that gingival bleeding, loss of periodontal attachment (indexes of periodontal disease) and serum *P. gingivalis* IgG were significantly associated with lower cognitive function even after extensive adjustments for confounders (Noble et al., 2009; Stewart et al., 2008).

Clinical PD has been linked to other systemic inflammatory conditions such as cardiovascular disease. Moreover, several studies (Beck et al., 2005; Beck and Offenbacher, 2005; Pussinen et al., 2003; Pussinen et al., 2007b) and one meta-analysis (Mustapha et al., 2007) showed that measures of periodontal infection such as bacterial burden and systemic antibody levels against periodontal bacteria associated stronger with cardiovascular disease than the clinical PD suggesting that the host response to periodontal bacteria (antibodies) is a relevant exposure to systemic diseases.

The consensus of the 1996 World Workshop in Periodontics identified 3 bacterial species causally related to PD status and progression; *Porphyromonas gingivalis* (*P. gingivalis)*, *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*; formerly known as *Actinobacillus actinomycetemcomitans*), and *Tannerella forsythia* (*T. forsythia*; formerly known as *Bacteroides forsythus*) (1996) and these species are capable of inducing a specific humoral immune response in the host (Ebersole et al., 1985). Since antibody response represents markers of infection with periodontopathic bacteria, we hypothesize that the presence of antibodies to periodontal bacteria is associated with AD.

The definition of biological markers based on the pathogenesis of AD has been the focus of many investigations. A landmark study (Ray et al., 2007) reported a molecular signature of 18 plasma proteins that could predict the development of AD and classified AD subjects with high sensitivity and specificity. Among the proteins that comprise the diagnostic signature for AD, TNF-α and several immune response proteins were significantly represented. Since TNF-α constitutes a central part of the AD molecular signature, and antibody response reflects host immune function, the immune markers of periodontal infection may improve the TNF-α discrimination of clinical AD. We hypothesized that AD patients would have both an elevated

cytokine expression and positive antibodies to *P. gingivalis, T. forsythia and A. actinomycetemcomitans,* and that these measures would contribute to clinical separation of AD from cognitively normal subjects. Our results support these hypotheses.

2. Methods

2.1 Study population

The study subjects, 18 with AD and 16 cognitively normal (NL), were selected from the New York University (NYU) Alzheimer's Disease Research Center (ADCC) and the affiliated Center for Brain Health (CBH) based on the following criteria: the diagnosis of cognitively normal (NL) or AD based on standardized criteria, the availability of frozen plasma, and availability of APOE ε4 carrier status. These patients participated in previous longitudinal and cross-sectional studies (de Leon et al., 2004; de Leon et al., 2006). NL and AD subjects were matched based on APOE ε4 carrier status. The diagnosis of NL and AD subjects was done by the Clinical Core of the NYU-ADCC or the CBH. Diagnostic classification was a multi-step procedure encompassing medical/neurological evaluations, psychiatric behavioral assessments and neuropsychological evaluation. All participants received extensive diagnostic screening to rule out confounding medical, neurological, and psychiatric conditions. A knowledgeable informant was interviewed for all participants suspected of impairment. The AD and NL were "pure" uncomplicated by other diseases that are known to affect cognition. Individuals with psychiatric or behavioral conditions (e.g. depression or substance abuse), or taking medications that affect cognition (e.g., benzodiazepines) were excluded. NL subjects did not demonstrate evidence of functional impairment. The determination was based on interviews with the subject and an informant and a structured clinical interview. The definition of AD included demonstration on structured clinical interviews of progressive impairments in multiple areas of cognition and difficulties with activities of daily living and the diagnosis of AD consistent with the NINCDS-ADRDA workgroup recommendations and DSM IV criteria (APA, 1994). The degree of cognitive impairment was evaluated using Mini-Mental State Exam (MMSE). MMSE is a 30-point scale for assessing attention, orientation, registration, and calculation.

2.2. ApoE Genotyping

APOE genotyping was conducted using frozen whole blood according to methods published (Main et al., 1991). Individuals with one or two ε 4 alleles were considered APOE ε4 positive carriers. 4 different genotypes were present in our sample: 4/4=2 ε4 alleles; 4/3 =one ε4 and one ε3 allele; 4/2= one ε4 and one ε2 allele and 3/3= 2 ε3 alleles.

2.3. Plasma IgG antibodies

Uniformly collect fasting plasma preserved in EDTA was obtained from NL and AD subjects and used to assess the IgG antibody and cytokine levels. Blood was collected into 10 mL EDTA polypropylene tubes, mixed thoroughly, centrifuged for 15 min. at ~3000 rpm, and aliquoted into 0.5mL labeled vials and frozen at −80°C. 100μL of plasma obtained from NL and AD subjects was assessed for the presence of antibodies against *A. actinomycetemcomitans* serotype b (ATCC 43718), *T. forsythia* (ATCC 43037), and *P. gingivalis* (ATCC 33277) by enzyme-linked immunosorbent assay (ELISA) as previously described (Craig et al., 2003). The bacterial species grown under anaerobic conditions [(at 37°C in broth base (BBL, Becton and Dickenson, Cockeysville, MD) supplemented with 5 μg/ml hemin and 0.3 μg/ml menadione)] were collected, washed in PBS pH 7.2 containing 1 mM ethylene diamine tetraacetic acid (EDTA), and then resuspended in PBS and 0.5% formalin overnight. Then, bacteria were stored in 0.02% formalin at 4°C until assay. The concentration of formalinized bacteria was adjusted to $OD_{580\ nm}$=0.3 and 200 μl of the cell suspension was added to each well of a 96 well polystyrene microtiter plate (Linbro, ICN Biomedical Inc. Aurora, OH)

excluding the peripheral wells. A series of three dilutions (1:100, 1:300 and 1:600) in duplicate of subject plasma and five dilutions in duplicate of standard sera were prepared in Tween/PBS and added to appropriate wells. The plate was incubated 2 h at room temperature and then washed three times for 5 min with Tween/saline. A 1:5000 dilution of affinity purified rabbit anti-human IgG (Calbiochem, La Jolla, CA) in Tween/PBS was added to each well and incubated at room temperature for 2 h. After washing, 100 μL of a 1:2500 dilution of affinity purified goat anti-rabbit IgG heavy and light chain specific alkaline phosphatase conjugate (Calbiochem) in Tween/PBS was added to each well. The plates were incubated for 2 h and washed three times for 5 min with Tween/saline. 200 μL of 1 mg/ml para nitrophenyl phosphate (Sigma 104 alkaline phosphatase substrate, Sigma Chemical Co., St. Louis, MO), in carbonate/magnesium buffer was added to each well. End point conversion of the enzyme substrate was measured by optical absorbance at 405 nm using a microtiter plate reader (Dynatech MRX, Dynatech, Chantilly, VA). Sample antibody reactivity was related to a standard reference serum curve for each organism. The highest standard concentration was assigned a value of 100 ELISA units (EU) and a reference curve relating to the $\log_{10}$ EU constructed for each plate using a curve-fitting software package (BioLinx, Dynatech). The results were expressed as the average EU value for the 6 serum dilutions of each sample. Plasma positively for a specific IgG antibody was defined as the value in the upper quartile as previously described in other studies on associations between PD and systemic diseases (Beck, 2005).

2.4. Cytokine assessment

Plasma TNF-α, IL-1β and IL-6 levels were assessed by Multiplex (performed by the General Clinical Research Center (GCRC) of NYU School of Medicine) using a Lincoplex kit from Millipore (Cat. #HCYTO-60K). Multiplex analysis combines the immunoassay principle with the fluorescent-bead-based technique for the simultaneous detection/measurement of multiple cytokines in a single well (25μL plasma). This method uses latex microbeads with unique fluorescent properties when excited by a laser beam. Each specific bead is coated with a specific antibody to the cytokine of interest and the coated beads are provided by the Lincoplex kits. Then, antibody-coupled beads are incubated with the plasma sample (antigen) after which they are incubated with biotinylated detection antibodies before finally being incubated with streptavidin–phycoerythrin for detection. Quantification is done using a standard curve of 5-fold dilutions of appropriate standards (provided by the kit). Cytokines are expressed in pg/ml. Each sample was assayed in duplicate and the multiplex analysis was performed on two separate occasions. The average was used for analysis.

2.5. Statistical methods

Statistical analyses were performed with SPSS version 12, Chicago Il. Between group differences for continuous variables (TNF-α, IL-1β and IL-6, age, MMSE score) were assessed using Student T-test and Mann-Whitney U test. The plasma samples for a specific IgG antibody were divided into highest and lowest quartiles. Positivity for an IgG antibody was defined when the levels of antibodies were in the upper quartile. Age was classified into three categories: ≤65, 66–79 and ≥80 (Hebert et al., 2003). For categorical variables, Chi-Square or Fischer's Exact Tests were used. Univariate and multivariate binary logistic regression analysis were used to model the classification of AD. A value of $p \leq 0.05$ was considered significant. ROC analysis was used to optimize the model for subjects' classification.

3. Results

Table 1 shows the characteristics of the study population. There were no differences found between AD and NL subjects with respect to gender, race, education, and carrier status. More subjects with AD were >80 years old (p=0.04). As expected, significant differences were found

in MMSE scores between NL and AD subjects. 17 AD subjects showed mild-moderate cognitive impairment. One subject had severe impairment.

Subjects with AD had higher number of positive IgG antibody tests (1.11±0.9) against periodontal bacteria (*A. actinomycetemcomitans, P. gingivalis* or/and *T. forsythia*) compared to NL (0.5±0.73) (Mann-Whitney U, p=0.04). Our results also showed that plasma samples from 72% of AD subjects were positive for at least one antibody to the periodontopathic bacteria tested (*A. actinomycetemcomitans, P. gingivalis* and *T. forsythia)* compared to only 38% of NL subjects (2=4.1, df=1, p=0.04) (Table 2).

Table 3 shows that the levels of TNF-α were significantly increased in AD subjects compared to NL (Mann-Whitney U test, p=0.007). IL-1β and IL-6 levels did not differ in AD subjects compared to NL. No statistical significant difference was found in cytokine levels between carriers of APOE 4ε and non-carriers.

To test the hypothesis that the markers of periodontal infection and systemic inflammation (TNF-α) contribute to the diagnosis of AD, we constructed binary logistic models. The first set of models tested the univariate associations of the number of positive IgG tests and TNF-α with AD. The results showed that the number of positive IgG tests and the level of TNF-α associated with AD even after adjusting for age (OR=1.3; 95% CI: 1.05–1.5 and OR=3.7; 95% CI: 1.1–12.9). When the number of positive IgG tests and TNF-α were entered simultaneously into the final model, both TNF-α (OR=1.4, 95% CI: 1.08–1.9) and the number of positive IgG antibodies to periodontal bacteria (OR=6.1, 95% CI: 1.16–33.1) independently associated with AD (Table 4). Age was not significant and therefore was not included into the final model. However, even if included, the statistical significance was maintained. These results suggest that AD subjects are more likely to have increased number of positive tests for IgG antibodies against periodontal pathogens (*A. actinomycetemcomitans, P. gingivalis* and *T. forsythia*) and high TNF-α levels.

Binary logistic models were also used to determine the contributions of specific inflammatory and immune markers to the clinical diagnosis of AD. The univariate analysis showed that TNF-α, the number of positive IgG tests and age were statistically significant (Table 5-Univariate). The second model including both TNF-α and the number of positive IgG antibody tests to periodontal bacteria showed a better fit (based on the change in the −2log likelihood ratio (LR=9, df=1, p=0.003) (Table 5-Multivariate). Since age was not statistically significant in the second model it was removed from the analysis. Table 5 also shows the contribution that the number of positive IgG antibody tests to periodontal bacteria and the levels of TNF-α add to the clinical diagnosis of AD. Including these 2 variables into the model classified clinical AD and cognitively normal subjects with an accuracy of 82%. These results suggest that biomarkers of periodontal infection and systemic inflammation may help improve the clinical diagnosis of AD.

4. Discussion

The results of our study showed that both the plasma TNF-α level and the number of positive tests for antibodies against periodontal bacteria were elevated in AD and independently associated with AD. The finding of increased plasma levels of TNF-α in the AD compared to NL subjects, concur with some previous studies (Alvarez et al., 2007; Bonotis et al., 2008; Zuliani et al., 2007). However, the findings of elevated number of positive tests for antibodies against periodontal pathogens in AD subjects are novel. These results are supported by a recently published study that showed that subjects with high levels of antibodies against *P. gingivalis* were more likely to have poor delayed verbal recall and impaired subtraction than

those with low levels even after adjusting for age, socioeconomic and vascular variables (Noble et al., 2009).

Inflammation is one of the key features of AD and provides the foundation for the hypothesis that inflammatory and infectious conditions may pose a risk factor for AD. PD, a chronic and prevalent infection could contribute to the expression of AD through mechanisms that have been described in detail previously (Kamer et al., 2008b). The role of peripheral inflammation and infections in the pathogenesis of AD has been investigated before (Holmes et al., 2003) (Lerner et al., 1997), (Schmidt et al., 2002) and several agents including oral bacteria have been implicated in AD (Balin et al., 2008; Itzhaki and Wozniak, 2008; Miklossy, 2008; Urosevic and Martins, 2008). One of the mechanisms by which bacterial species may affect AD is by systemic dissemination from local infections (Miklossy, 2008). For example, oral bacteria including periodontal species gain access to the systemic circulation during surgical, nonsurgical procedures and normal functioning and colonize at distant anatomical sites inducing significant pathology. Endocarditis, brain and lung abscesses are just a few examples (Zijlstra et al., 1992). Similar mechanisms may be implicated in AD pathogenesis. Supportive of this mechanism are the findings that spirochetes were reported in blood, CSF and brain samples from 14 AD cases but were absent in 13 controls lacking Alzheimer's symptoms (Miklossy, 1993; Miklossy et al., 1994); *Treponema* species including *Treponema denticola (T. denticola)*, a pathogenic periodontal bacterium were detected in 14/16 AD and 4/18 non-AD brains by molecular techniques (Riviere et al., 2002). *T. denticola* has been found to have high disseminating capacity as in a model of endodontic infection *T. denticola* DNA was detected at several distant sites including brain (Foschi et al., 2006). This finding is not surprising since it belongs to the same class as *Treponema pallidum* which is known to invade the brain and induce a pathology comparable with the pathology found in AD (Miklossy, 2008). Our results of significant higher number of positive IgG antibody tests against periodontal pathogens are interesting. *T. denticola*, colonize the subgingival biofilm with *P. gingivalis* and *T. forsythia* as part of the highly pathogenic red-complex (Socransky et al., 1998) It is possible that AD subjects may have also high IgG antibodies to *T. denticola.* To date there are no clinical studies to directly link PD and AD. However, several studies with different designs and populations reported that tooth loss was associated with cognitive impairment, dementia and AD (Stein et al., 2007). For example, one study reported in monozygotic twins a strong association between tooth loss and the presence of AD with an OR=5.5 (Gatz M, 2006). Another study, examining oral health in a longitudinal study of aging in Catholic Nuns, reported that a lower number of teeth increased the risk of dementia with an OR=6.4 (Stein et al., 2007). Tooth loss may occur due to multiple factors including PD (Al-Shammari et al., 2005; Desvarieux et al., 2003; Jovino-Silveira et al., 2005). Other causes may include carious lesions, trauma, and extractions due to prosthetic/orthodontic indications or impacted teeth. Severe nutritional deficiencies such as those seen in kwashiorkor may also result in tooth loss but they are very seldom occurrences (Enwonwu, 2006). In a large population survey that included over 6,000 subjects (NHANES III) with a broad age range (20–59 and ≥70), associations were found between measures of periodontal disease (extent of bleeding on probing and extent of attachment loss ≥3mm) and cognitive function. In another study analyzing subjects from NHANES III, an association was found between high levels of antibodies against *P. gingivalis* and lower cognitive functions in subjects >60 year old (Noble et al., 2009). These associations remained significantly even after adjustments for socioeconomic and cardiovascular risk factors (Stewart et al., 2008). Consistent with these findings, our study showed that AD subjects are more likely to have infections with periodontal bacteria than NL controls suggesting that perhaps periodontal bacterial infection may be linked to the pathogenesis of AD (Kamer et al., 2008b).

The biological reason for an association between antibody levels to periodontal bacteria and AD is not known. AD subjects have been shown to have poor oral hygiene (Ship, 1992) and

therefore AD subjects may have higher antibody levels representing increased bacterial colonization. Over 500 bacterial species can colonize the supragingival and subgingival (above and below the gingival margin) environment around teeth (Haffajee and Socransky, 2006). In addition, even pathogenic species may be found in healthy subjects. For example, A. *actinomycetemcomitans* may occur in approximately 20% of healthy subjects compared to 83% of periodontitis subjects (Slots, 1999), while *P. gingivalis* may occur in up to 80% of periodontally healthy subjects compared to up to 100% of periodontitis subjects (Papapanou et al., 2000; Slots, 1999). Antibodies to periodontal pathogens represent bacterial-host immune responses and bacterial colonization is only one component of this interaction. Recent studies suggest that host inflammatory response may be more important in bacterial biofilm formation then originally thought (Van Dyke and Kornman, 2008). And finally, the recent study of association between measures of periodontal infections and cognitive functions in normal population suggests that this association may occur before the AD onset (Noble et al., 2009; Stewart et al., 2008).

Studies reported that elevated antibody levels to periodontopathic species were associated with periodontitis (Craig et al., 2002; Ebersole et al., 1985; Pussinen et al., 2002). Although, a composite index of *A. actinomycetemcomitans* and *P. gingivalis* antibodies resulted in a sensitivity of 71% and 90% specificity for the diagnosis of periodontitis (Pussinen et al., 2002), antibodies against periodontal bacteria cannot diagnose periodontal disease.

Studies of association between periodontal disease and other systemic diseases such as cardiovascular diseases have also used antibodies to periodontal bacteria as an exposure index (Beck and Offenbacher, 2005; Kshirsagar et al., 2007; Pussinen et al., 2007a). A recent meta-analysis showed that elevated antibodies *A. actinomycetemcomitans* and *P. gingivalis* better associated with cardiovascular disease than clinical periodontal disease measures (Mustapha et al., 2007) suggesting that antibodies to periodontopathic species may be good measures of exposure when investigating relationships between periodontal disease and systemic diseases. Studies also showed that subjects with simultaneous expression of high serum antibody levels to periodontal bacteria (*A. actinomycetemcomitans* and *P. gingivalis*) and cytokine levels (TNF-α) significantly increased the risk of cardiovascular disease compared to subjects with low antibody and cytokine levels (Pussinen et al., 2007b). This suggests that periodontal infection may render increased risk to systemic diseases when there is also systemic inflammation. Our study is in line with these findings since both TNF-α and IgG antibodies against periodontal pathogens associated independently with AD suggesting that TNF-α may not be related to the number of positive antibody response. Age and the presence of teeth may influence the antibody levels (Papapanou et al., 2004). Age may decrease the antibody response (McArthur, 1998; Papapanou et al., 2000). Therefore, since AD subjects are older than NL controls the level of antibody response in AD may actually be underestimated. Since there is no data on bacterial colonization or periodontal disease status, we can only interpret antibody levels to be a measure of host systemic exposure and immune response to periodontal bacterial infection.

The characterization of biological markers as predictors of AD has been the focus of many investigations. Ray and colleagues (Ray et al., 2007) developed a molecular signature composed of 18 plasma proteins that predicted AD development and classified AD subjects with 95-90% sensitivity and 88-83% specificity. This study reported that the diagnostic signature was composed of TNF-α occupying a central role and several immune associated plasma proteins having significant roles. Since antibody response reflects host immune function, we hypothesized that antibody titers to periodontal bacteria may compliment plasma TNF-α levels in helping to improve the clinical diagnosis of AD patients and differentiate them from cognitively normal subjects. Our current study investigated the plasma reactivity for antibodies against periodontopathic bacteria and TNF-α, IL-1β and IL-6 levels in AD and NL

subjects. We examined their role as classifiers of clinical AD vs normal. We found that the number of positive IgG antibodies against periodontal bacteria significantly added to TNF-α values in discriminating between clinical AD and NL subjects. However, if antibodies against periodontal bacteria are to be part of the AD conversion signature, further longitudinal studies would need to be performed.

Caution needs to be applied when interpreting the results of this study due to the limitations of the study design. The modest sample size and case-control design are major limitations. The case-control design of our study does not enable us to infer whether the antibodies to periodontal bacteria occurred prior to the onset of AD or occurred following it. Both possibilities are feasible since antibodies to periodontal bacteria are quite stable over time (Papapanou et al., 2004), and resolution will await the results of future investigations. Although, data on the subjects' oral health and periodontal bacterial colonization measures would have been interesting to have their presence is not essential. Initial studies investigating the associations between PD and other systemic diseases such as cardiovascular disease (CVD) reported associations between clinical periodontal disease and CVD (Janket et al., 2003). These studies used only clinical periodontal disease parameters and they were criticized for the lack of direct measures of periodontal infection such as bacterial burden or systemic antibody levels to periodontal bacteria (Danesh, 1999). A cross sectional data of more than 6000 subjects showed that severe periodontal disease associated with carotid artery intima-media wall thickness (IMT) ≥ 1mm with OR of 1.3. However, when the measures of exposure were antibodies to periodontal bacteria the association was stronger suggesting that the host response to periodontal bacteria is relevant to systemic diseases (Beck et al., 2005; Beck and Offenbacher, 2005). Several other investigations had similar findings (Pussinen et al., 2003; Pussinen et al., 2007b). These studies suggest that antibodies against periodontal bacteria constitute adequate measures of exposure for systemic diseases (Mustapha et al., 2007).

In conclusion, these results suggest that antibody levels to periodontal bacteria associate with AD and may help improve the clinical diagnosis of AD. These preliminary observations warrant prospective longitudinal studies that in addition to antibodies to periodontal bacteria they will assess dental plaque, bacterial colonization, oral and periodontal disease status.

Acknowledgments

This project was supported by NIH grants This study was supported by the following grants: NIH-NIA AG12101, AG08051, AG03051 and CRR MO1RR0096, and NYUCD Fund R7173.

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Table 1

Characteristics of AD and NL subjects. Among the 18 AD and 16 NL subjects participating in our study, there were no differences with respect to gender, race, education, and carrier status. Significant differences were found in age and MMSE scores between NL and AD subjects.

		NL (n=16)	AD (n=18)	p value
Age group	40–65	7 (21%)	2 (6%)	
	66–79	6 (18%)	6 (18%)	
	>80	3 (9%)	10 (53%)	0.04
Gender	Female	94%	78%	NS
Race	White	81%	83%	NS
Education (mean±sd)		15.6±3	14.4±4	NS
MMSE score		29.3(0.77)	19.5(5.2)	<0.000

NS=not significant

Table 2

Number (percentage) of subjects testing positive for IgG antibodies to the periodontal bacteria. Patients' plasma samples were assessed for the levels of IgG antibody against *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia.* IgG levels in the highest quartile were defined as "positive". Note: higher percentage of AD subjects tested positive for at least one IgG compared to NL controls.

	Controls (%)n=16	**AD (%)n=18**	**p value**
Positive for 1 IgG antibody	4 (25)	7 (39)	
Positive for 2 IgG antibodies	2 (12.5)	5 (28)	
Positive for 3 IgG antibodies	0 (0)	1 (6)	
Positive for ≥1 IgG antibody	6 (38)	13 (72)	0.042*

Table 3

Cytokine levels (pg/ml) in AD and NL subjects (means±SD). Cytokine levels in patients' plasma samples were assessed by Multiplex assay. Note: the TNF-α is significantly increased in AD compared to NL controls.

	Normal	AD
Interleukin-1β	11.6±15.9	6.9±3.5
Interleukin-6	30.2±55.0	8.1±11.4
Tuomor Necrotic Factor-α	8.2±4.7	13.0±4.3*

* $p<0.01$

Table 4

Multivariate binary logistic model of association between the number of IgG antibodies to periodontal bacteria and TNF-α and AD. Note: when TNF-α and IgG antibodies against *A. actinomycetemcomitans, P. gingivalis* and *T. forsythia* were simultaneously included into the model both variables associated with AD independently. IgG=number of positive IgG antibody tests in the highest quartile. Since age was not significant, it was not included into this model.

Predictor	Odds Ratio	95% Confidence Interval	p value
TNF-α	1.4	1.08–1.9	0.01
IgG	6.1	1.16–33	0.04

Table 5

Univariate and multivariate logistic regression analyses predicting clinical diagnosis of Alzheimer's disease. Binary logistic models showed that the inclusion of the TNF-α and the number of IgG antibodies against *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* classified clinical AD and cognitively normal subjects with an accuracy of 82%. IgG =number of positive IgG antibody tests against *A. actinomycetemcomitans*, *P. gingivali* and *T. forsythensis*. Positive test is defined as levels of IgG in the highest quartile. Age = as defined in the text. P value is for Omnibus tests of model coefficients (effect on the dependant variable–disease). Since age group was not significant into the multivariate model, in the final model, it was not included.

	−2 Log likelihood (χ^2)	Specificity (%)	Sensitivity (%)	Accuracy (%)	p
Univariate:					
TNFα	38.5 (8.5)	62.5	77.8	70.6	0.00
IgG	42.0 (4.6)	62.5	72.2	67.6	0.03
Age	40.2 (6.8)	43.8	88.9	67.6	0.01
Multivariate:					
TNFα +IgG	30.0 (16.5)	81.3	83.3	82	0.00

J Neuroimmunol. Author manuscript; available in PMC 2010 November 30.