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# Complement Biomarkers as Predictors of Disease Progression 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 (MCI) and early Alzheimer's disease (AD). The growing appreciation of the importance of inflammation in early AD has focussed attention on inflammatory biomarkers in cerebrospinal fluid or plasma; however, non-specific inflammation markers have disappointed to date. We have adopted a targeted approach, centered on an inflammatory pathway already implicated in the disease. Complement, a core system in innate immune defense and potent driver of inflammation, has been implicated in pathogenesis of AD based on a confluence of genetic, histochemical, and model data. 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 apply a novel multiplex assay to measure five complement proteins and four activation products in plasma from donors with MCI, AD, and controls. Only one complement analyte, clusterin, differed significantly between control and AD plasma (controls, 295 mg/l; AD, 388 mg/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 MCI individuals who had converted to dementia one year later compared to non-converters; a model combining these three analytes with informative co-variables was highly predictive of conversion. The data confirm the relevance of complement biomarkers in MCI and AD and build the case for using multi-parameter models for disease prediction and stratification.

**Keywords:** Alzheimer's disease, biomarker, complement, inflammation

## 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 patient selection for trials of novel therapies

[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 few plasma markers have been described but are untested in preclinical disease and likely unsuitable for early diagnosis [3]. The goal for current studies is

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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, focusing 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 in the pathogenesis of AD 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 a 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, and 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.

The findings further implicate complement as a contributor to disease progression in AD and make the case for building multi-parameter models including informative complement biomarkers, non-complement biomarkers and other patient data that enable patient stratification and prediction of progression.

## MATERIALS AND METHODS

### *Samples*

All samples were first visit samples 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 mean age for the AD samples was 74.7 years and mean age of controls was 78.1 years. 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. Note that all measurements here were on this first sample when all were classified as MCI. Upon re-assessment 12 months later, 49 of these patients had converted to AD while 140 had not converted. The mean age at first presentation was 75.2 years for the convertors and 76.3 years for non-convertors. These 189 MCI samples were randomly 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, an essential quality control in multiplex assays, and CVs for each analyte re-tested.

The assay protocol was as follows: Printed ten-plex plates were blocked with 150  $\mu$ l/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); 25  $\mu$ l 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 room temperature 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, 150  $\mu$ l 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 modeling (using the lme4 and lmerTest R packages) was used to explore the associations between analyte concentration and disease status. Center of sampling was included as a random effects variable, and complement analyte, APOE- $\epsilon$ 4 status (negative, heterozygous, homozygous), age at onset, and gender included as fixed effects variables. Variables that 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. AUC was calculated, and 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 as assessed by calculating CVs for each analyte. All analytes were accurately

Table 1

Antibody pairs for ELISA and multiplex. The table lists the antibody pairs used in the multiplex set and the sources of the antibodies. Quidel, <https://www.quidel.com/>; Hycult, <http://www.hycultbiotech.com/>; Comptech, <http://www.complementtech.com/>; Millipore, [www.emdmillipore.com](http://www.emdmillipore.com)

Analyte/assay	Capture antibody (source)	Detection antibody (source)
C1s	MM Anti-C1s (M81, Hycult)	MM Anti-C1s (F33, in house)
C9	MM Anti-C9 (B7, in house)	MM Anti-C9 (6D4, in house)
Clusterin	RP Anti-Apolipoprotein J/Clusterin (AB825, Millipore)	MM Anti-Clusterin (MBI-40, in house)
FH-Y	MM Anti FH-Y402 (MBI-6, in house)	MM Anti-FH (OX-24)
FH-H	MM Anti FH-H402 (MBI-7, in house)	MM Anti-FH (OX-24)
FI	MM Anti-FI (7B5, in house)	RP Anti-FI (in house)
C4d	MM Anti-neo-C4d (A251, Quidel)	MM Anti-C4d (A213, Quidel)
TCC	MM Anti-neo-C9 (aE11, Hycult)	MM Anti-C8 (E2, in house)
iC3b	MM Anti-neo-iC3b (A209, Quidel)	MM Anti-C3b (C3-30, in house)
Bb	MM Anti-neo-Bb (A252, Quidel)	MM Anti-FB (JC1, in house)

MM, mouse monoclonal antibody; RP, rabbit affinity purified polyclonal antibody. Neo denotes neopeptide-specific antibody.

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

Analyte	R <sup>2</sup>	o
C1s	-0.016	0.82
C9	0.23	0.0009
FH	0.059	0.40
Clusterin	-0.08	0.26
FI	0.13	0.07
TCC	0.17	0.02
iC3b	-0.059	0.40
Bb	-0.013	0.85
C4d	0.11	0.13

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, confirming the suitability of the assay sets chosen for multiplexing. CVs were < 10% for all analytes in the multiplex (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 clus-

terin concentration in controls was 295 mg/l and in AD was 388 mg/l, a highly significant difference ( $p = 2.32 \times 10^{-6}$ ). 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; Fig. 1A). At 70% sensitivity, the predicted specificity of the model was 75%.

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

From the analysis of MCI converters versus non-convertors, three of the nine complement analytes were significantly different between the groups: clusterin, TCC, and FI (Table 4; Fig. 1B). Of these, clusterin was the most significant; the mean clusterin level in non-convertors was 309 mg/l and in converters was 418 mg/l. TCC was significantly lower in MCI converters compared to non-convertors (0.7 mg/l versus 3.6 mg/l), while FI was significantly reduced in MCI converters compared to non-convertors (27.7 mg/l versus 50.7 mg/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, Fig. 1B). At 80% sensitivity, the predicted specificity of the model was 79%.

#### *Measurement of FH Y402 H allotypic variants predicts progression in MCI*

In the selected model, total FH concentration was not significantly reduced in AD compared to controls (335.3 mg/l versus 350.8 mg/l; Table 3)

Table 3

Mixed effects linear model for complement analyte difference between AD and controls. Clustered mixed-effects linear modeling (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

Initial Model	AD (mean $\pm$ SD; mg/l)	Controls (mean $\pm$ SD; mg/l)	$\beta$ (95% CI)	$p$
C1s	102.2 $\pm$ 19.4	104.1 $\pm$ 20.4	-0.001 (-0.005 - 0.003)	0.54
C4d	3.8 $\pm$ 4.5	2.9 $\pm$ 6.2	0.014 (-0.017 - 0.05)	0.39
C9	52.0 $\pm$ 17.1	51.2 $\pm$ 14.8	0.001 (-0.003 - 0.006)	0.65
Clusterin	387.6 $\pm$ 113.9	295.0 $\pm$ 128.5	-0.001 (-0.002 - -0.0004)	<b>2.32 <math>\times 10^{-6}</math></b>
FI	51.5 $\pm$ 37.8	50.7 $\pm$ 38.9	0.001 (-0.002 - 0.004)	0.32
TCC	3.2 $\pm$ 4.3	2.8 $\pm$ 2.3	-0.016 (-0.043 - 0.011)	0.27
iC3b	1.8 $\pm$ 1.2	1.6 $\pm$ 1.1	0.003 (-0.062 - 0.068)	0.93
Bb	21.2 $\pm$ 9.3	18.7 $\pm$ 8.8	0.003 (-0.005 - 0.010)	0.51
FH	335.3 $\pm$ 81.0	350.8 $\pm$ 99.0	0.0004 (-0.0004 - 0.001)	0.34
Gender (male)			-0.082 (-0.21 - 0.05)	0.23
APOE4			-0.12 (-0.23 - -0.008)	0.039
Age at sample			-0.021 (-0.03 - -0.01)	<b>4.75 <math>\times 10^{-5}</math></b>
Final model AD versus control			$\beta$ (95% CI)	$p$
Clusterin			-0.001 (-0.002 - -0.0008)	8.1 $\times 10^{-7}$
APOE4			-0.13 (-0.2378 - -0.02)	0.02
Age at sample			-0.02 (-0.03 - -0.01)	2.4 $\times 10^{-5}$

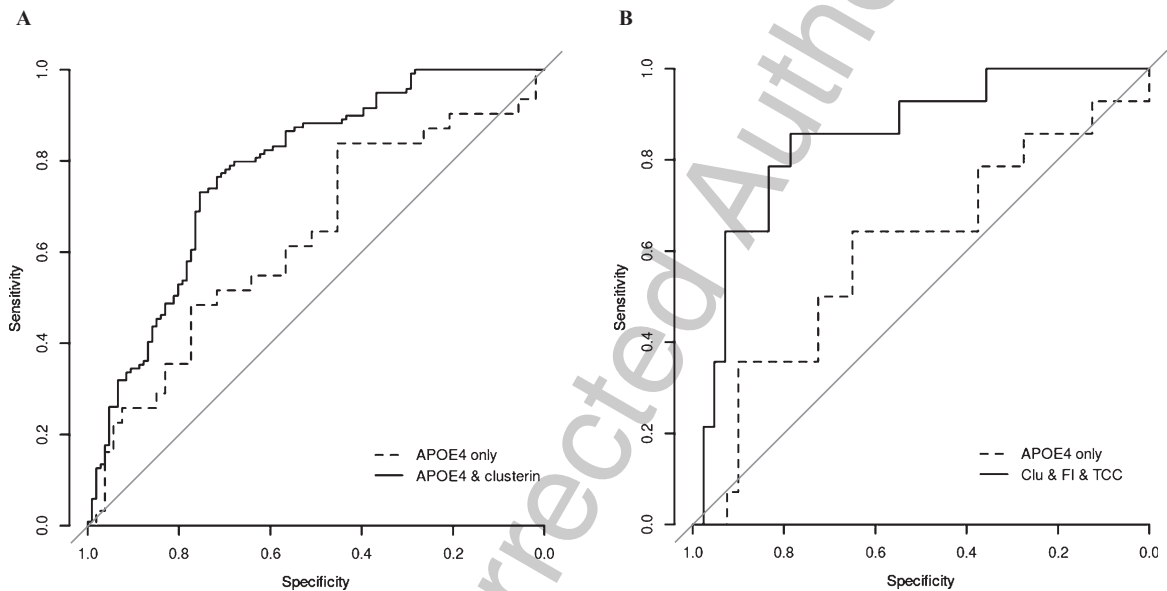


Fig. 1. Receiver-operating characteristic (ROC) curves representing models which differentiate AD from controls (A) and MCI converters from non-converters (B). ROC curves were drawn for the final models distinguishing AD from controls (A; clusterin and APOE4) and MCI converters from non-converters (B; 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.

294 or in MCI converters compared to non-converters  
 295 (297.9 mg/l versus 351.4 mg/l; Table 4). When the  
 296 levels of the Y402 and H402 variants of FH, measured  
 297 separately using variant-specific capture antibodies  
 298 and corrected for allele number, were compared  
 299 between AD and control groups, there was no significant  
 300 difference (Y variant, AD, 170.4 mg/l, controls,

172.5 mg/l; H variant, AD, 167 mg/l, controls,  
 175.5 mg/l; Table 5). A similar analysis comparing  
 MCI non-converters and converters revealed a significantly  
 lower level of the H variant in the converters  
 (Y variant, non-converters, 166.3 mg/l, converters,  
 164.4 mg/l; H variant, non-converters, 172.5 mg/l,  
 converters, 142.1 mg/l; Table 5;  $p=0.0056$ ).

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Table 4

Mixed effects linear model for complement analyte difference between MCI converters and non-converters. Clustered mixed-effects linear modeling (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

Initial Model	Converted to AD (mean $\pm$ SD; mg/l)	Not converted (mean $\pm$ SD; mg/l)	$\beta$ (95% CI)	$p$
C1s	88.9 $\pm$ 15.1	103.2 $\pm$ 24.8	-0.003 (-0.006– 0.0009)	0.15
C4d	2.2 $\pm$ 2.1	3.6 $\pm$ 3.2	-0.017 (-0.042– 0.008)	0.18
C9	42.8 $\pm$ 16.6	50.5 $\pm$ 14.1	0.002 (-0.0027 – 0.006)	0.43
Clusterin	417.5 $\pm$ 88.5	308.7 $\pm$ 115.2	0.002 (0.001 – 0.002)	<b>2.43 <math>\times</math> 10<sup>-7</sup></b>
FI	27.7 $\pm$ 7.9	50.7 $\pm$ 26.6	-0.006 (-0.009 – -0.002)	<b>0.0025</b>
TCC	0.7 $\pm$ 2.5	3.6 $\pm$ 3.4	-0.03 (-0.04 – -0.01)	<b>0.0027</b>
iC3b	1.7 $\pm$ 1.2	1.7 $\pm$ 0.9	0.02 (-0.05 – 0.08)	0.67
Bb	18.3 $\pm$ 9.3	18.4 $\pm$ 8.8	0.003 (-0.005 – 0.01)	0.51
FH	297.9 $\pm$ 75	351.4 $\pm$ 96.8	-0.0001 (-0.001 – 0.0008)	0.76
Gender (male)			0.11 (-0.02 – 0.24)	0.10
APOE- $\epsilon$ 4			-0.02 (-0.11 – 0.08)	0.71
Age at sample			0.0004 (-0.009 – 0.01)	0.94
Final model convertor versus non-convertor			$\beta$ (95% CI)	$p$
Clusterin			0.002 (0.001 – 0.002)	1.26 $\times$ 10 <sup>-9</sup>
FI			-0.006 (-0.009 – -0.003)	1.42 $\times$ 10 <sup>-5</sup>
TCC			-0.024 (-0.04 – -0.008)	0.005

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,  $p=0.00330$ ). Allele number-corrected allotype levels in FH-Y402H heterozygotes were then compared between AD and controls (top) and MCI non converters and converters (bottom); FH-H402 levels were significantly lower (\*\*) in MCI patients who subsequently converted to AD when compared to those who did not convert

FH allotype levels in AD and controls (mean $\pm$ SD; mg/l)			
diagnosis	Y402	H402	$p$
AD	170.4 $\pm$ 45.1	167.0 $\pm$ 39.8	0.71
control	172.5 $\pm$ 42.7	175.5 $\pm$ 38.2	0.71
$p$	0.81	0.26	
FH allotype levels in MCI converters and non-convertors (mean $\pm$ SD; mg/l)			
converted	Y402	H402	$p$
no	166.3 $\pm$ 47.2	172.5 $\pm$ 41.6	0.44
yes	164.4 $\pm$ 57.3	142.1 $\pm$ 34.5	0.18
$p$	0.90	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 and inform selection of participants into future clinical trials, particularly those targeting immune system involvement and inflammation. 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 significantly differentiated AD patients from matched controls, while clusterin, FI, and TCC were all significantly different between individuals with MCI who subsequently either converted to AD or remained stable when reassessed 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 reported 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

339 for AD that is replicated across different assay plat-  
340 forms. In light of this, it is somewhat paradoxical that  
341 two disease-associated SNP in clusterin are reported  
342 to associate with decreased plasma levels [29, 30].  
343 Precisely how plasma clusterin levels impact disease  
344 risk remains uncertain. Clusterin is a multifunctional  
345 molecule, an inhibitor of the complement terminal  
346 pathway but also a professional molecular chaperone  
347 involved in clearance of debris [31]. Amyloid plaques  
348 in AD are richly decorated with clusterin and a role  
349 in clearance of amyloid has been proposed [32, 33].  
350 Clusterin has also been shown to reduce  $A\beta_{42}$  toxicity  
351 in a rat model of AD [34].

352 Association of plasma clusterin levels with rate of  
353 cognitive decline has been reported both in MCI and  
354 AD [17, 20, 24]; in each of these studies, higher  
355 clusterin levels predicted more rapid decline. Our  
356 data demonstrating substantially higher plasma clusterin  
357 in MCI donors who subsequently convert to AD  
358 compared to non-convertors robustly support these  
359 findings and show that elevated plasma clusterin is  
360 a powerful predictor of progression. The functional  
361 basis of this association is problematic; if clusterin  
362 is involved in reducing  $A\beta$  toxicity and accelerat-  
363 ing amyloid clearance, then increased plasma levels  
364 might be expected to restrict the development of  
365 pathology. It is possible that increased clusterin  
366 production, reflected in increased plasma levels, rep-  
367 represents a failed protective response to the disease  
368 process.

369 TCC, a marker of complement terminal pathway  
370 activation, is present on neurons, plaques, and adja-  
371 cent blood vessels in AD brain [32–35]; fluid-phase  
372 TCC (also termed sC5b-9) has been measured in AD  
373 CSF [36], but plasma levels of TCC have not pre-  
374 viously been reported in AD or MCI. Plasma TCC  
375 levels were not different between AD and controls  
376 but were significantly lower in MCI donors who  
377 subsequently converted to AD compared to non-  
378 convertors; this finding is somewhat counterintuitive  
379 in that it implies lower levels of terminal pathway  
380 activation in the convertors despite clear evidence  
381 that the terminal pathway is abundantly activated in  
382 AD brain. We suggest that the demonstrated elevated  
383 levels of clusterin, an efficient inhibitor of the termi-  
384 nal pathway, in the convertor group might suppress  
385 terminal pathway activation and TCC generation in  
386 plasma. Notably, plasma levels of activation pathway  
387 products (iC3b, C4d, Bb) were not different between  
388 the groups, suggesting that any difference in central  
389 complement activation between MCI convertors and  
390 non-convertors was not reflected in the periphery.

391 FI is the enzyme responsible for regulating the  
392 activation pathway convertases; complement recep-  
393 tor 1, linked through GWAS studies to AD [9, 37,  
394 38], is the major cell-associated cofactor for FI-  
395 mediated cleavage of C3b/C4b. Plasma FI has  
396 previously been reported as a biomarker of brain  
397 atrophy [22]. Here we show that plasma FI level was  
398 significantly reduced in MCI convertors compared to  
399 non-convertors (27.7 mg/l versus 50.7 mg/l; the latter  
400 identical to healthy controls). Lower levels of FI will  
401 impact capacity to control complement activation  
402 once triggered and favor dysregulation [39].

403 In the mixed effects linear models described here,  
404 FH was not significantly different between AD  
405 patients compared to controls (335.3 mg/l versus 350.  
406 8 mg/l; Table 3) or between MCI convertors com-  
407 pared to non-convertors (297.9 mg/l versus 351.  
408 4 mg/l; Table 4). The common FH-Y402 H poly-  
409 morphism is a major risk factor for AMD [12, 13],  
410 but does not associate with AD in multiple studies  
411 [14–16]. Here we separately measured plasma lev-  
412 els of the products of the two allotypic variants and  
413 showed that levels of the FH-H402 variant were sig-  
414 nificantly lower in MCI donors who subsequently  
415 converted to AD compared to non-convertors. These  
416 data could be explained by decreased expression of  
417 the FH-H402 allele in the convertor group or, more  
418 likely, by increased consumption of the FH- H402  
419 allotype protein in response to the disease in MCI  
420 convertors. Differential binding of the different FH-  
421 Y402 H allotypes at sites of pathology has previously  
422 been described in the context of AMD [40], and our  
423 unpublished data suggest preferential binding of the  
424 FH-H402 allotype in AD brain.

425 From our data we generated two models. The first  
426 compared AD patients with controls and included  
427 clusterin with the co-variables age and APOE status;  
428 ROC curves constructed from this set gave an AUC  
429 of 0.78 for the entire set, considered “moderately  
430 predictive” [41]. The second model compared MCI  
431 convertors and non-convertors and included clusterin,  
432 TCC, and FI with the sole co-variable associated with  
433 MCI conversion (APOE status); ROC curves gave an  
434 AUC of 0.85 for the entire sample set, considered  
435 “highly predictive”. Although levels of the FH-H402  
436 allotype were significantly predictive of MCI conver-  
437 sion when measured in FH-Y402 H heterozygotes,  
438 this variable was not included in the model because  
439 it applied only to the subset of the population that  
440 possessed one or more H402 alleles; nevertheless,  
441 the data demonstrate that measuring plasma allotype  
442 levels for a common complement polymorphism can



help predict disease and raise the prospect that other complement polymorphisms might also be predictive, as is the case in AMD [12, 13].

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 and provide further information on precisely how complement contributes to AD pathology. 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.

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