Complement Biomarkers as predictors of disease progression in Alzheimer’s disease.

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Running title: Complement biomarkers in Alzheimer’s disease

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Abstract:

There is a critical unmet need for reliable markers of disease and disease course in mild cognitive impairment and early stage dementia. The growing appreciation of the importance of inflammation in the early stages of dementia has focussed attention on the utility of inflammatory markers in cerebrospinal fluid or plasma; however, non-specific inflammation markers have disappointed to date. A targeted approach, centred on inflammatory pathways already implicated in the disease, might prove more useful. Complement is a core system in innate immune defence and potent driver of inflammation; a confluence of genetic, histochemical and model data has implicated complement in pathogenesis of Alzheimer’s disease, encouraging a search for complement biomarkers. Numerous studies have suggested that measurement of individual complement proteins or activation products in cerebrospinal fluid or plasma is useful in diagnosis, prediction or stratification, but few have been replicated. Here we measured in a single multiplex assay a panel of five complement proteins and four activation products spanning key activation pathways in plasma from donors with cognitive impairment and dementia and compared with matched controls. Only one of these analytes, clusterin, differed significantly between controls and Alzheimer’s disease (controls, 295mg/l; AD, 388mg/l: p<10⁻⁵). A model combining clusterin with relevant co-variables was highly predictive of disease. Three analytes (clusterin, factor I, terminal complement complex) were significantly different between mild cognitively impaired individuals who had converted to dementia one year later compared to non-converters at one year; a model combining these three analytes with informative co-variables was highly predictive of conversion.

Key words: Alzheimer’s disease, Inflammation; Complement; Biomarker.
Introduction

The current lack of plasma biomarkers for diagnosis, stratification or prediction of outcome in AD is a major deficit that compromises early diagnosis and informed therapeutic choice [1-3]. In particular, biomarkers that aid early diagnosis and/or predict progression from MCI to AD are a critical need. Primarily of value in the near future to aid in the recruitment to secondary prevention trials, such markers predictive of progression in prodromal states might become of clinical value in future as disease modification therapies become available. A handful of plasma markers have been described but are untested in preclinical disease and likely unsuitable for early diagnosis [3]. The goal for current studies is to deliver a highly informative plasma biomarker or set of markers that enable early diagnosis and predict disease course [4, 5]. The recognition that inflammation is an important player in AD and likely an early event in disease pathogenesis brings to the fore the potential use of markers of inflammation [6]. Non-specific indicators of peripheral inflammation such as C-reactive protein and inflammatory cytokines have proved unreliable as markers of disease or disease progression [7, 8], suggesting that a more targeted approach, focussing on specific inflammatory pathways might be more rewarding.

Complement, a pillar of innate immunity and key player in driving inflammatory responses to injury and infection, is a prime target pathway, implicated through genetic, pathological and animal model evidence [9-11]. Several published studies have explored whether plasma levels of complement components, regulators or activation products are altered in AD or predict progression in the disease. In one of the first untargeted proteomics analyses of plasma in AD, complement factor H (FH) was found to be elevated in AD plasma compared to controls [1]; an intriguing observation given the genetic association between FH and age related macular degeneration (AMD), another amyloid related condition [12, 13]. However, one other, targeted study using a different methodology did not find association of FH with AD [14], and the common polymorphism in FH (Y402H) that is strong risk for AMD does not significantly impact AD risk when assessed at the genetic level [15,16]. Levels of plasma clusterin, a modulator of the terminal complement pathway, have been associated with disease, disease subtype and rate of progression in several studies [17-20], although as with FH, negative findings have also been reported [21]. Plasma factor I (FI), measured semi-quantitatively, was highly predictive of brain atrophy in AD [22]. C1s and C9 have been implicated at the genetic level in pathway analysis studies [23].

We used these published findings, together with relevant genetic data, to identify a candidate complement biomarker set. Here we describe the use of a custom-made ten-analyte multiplex set on the MSD platform to measure selected candidate AD biomarker complement proteins and activation
products. The set comprised FH (measured as the individual Y402 and H402 alleles [22]), clusterin, FI, C1s, C9, C4d, Bb, iC3b, TCC. The four complement activation products selected for measurement included markers of classical (C4d, iC3b), alternative (Bb, iC3b) and terminal (terminal complement complex; TCC) pathway activation. The study comprised two arms, one in which AD samples were compared to matched controls, and the other in which enrolment samples from individuals with MCI who had subsequently converted to AD when re-assessed 12 months later (convertors) or who had remained stable over the period of assessment (non-convertors) were compared. Of the analytes measured, only clusterin differed significantly between matched controls and AD patients, while three analytes, clusterin, FI and TCC, differed significantly between MCI convertors and non-convertors. For each study arm, models were built comprising the analytes that differed significantly together with relevant co-variables (APOE status, age). Each of the models was highly predictive with overall predictive power (from area under the curve [AUC] in receiver-operating characteristic [ROC] analysis) of 0.78 for AD versus control and 0.85 for MCI convertor versus non-convertor.

Materials and methods

Samples

All samples were obtained from the previously reported AddNeuroMed and Dementia Case Register studies [24, 25]. For comparison of AD and control groups a total of 292 first visit samples (106 AD, 186 controls) were selected. The sample set was randomly divided into a training set comprising 206 samples (75 AD, 131 controls) to generate the model, and a testing set comprising 86 samples (31 AD, 55 controls) to assess the accuracy of the model. In a separate analysis, 189 samples obtained from patients diagnosed with MCI at the point of sampling were tested. Upon re-assessment 12 months later, 49 of these patients had converted to MCI while 140 had not converted. These were divided into a training set of 133 (98 not converted, 35 converted), and testing set of 56 (42 not converted, 14 converted) for analysis.

Assay development and multiplexing

Ten complement analytes were selected for this study, six components or regulators (C1s, C9, clusterin, FI, FH-Y402, FH-H402), and four activation products (iC3b, C4d, Bb and TCC). Analyte choice was informed by reference to previous studies of complement biomarkers in AD, and availability of reagents; the activation marker set was chosen to interrogate classical (C4d, iC3b), alternative (Bb, iC3b) and terminal (TCC) activation pathways. The FH-Y402 and FH-H402 allotypes were measured separately using highly specific monoclonal antibodies as described previously [26], and total FH concentration was obtained by summing the concentrations of the two...
allotypes. For each analyte, an antibody pair was selected from commercial or in-house sources (Table 1) and tested in ELISA for capacity to detect the analyte in plasma using purified proteins as standards. Selected antibody pairs were then tested in single-plex assays using high-bind plates from “ELISA Conversion Pack I” (MesoScale Discovery Platform [MSD], Rockville, Maryland, USA). Detection antibodies were conjugated to SULFO-TAG with ratio 1:12 according to the manufacturer’s instructions. Single-plex assays were validated for reproducibility (intra- and inter-assay Coefficient of Variation [CV] <10%), sensitivity and dynamic range. For each analyte the range of plasma dilutions that enabled accurate quantitation was assessed; the optimal plasma dilution for measurement of all analytes in the set was then selected. Ten-plex plates (all analytes measured in a single well) were then printed by MSD using the supplied capture antibodies, and re-validated for reproducibility, sensitivity and dynamic range and to confirm that all included analytes could be measured at a single plasma dilution. Ten-plex plates were also tested with mixtures of the analyte standards to ensure that there was no “cross-talk” between assays.

The assay protocol was as follows: Printed ten-plex plates were blocked with 150µl/well 3% BSA in PBS at 4°C overnight. Plasma samples were diluted 1:300 in assay buffer (PBS containing 1% BSA and 10 mM EDTA); 25µl aliquots were then added in duplicate to wells. To calibrate the assays, a standard plasma was generated comprising a mixture of normal plasma and complement-activated plasma in which levels of all analytes were pre-calibrated against pure proteins using the single-plex assays. A calibration curve comprising a series of 5-fold dilutions of the standard plasma (1:5 to 1:6250) was run in duplicate on each plate. Two additional dilutions of standard plasma (1:250, 1:2500) in duplicate were used as inter-plate controls. Plates were incubated while shaking at RT for 60 min. After washing in PBS containing 0.01% Tween20, a mixture of the relevant SULFO-TAG-labelled detection antibodies diluted in assay buffer (1:100) was added and incubated as before. After washing, 150µl of 2x reading buffer was added to each well and electrochemiluminescence (ECL) signal was immediately registered in a Sector S600 plate reader (MSD). ECL values in plasma samples were automatically converted to analyte concentration by reference to the calibration curve.

Statistical methods
All statistical analysis was conducted in R version 3.0.2. Correlation of individual analyte concentration with age at time of sampling was tested using Pearson correlation.

In both the AD : control comparison and the MCI convertor : non-convertor comparison, samples were split into training and testing sets as described above in order to reduce over-fitting of the model. Clustered mixed-effects linear modelling (using the lme4 and lmerTest R packages) was used to explore the associations between analyte concentration and disease status. Centre of
sampling was included as a random effects variable, and complement analyte, APOE-ε4 status (negative, heterozygous, homozygous), age at onset, and gender included as fixed effects variables. Variables which were found to be significant in the training set were retained in a refined model, which was tested for accuracy by applying to the test group. Area under the curve (AUC) was calculated, and receiver-operating characteristic (ROC) curves drawn to define the predictive power of the model.

**Results**

*Complement protein assays are sensitive and specific in multiplex formats*

Each of the complement analyte assays translated from ELISA, through single-plex to multiplex without loss of performance. All analytes were accurately measured at a plasma dilution of 1:300. There was no detectable inter-assay interference between the different analytes in the multiplex and intra- and inter-assay CVs were <10% for all analytes (data not shown).

*C9, FI and TCC levels correlate with age*

Correlation with donor age at sampling was tested for all complement analytes in the complete set of samples (Table 2). C9 levels showed a strong positive correlation with donor age at time of sampling. Levels of FI and TCC demonstrated weak but significant positive correlations with donor age at time of sampling. Other complement analytes did not significantly correlate with donor age.

*Clusterin is the sole plasma complement biomarker that distinguishes AD from control*

Of the nine complement analytes measured (FH variants combined to give total FH), only one, clusterin, was significantly different between AD and control populations (Table 3). The mean plasma clusterin concentration in controls was 295mg/l and in AD was 388mg/l, a highly significant difference (p= 2.32 x 10^{-6}). A model combining clusterin with co-variables associated with AD (APOE status and age) was highly predictive with an AUC of 0.66 for the test set and 0.78 for the entire sample set (Table 3; Figure 1A). At 70% sensitivity, the predicted specificity of the model was 75%.

*Three complement analytes differentiate MCI convertors from non-convertors*

From the analysis of MCI convertors versus non-convertors, three of the nine complement analytes were significantly different between the groups: clusterin, TCC and FI (Table 4; Figure 1B). Of these, clusterin was the most significant; the mean clusterin level in non-convertors was 309mg/l and in convertors was 418mg/l. TCC was significantly lower in MCI convertors compared to non-
convertors (0.7mg/l vs. 3.6mg/l), while FI was significantly reduced in MCI convertors compared to non-convertors (27.7mg/l vs. 50.7mg/l; the latter identical to healthy controls). From these data, a model was constructed combining clusterin, TCC and FI with the sole co-variable associated with MCI conversion (APOE status); the model was highly predictive of conversion with an AUC of 0.85 for the entire sample set (Table 4, Figure 1B). At 80% sensitivity, the predicted specificity of the model was 79%.

**Measurement of FH Y402H allotypic variants predicts progression in MCI**

Total FH concentration was reduced in MCI convertors compared to non-convertors (297.9mg/ml versus 351.4mg/ml; Table 4), although this failed to reach significance in the model. In FH-Y402H heterozygous samples, measurement of the Y402 and H402 variants of FH separately using variant-specific capture antibodies revealed that this difference was largely due to a decrease in MCI convertors of the concentration of the H402 variant; average expression from each Y allele was 164.4mg/l in convertors and 166.3mg/l in non-convertors, while expression from each H allele was 142.1mg/l in convertors and 172.5mg/l in non-convertors (Table 5; p=0.0056).

**Discussion**

A plasma marker or marker set that is indicative of pathology or predictive of conversion to AD in individuals with MCI, or disease course in patients with early AD is sorely needed to facilitate early diagnosis, inform selection of participants in future clinical trials and to guide choice of therapy. The abundant evidence implicating inflammation, and specifically complement, in pathogenesis led us to explore the complement system as a source of biomarkers. Guided by literature evidence and reagent availability, we selected ten complement analytes and designed a multiplex assay to measure all simultaneously. Our data demonstrate that clusterin alone among the analytes tested differentiated AD patients from matched controls, while clusterin, FI and TCC were all significantly different between MCI convertors and non-convertors at one year post-sampling. In our sample set, 26% of the MCI cases progressed to dementia at one year; this is markedly higher than published annual conversion rates, typically around 10%, although considerable variation between sample sets has been noted [27]. Several published studies have report elevated plasma levels of clusterin in AD compared to controls in diverse ethnic groups [17-20; 28-30]. Taken together with our findings, these data demonstrate that elevated plasma clusterin level is a robust marker for AD that is replicated across different assay platforms. In light of this, it is somewhat paradoxical that two disease-associated SNP in clusterin are reported to associate with decreased plasma levels [29,30]. Precisely how plasma clusterin levels impact disease risk remains uncertain. Clusterin is a multifunctional
molecule, an inhibitor of the complement terminal pathway but also a professional molecular chaperone involved in clearance of debris [31]. Amyloid plaques in AD are richly decorated with clusterin and a role in clearance of amyloid has been proposed [32,33]. Clusterin has also been shown to reduce Aβ42 toxicity in a rat model of AD [34]. Association of plasma clusterin levels with rate of cognitive decline has been reported both in MCI and AD [17, 20, 24]; in each of these studies, higher clusterin levels predicted more rapid decline. Our data demonstrating substantially higher plasma clusterin in MCI convertors compared to non-convertors robustly support these findings and show that elevated plasma clusterin is a powerful predictor of progression. The functional basis of this association is problematic; if clusterin is involved in reducing Aβ toxicity and accelerating amyloid clearance, then increased plasma levels might be expected to restrict the development of pathology. It is possible that increased clusterin production, reflected in increased plasma levels, represents a failed protective response to the disease process.

TCC, a marker of complement terminal pathway activation, is present on neurones, plaques and adjacent blood vessels in AD brain [32-35]; fluid-phase TCC (also termed sC5b-9) has been measured in AD CSF [36], but plasma levels of TCC have not previously been described in AD or MCI. Plasma TCC levels were not different between AD and controls but were significantly lower in MCI convertors compared to non-convertors; this finding is somewhat counterintuitive in that it implies lower levels of terminal pathway activation in the convertors despite clear evidence that the terminal pathway is abundantly activated in AD brain. We suggest that the demonstrated elevated levels of clusterin, an efficient inhibitor of the terminal pathway, in the convertor group might suppress terminal pathway activation and TCC generation in plasma. Notably, plasma levels of activation pathway products (iC3b, C4d, Bb) were not different between the groups, suggesting that any difference in central complement activation between MCI convertors and non-convertors was not reflected in the periphery.

FI is the enzyme responsible for regulating the activation pathway convertases; complement receptor 1, linked through GWAS studies to AD [9,37,38], is the major cell-associated cofactor for FI-mediated cleavage of C3b/C4b. Plasma FI has previously been reported as a biomarker of brain atrophy [22]. Here we show that plasma FI level was significantly reduced in MCI convertors compared to non-convertors (27.7mg/l vs. 50.7mg/l; the latter identical to healthy controls). Lower levels of FI will impact capacity to control complement activation once triggered and favour dysregulation [39].

FH was lower in MCI convertors compared to non-convertors (297.9mg/ml versus 351.4mg/ml), although this difference was not significant in the mixed effects linear model (Table 4). The common FH-Y402H polymorphism is a major risk factor for AMD [12, 13], but does not associate
with AD in multiple studies [14-16]. Here we separately measured plasma levels of the products of the two allotypic variants and showed that low levels of the FH-H402 allotype significantly differentiated MCI convertors from non-convertors. These data could be explained by decreased expression of the FH-H402 allele in the convertor group or, more likely, by increased consumption of the FH-H402 allotype protein in response to the disease in MCI convertors. Differential binding of the different FH-Y402H allotypes at sites of pathology has previously been described in the context of AMD [40], and our unpublished data suggest preferential binding of the FH-H402 allotype in AD brain.

From our data we generated two models. The first compared AD patients with controls and included clusterin with the co-variables age and APOE status; ROC curves constructed from this set gave an AUC of 0.78 for the entire set, considered “moderately predictive” [41]. The second model compared MCI convertors and non-convertors and included clusterin, TCC and FI with the sole co-variable associated with MCI conversion (APOE status); ROC curves gave an AUC of 0.85 for the entire sample set, considered “highly predictive”. Although levels of the FH-H402 allotype were significantly predictive of MCI conversion when measured in FH-Y402H heterozygotes, this variable was not included in the model because it applied only to a subset of the samples; nevertheless, the data demonstrate that measuring plasma allotype levels for common complement polymorphisms can help predict disease.

In summary, we show that combinations of complement biomarkers can aid diagnosis and prediction of outcome in MCI and AD. The results described were from an initial set of just ten complement analytes and from these only one was predictive for distinguishing AD from controls and three for predicting progression in MCI. Expanding the test set of complement biomarkers will add other predictive analytes that will strengthen the predictive power of the marker set. Adding in non-complement biomarkers will likely further strengthen and contribute to an optimum multiplex for diagnosis and prediction of outcome. The demonstration that complement activation occurs in MCI and predicts conversion strengthens the case for testing anti-complement therapies in this group.

Acknowledgments:

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References:


### TABLES:

<table>
<thead>
<tr>
<th>Analyte/assay</th>
<th>Capture antibody (source)</th>
<th>Detection antibody (source)</th>
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<tbody>
<tr>
<td>C1s</td>
<td>MM Anti-C1s (M81, Hycult)</td>
<td>MM Anti-C1s (F33, in house)</td>
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<td>C9</td>
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<td>MM Anti-C9 (6D4, in house)</td>
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<td>MM Anti FH-Y402 (MBI-6, in house)</td>
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<td>Bb</td>
<td>MM Anti-neo-Bb (A252, Quidel)</td>
<td>MM Anti-FB (JC1, in house)</td>
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Abbreviations: MM, mouse monoclonal antibody; RP, rabbit affinity purified polyclonal antibody.

Neo denotes neoepitope-specific antibody.

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<th>$P$</th>
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<td>C9</td>
<td>0.23</td>
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<td>C4d</td>
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**Table 2.** Correlation between complement analyte concentration and age at time of sample. C9, FI and TCC all showed a significant positive correlation with age in the populations sampled.
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<thead>
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<th>Initial Model</th>
<th>AD (mean ± SD; mg/l)</th>
<th>Controls (mean ± SD; mg/l)</th>
<th>β (95% CI)</th>
<th>P</th>
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<td>C4d</td>
<td>3.8±4.5</td>
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<td>0.014 (-0.017 - 0.05)</td>
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<td>C9</td>
<td>52.0±17.1</td>
<td>51.2±14.8</td>
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<td>Clusterin</td>
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<td>295.0±128.5</td>
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<td>FI</td>
<td>51.5±37.8</td>
<td>50.7±38.9</td>
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<td>TCC</td>
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<tr>
<td>iC3b</td>
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<td>1.6±1.1</td>
<td>0.003 (-0.062 - 0.068)</td>
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<td>Bb</td>
<td>21.2±9.3</td>
<td>18.7±8.8</td>
<td>0.003 (-0.005 - 0.010)</td>
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<td>Age at sample</td>
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<td>-0.021 (-0.03 - -0.01)</td>
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**Final model AD versus control**

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<thead>
<tr>
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<td>Clusterin</td>
<td>-0.001 (-0.002 - -0.0008)</td>
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<td>APOE4</td>
<td>-0.13 (-0.2378 - -0.02)</td>
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<td>Age at sample</td>
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**Table 3.** Mixed effects linear model for complement analyte difference between AD and controls. Clustered mixed-effects linear modelling (using the lme4 and lmerTest R packages) was used to explore the associations between each variable and disease status. The variables which were most strongly associated with diagnosis (based on p value) were then combined into one model (final model). Any variables which were not significant after inclusion in the model were discarded. Note that final model for AD versus controls comprises Clusterin, APOE4 and age.
### Table 4. Mixed effects linear model for complement analyte difference between MCI convertors and non-convertors. Clustered mixed-effects linear modelling (using the lme4 and lmerTest R packages) was used to explore the associations between each variable and disease status. The variables which were most strongly associated with diagnosis (based on p value) were then combined into one model (final model). Any variables which were not significant after inclusion in the model were discarded. Note that final model for conversion versus non-conversion comprises Clusterin, FI and TCC.
Table 5. FH allotypes in AD and MCI. In an initial analysis there was no association between FH-Y402H genotype or diagnosis and FH plasma levels, but plasma FH levels did predict whether patients convert from MCI to AD (by ANOVA, p=0.0033). Allele number-corrected allotype levels in FH-Y402H heterozygotes were then compared between AD and controls (top) and MCI non-convertors and convertors (bottom); FH-H402 levels were significantly lower in FH-Y402H heterozygous MCI patients who subsequently converted to AD than in those who did not convert.
Figures

Figure 1. ROC curves.

A

B

Figure Legend.

Figure 1. Receiver-operating characteristic (ROC) curves representing models which differentiate AD from controls (A) and MCI convertors from non-convertors (B). ROC curves were drawn for the final models distinguishing AD from controls (A; clusterin and APOE4) and MCI convertors from non-convertors (B; clusterin, Fi and TCC). The area under the curve (AUC) for the final model was calculated, and compared to that for APOE4 alone. AUC was used to define the predictive power of the analyte or analyte set that comprised the model; the predictive power of the model for distinguishing AD from controls was 0.78, and for predicting conversion was 0.85.