Longitudinal protein changes in blood plasma associated with the rate of cognitive decline in Alzheimer's disease

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Abstract

Biomarkers of Alzheimer's disease (AD) progression are needed to support the development of urgently needed disease modifying drugs. We employed a SOMAscan assay for quantifying 1,001 proteins in blood samples from 90 AD subjects, 37 stable mild cognitive impaired (MCI) subjects, 39 MCI subjects converting to AD within a year and 69 controls at baseline and one year follow up. We used linear mixed effects models to identify proteins changing significantly over one year with the rate of cognitive decline, which was quantified as the reduction of Mini Mental State Examination (MMSE) scores. Additionally we investigated proteins changing differently across disease groups and during the conversion from MCI to AD. We found that levels of proteins belonging to the complement cascade increase significantly in fast declining AD patients. Longitudinal changes in the complement cascade might be a surrogate biomarker for disease progression. Additionally we found that members of the cytokine-cytokine receptor interaction pathway change during AD when compared to healthy aging subjects.

1   Introduction

Alzheimer's disease (AD), the most common form of dementia, is a devastating illness characterized by progressive short-term memory loss, followed by the inability of patients to care for themselves and leading to eventual death 10-15 years after diagnosis. To date there is no cure and available medication can only temporarily alleviate some symptoms or slow down progression in a subset of patients. Thus new drugs are urgently needed.

To support the development of disease modifying drugs, biomarkers for early diagnosis and disease progression are required [1] and blood based biomarkers have been the focus of much recent work as blood can be accessed for repeated measures relatively easily. Of particular note is the growing body of work around changes in plasma proteins in relation to AD and/or MCI-related protein biomarkers using Mass Spectrometry and antibody capture technologies [2-10].

The majority of studies have investigated the potential of blood proteins by only considering cross sectional measures. In this study we investigated longitudinal protein changes associated with the rate of cognitive decline in AD patients. Additionally, we compared longitudinal protein changes between normal aging and changes during AD, and changes occurring during the transition from MCI to AD.

2   Methods

2.1   Subjects

We obtained protein measures for 235 subjects (69 controls, 37 'stable' MCI (MCIs), 39 MCI converting to AD within a year (MCIc) and 90 AD) from the EU funded AddNeuroMed (ANM) biomarker study [11, 12]. Informed consent was obtained for all subjects according to the Declaration of Helsinki (1991), and protocols and procedures were approved by the relevant local ethical committees at each site. All subjects were assessed with a standardised assessment protocol including informant interview for diagnosis, cognitive assessment such

as the Mini Mental State Examination (MMSE) together with standardised assessment of function, behaviour and dementia severity as previously reported [3, 9, 10].

2.2 Blood samples

At the time of assessment, all blood samples were drawn by venipuncture and collected into EDTA glass tubes. Subjects were required to fast for at least 2 hours prior to collection. All samples were centrifuged at 2000g for 10min at 4□C within approximately 2h of collection. Plasma supernatant was collected, divided into aliquots, and frozen at -80□C until further use.

2.3 Protein measures

Proteins were measured using a Slow Off-rate Modified Aptamer (SOMAmer)-based capture array called 'SOMAscan' (SomaLogic, Inc, Boulder, Colorado). This approach uses chemically modified nucleotides to transform a protein signal to a nucleotide signal that can be quantified using relative florescence on microarrays. Therefore all gathered SOMAscan measures are relative fluorescence units (RFU). This assay has been shown to have a median intra- and inter-run coefficient of variation of ~5%. The median lower and upper limits of quantification were ~1 pM and ~1.5 nM in buffer, and ~2.95 pM and ~1.5 nM for a subset of the somamers in plasma, full details are given in Gold et al. [13].

Quality control was performed at the sample and SOMAmer level, and involved the use of control SOMAmers on the microarray and calibration samples. At the sample level, hybridization controls on the microarray were used to monitor sample-by-sample variability in hybridization, while the median signal over all SOMAmers was used to monitor overall technical variability. The resulting hybridization scale factor and median scale factor were used to normalize data across samples. The acceptance criteria for these values was 0.4-2.5, based on historical trends in these values. SOMAmer-by-SOMAmer calibration occurred through the repeated measurement of calibration samples, these samples were of the same matrix as the study samples, and were used to monitor repeatability and batch to batch

variability. Historical values for these calibrator samples for each SOMAmer were used to generate a calibration scale factor. The acceptance criteria for calibrator scale factors was 95% of SOMAmers having a calibration scale factor within ±0.4 of the median.

The assay required 8μL of plasma from each sample. A single assay was used per plasma sample, and thus no technical replicates were performed. All measurements were log2 transformed.

The assay measures the level of 1,001 human proteins representing different molecular pathways and gene families. The majority of proteins are involved in the following processes: signal transduction pathways, stress response, immune process and phosphorylation, but in addition proteolysis, cell adhesion, cell differentiation and intracellular transport proteins are represented.

2.4   Statistical analysis

All statistical analyses were performed using R and the library 'nlme' version 3.1 [14]. Firstly, we investigated the association between change in protein level (follow-up minus baseline) and the rate of cognitive decline in AD subjects at the single analyte level. Cognitive decline was calculated by fitting a multi-level linear mixed model to the longitudinal Mini Mental State Examination (MMSE) assessments, which were gathered over five visits during a one year follow-up period. Subjects and centre were included as random effects in the model. Further covariates including age of onset, disease duration at baseline, gender, presence of apolipoprotein (*APOE*) ε4 allele, living in a nursing home and years of education were added as fixed effects. The associations between protein changes and the rate of MMSE decline was assessed by including a protein change by time interaction in within the multi-level model.

Additionally we aimed to identify proteins changing over time in the diagnostic groups relative to controls, and therefore possibly associated with disease progression. For each individual

protein we estimated the rate of change by using a multi-level linear model with random intercepts for subject and centre level clustering. The slope was fixed as there were only protein measures at two time points, namely baseline and follow-up. Age, gender and apolipoprotein (*APOE*) ε4 allele presence were included as fixed covariates. In order to estimate and test the association between protein changes and disease status, we included a time by diagnosis interaction term in the model. Control subjects were set as the reference level to which all the disease groups were compared. Dummy coding was applied for the three disease groups (MCIs, MCIc and AD). We used the same approach to identify proteins, which are changing during the transition from MCI to AD relative to unchanging MCI subjects. Stable MCI subjects were set as the reference to which MCI subjects converting to AD were compared (MCIs = 0, MCIc = 1).

All p-values were FDR (false discovery rate) adjusted and an association was considered significant if it passed a threshold of q-value < 0.05. However, we also considered associations passing a threshold of p-value < 0.01 as nominally significant.

2.5   Enrichment analysis

Enrichment analysis for pathways (KEGG) and Gene Ontologies (molecular function, cellular component and biological process) was performed on significantly associated proteins (q-value < 0.05) using the DAVID knowledgebase [15, 16]. In the case that only small number of proteins passed the threshold a more exploratory threshold of p-value < 0.01 was used. The background for enrichment was set to the full list of proteins measured on the SomaLogic panel.

Again, all p-values were FDR (false discovery rate) adjusted and enrichment was considered significant if it passed a threshold of q-value < 0.05 and nominally significant at a p-value < 0.01.

3   Results

3.1   Demographics

Demographic characteristics stratified by diagnostic group are provided in Table 1. Differences in demographic characteristics according to clinical diagnosis were assessed using a one-way ANOVA. Significant differences were found for age, number of *APOE* ε4 alleles and MMSE score at baseline; no significant differences in gender were observed.

< Table 1>

3.2   Protein changes associated with the rate of decline in AD patients

Twelve proteins were found to be significantly associated with rate of progression on an exploratory level (p-value < 0.01) (See Table 2): C2 (Complement C2), SAA (Serum amyloid A-1 protein), C9 (Complement C9), MBL (Mannose-binding protein C), SAP (Serum amyloid P-component), α2-Antiplasmin, CHK1 (Serine/threonine-protein kinase Chk1), IL-17 (Interleukin-17A), eIF-5A-1 (Eukaryotic translation initiation factor 5A-1), Hemopexin, CDC37 (C-C motif chemokine 19) and Complement factor H-related protein 5. Both C2 and SAA passed multiple testing corrections at a q-value threshold of <0.05. Ten out of 12 proteins showed a negative association with rate of cognitive decline and therefore increasing levels of these proteins can be associated with high loss in MMSE scores per year or in other words with fast decline. Only eIF-5A-1 and CDC37 showed a positive association with the rate of cognitive decline and therefore increasing levels of these proteins are associated with lower loss in MMSE scores per year or in other words with slow decline. Scatter plots of the four proteins showing the most significant change with rate of cognitive decline are shown in Figure 1.

< Table 2 >

< Figure 1 >

The 12 proteins passing the threshold of p-value < 0.01 (Table 2) were significantly enriched in members of the complement and coagulation cascades (q-value = 0.005, KEGG hsa04610). These proteins are: MBL, C9, C6, and α2-Antiplasmin. We also found significant enrichment (q-value<0.05) of proteins involved in 24 biological processes, including acute inflammatory response (q-value=0.0003), inflammatory response (q-value=0.002), defense response (q-value=0.011) and complement activation (q-value=0.015).

3.3   Protein changes across disease groups

X Proteins were found to be changing (q-value<0.05) in MCIs and in AD over time relative to controls (See Table 3). HCC-1 (C-C motif chemokine 14) was the only protein showing significant changes when comparing in the MCIs group. Changes in HCC-1 levels were also significant at a suggestive p-value of < 0.05 between the AD and control group. Ten proteins showed a significant change in levels between the AD and control group. Notably protein levels gradually decrease over time with more established disease and thus AD patients showed a generally larger decrease in protein levels than MCIs over a year. Raw differences in plasma levels are shown in Figure 2 for the four strongest associations.

The 11 proteins changing in   in AD did not show significant enrichment, not even at the exploratory level (p-value<0.01), for KEGG pathways, GO molecular function, cellular components or biological processes.

< Table 3 >

< Figure 2 >

3.4   Protein level changes associated with conversion from MCI to AD

No protein was found to show significant enough changes during conversion from MCI to AD to pass multiple testing correction (q-value < 0.05) when compared to stable MCI . However

nine proteins, SDF-1α (Stromal cell-derived factor 1 alpha, p-value=3.3E-5), SLPI (Antileukoproteinase, p-value=8.5E-4), HCC-1 (C-C motif chemokine 14, p-value=0.002), BCMA (Tumor necrosis factor receptor superfamily member 17, p-value=0.005), LTA-4 hydrolase (Leukotriene A-4 hydrolase, p-value=0.006), Endostatin (p-value=0.006), Trypsin (p-value=0.006), IL-6 sRα (Interleukin-6 receptor subunit alpha, p-value=0.007) and Albumin (p-value=0.010) passed a suggestive threshold of p-value < 0.01 (Table 4).

The nine proteins were not significantly enriched for any pathway or Gene Ontologies at an uncorrected p-value.

< Table 4 >

3.5 Assay performance

In order to assess the quantitative performance of the assay for C2, C9, MBL and SAA, limit of quantification (LOQ) six-point standard curves spanning six logs in concentration, from 10 nM to 10 fM, in buffer were generated. The upper limit of quantification (ULOQ), the lower limit of quantification (LLOQ) and the dynamic range of quantification (ROQ) were measured. Standard curves and precision profiles for all four analytes are shown in Supplementary Material 1.

4   Discussion

In this study, we investigated the relationship between changes in 1,001 proteins and rate of Alzheimer's disease progression, as measured by change in MMSE scores. As we had longitudinal measures only for a subset of samples (69 controls, 37 'stable' MCI (MCIs), 39 MCI converting to AD within a year (MCIc) and 90 AD) cross-sectional results for the complete data set comprising protein measures for 691 subjects (211 controls, 106 MCI patients, 43 MCI patients converting to AD within a year, and 331 AD patients) wereWe previously reported a cross sectional analysis in a superset of these samples [4, 7].

We found that proteins exhibiting rate of cognitive decline associated changes were enriched with proteins from the complement cascade,namely MBL (Mannose-binding protein C), C2 (Complement C2), and C9 (Complement C9). All three were shown to be increasing more rapidly in AD patients with faster cognitive decline. They are found at the earliest stages of amyloid deposition and their activation coincides with the clinical expression of AD [17, 18]. The complement cascade is activated through three pathways: the classical, alternative and lectin pathway [19]. Target recognition of the three pathways varies, but they all share the common step of activating the central component C3 [20]. Mannose-binding protein C (MBL) activates the lectin pathway following the recognition and binding of pathogen-associated molecular patterns [20]. MBL activates the MBL-associated serine protease that leads to activation of Complement C4 and Complement C2 [21]. We found strong associations between C2, and MBL changes and the rate of cognitive decline and a statistically significant change in C9, also a component of the lectin complement pathway. Therefore our results suggest that changes in products of the lectin complement pathway are associated with fast cognitive decline. To our knowledge this is the first study to report an association between changes in the lectin complement pathway and the rate of cognitive decline in AD patients. C2 and C9 are also members of the classic pathway, which has an established association with AD as it was found to be directly activated *in vitro* by fibrillar Aβ40 and Aβ42 by binding to C3 and the globular heads of C1q [22].

We also found that Serum amyloid P-component (SAP) levels increase more in individuals with a fast cognitive decline. SAP is reported to co-localise with Aβ plaques in human AD brain [23, 24], exhibit up-regulated synthesis in AD affected brain regions [25], induce neuronal apoptosis in vitro [26, 27] and protect senile plaques from proteolysis [28]. Increasing levels of SAP could potentially create an increasingly neurotoxic environment and therefore result in a faster cognitive decline in AD patients. Interestingly SAP can also activate the classical complement pathway via C1q [29]. Thus, greater increase of SAP, together with increased levels of Complement C2 and Complement C9 levels could also be an indication of increased levels of components of the classic complement pathway components in AD patients with fast cognitive decline.

To our knowledge this is the first study to report a correlation in the level of members of the complement cascade associated with and rate of decline. Although our study is limited by the fact that we only observed protein changes over a year, a short timeframe for a slow developing disease such as AD, and a longer follow up of cognitive as well as protein changes in plasma would be needed to further support our findings.

SAP has been previously reported as a diagnostic AD candidate biomarker in the literature [4] albeit it with conflicting direction of association [3, 10, 30, 31]. Although we found changes in SAP to be associated with rate of decline we did not observe significant changes of SAP when comparing diagnostic groups against controls.

The comparison of protein changes across clinical diagnostic groups showed that the most significant changes are in AD subjects when compared to controls. However, the sample number for stable MCI and converting MCI patients was smaller than the AD group, resulting in lower statistical power for these groups. We found that proteins passing multiple testing correction for the AD group were generally reducing over time. Among the proteins four were identified as being involved in cytokine-cytokine receptor interactions: Ck-β-8-1, MIP-1α, VEGFA, BMP RII and HCC-1. A number of studies have previously identified cytokine proteins as being predictive of clinical AD diagnosis and progression from MCI to AD with high accuracy [6, 32-34]. Leung *et al.* [35] investigated plasma levels of cytokines changing with the rate of cognitive decline. This study investigated 27 cytokines of which 14 were also measured on our panel. They found a significant increase (p-value<0.05) in the levels of IL-4, IL-10, and granulocyte-colony stimulating factor in AD patients with a fast cognitive decline compared to slow cognitive decline. IL-4 was not measured on our panel. IL-10 and granulocyte-colony stimulating factor were not found to be significant in our study.

No significant enrichment for pathways or gene ontology terms was found in protein changes during the conversion from MCI to AD. Generally associations were weak with no proteins passing multiple testing correction, possibly in part due to the low sample number. Protein

levels of SDF-1α (Stromal cell-derived factor 1 alpha) showed the most significant increase and it has been suggested that SDF-1α could act in the central nervous system as a classical neuromodulator under normal conditions [36, 37]. Enhanced production of SDF-1α is suspected to affect neuronal and neuroendocrine activities, consequently alter brain function and lead to pathological behaviors and/or neurotoxicity [38]. Our findings might support the idea that increasing levels of SDF-1α are occurring during the transition from MCI to AD. We found a suggestive association between Albumin and the conversion from MCI to AD Albumin (p-value=0.010). Albumin has been previously associated with AD [3, 9, 32, 39]. In a recent review, we found that Albumin was associated with an AD-related phenotype in four independent research cohorts [4]. We also found that Albumin was also associated with Parkinson's Disease, Schizophrenia and Depression [40]. The majority of associations between AD and albumin is positive, while association with Parkinson's Disease [41-44] Schizophrenia [45-47] and Depression [48-53] were mainly negative [40]. Albumin is an acute-phase protein, which can increase or decrease in plasma concentrations as part of an inflammatory response. In addition Albumin, is also generally used as a nutritional marker [49] and thus might only reflect nutritional status.

Another protein found to be associated on a suggestive level with conversion from MCI to AD was Trypsin (p-value=0.006). Literature suggests that Aβ may play a direct role in protease activities, for instance it was shown that fibrillar Aβ, a key component of AD plaques, is resistant to proteolytic digestion [54]. Further it was reported that fibrillar Aβ inhibits the proteolytic activity of Trypsin and that soluble Aβ is a substrate for Trypsin [55]. We found higher Trypsin levels in subjects converting to AD than stable MCI subjects and therefore our findings may support hypothesis that that Trypsin is involved in the Aβ catabolism.

We have presented the largest longitudinal study to date of changes in plasma protein levels associated with rate of disease progression and clinical diagnosis. Our results suggest that levels of complement pathway products are elevated in faster declining AD patients Thus longitudinal changes in the complement cascade might be a surrogate biomarker for disease progression and also be a promising target for AD drug discovery. However, we only

investigated protein plasma levels changing over one year, therefore studies with a longer follow up time are needed for further validation.

5   Acknowledgement

6   Disclosure statement

Intellectual property has been registered on the use of plasma proteins for use as biomarkers for AD by King's College London and Proteome Sciences, with Simon Lovestone named as an inventor. Stephen Williams is employee of SomaLogic Inc. and the proteomic assay reported in this manuscript was performed using reagents supplied by SomaLogic, Inc.

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Table 1 Sample characteristics stratified by clinical diagnostic group. P-values comparing the different diagnostic groups were calculated using a one-way ANOVA. (MCI – Mild Cognitive Impaired, AD – Alzheimer's disease)

	Control	MCI stable	MCI converting	AD	P-Value
	69	37	39	90	
Age (median [IQR])	74.0 [9.00]	75.0 [8.00]	77.0 [10.00]	76.0 [9.75]	0.003
Gender (male/female)	33/36	15/22	17/22	29/61	0.234
Number of *APOE* e4 alleles (0/1/2)	48/18/3	24/12/1	15/21/3	39/36/15	< 0.001
Baseline MMSE (median [IQR])	29 [2]	28[2]	27 [3]	22 [7]	< 0.001
Missing baseline MMSE	0	2	3	4	

Table 2: Proteins whose level changes over time are associated with the rate of cognitive decline. A negative Beta value indicates higher levels in AD patients with fast cognitive decline; a positive Beta value indicates a lower levels in rapidly declining patients.

Protein	Short name	UniProt	Beta	p-Value	q-Value
Complement C2	C2	P06681	-0.031	1.9E-5	0.019
Serum amyloid A-1 protein	SAA	P02735	-0.003	5.4E-5	0.027
Complement C9	C9	P02748	-0.014	1.8E-4	0.055
Mannose-binding protein C	MBL	P11226	-0.010	2.2E-4	0.055
Serum amyloid P-component	SAP	P02743	-0.014	3.9E-4	0.079
α2-Antiplasmin		P08697	-0.021	0.003	0.377
Serine/threonine-protein kinase Chk1	Chk1	O14757	-0.027	0.003	0.377
Interleukin-17A	IL17	Q16552	-0.021	0.004	0.500
Eukaryotic translation initiation factor 5A-1	eIF-5A-1	P63241	0.011	0.006	0.645
Hemopexin		P02790	-0.009	0.007	0.645
C-C motif chemokine 19	CDC37	Q16543	0.021	0.008	0.645
Complement factor H-related protein 5		Q9BXR6	-0.010	0.008	0.645

Table 3. Proteins shown to be changing over time at least one of the disease groups (q-value<0.05). Beta (B) coefficients represent diagnosis x time interactions and thus a negative Beta means that the protein levels decrease as disease progresses.. [DC-SIGNR (C-type lectin domain family 4 member M), MIP-1α (C-C motif chemokine 3), RGM-A (Repulsive guidance molecule A), BMP-RII (Bone morphogenetic protein receptor type-2), CD39 (Ectonucleoside triphosphate diphosphohydrolase 1), Ck-β-8-1 (C-C motif chemokine 23), VEGF (Vascular endothelial growth factor A), EphA1 (Ephrin type-A receptor 1) and HCC-1 (C-C motif chemokine 14)]

Protein	UniProt	MCI stable			MCI converter			Alzheimer's Disease		
		Beta	p-Value	q-Value	Beta	p-Value	q-Val	Beta	p-Value	q-Value
DC-SIGNR	Q9H2X3	-1.6E-4	0.224	0.994	-2.9E-4	0.026	0.999	-5.9E-4	3.2E-8	3.E-5
Aurora kinase A	O14965	1.6E-5	0.831	0.994	-1.4 E-4	0.069	0.999	-2.8E-4	3.8E-6	0.002
MIP-1α	P10147	-2.0E-5	0.834	0.994	-4.0E-5	0.668	0.999	-3.4E-4	9.9E-6	0.003
RGM-A	Q96B86	-2.2E-5	0.809	0.994	-1.7E-4	0.060	0.999	-3.2E-4	1.3E-5	0.003
BMP-RII	Q13873	9.7E-5	0.292	0.994	-1.6E-4	0.086	0.999	-3.1E-4	2.4E-5	0.004
CD39	P49961	2.5E-5	0.731	0.994	-8.3E-5	0.244	0.999	-2.4E-4	2.5E-5	0.004
Kallikrein 14	Q9P0G3	3.9E-5	0.605	0.994	-1.8E-4	0.017	0.999	-2.5E-4	5.3E-5	0.008
Ck-β-8-1	P55773	-7.7E-5	0.434	0.994	-9.3E-5	0.339	0.999	-3.1E-4	9.6E-5	0.011
VEGF	P15692	-8.0E-5	0.220	0.994	-8.9E-5	0.171	0.999	-2.1E-4	9.2E-5	0.011
EphA1	P21709	4.9E-6	0.972	0.994	-6.7E-5	0.628	0.999	-4.3E-4	1.1E-4	0.011
HCC-1	Q16627	-8.2E-4	5.9E-6	0.006	-9.3E-5	0.598	0.999	-4.9E-4	6.1E-4	0.051

Table 4. Proteins that were found to change (p-value < 0.01) during the conversion from MCI to AD relative to the stable MCI group

Protein name	Short name	UniProt	Beta	p-value	q-value
Stromal cell-derived factor 1	SDF-1α	P48061	3.3E-4	9.6E-5	0.098
Antileukoproteinase	SLPI	P03973	4.8E-4	8.5E-4	0.434
C-C motif chemokine 14	HCC-1	Q16627	7.3E-4	0.002	0.695
Tumor necrosis factor receptor superfamily member 17	BCMA	Q02223	3.8E-4	0.005	0.911
Leukotriene A-4 hydrolase	LTA-4 hydrolase	P09960	2.1E-4	0.006	0.911
Endostatin		P39060	4.0E-4	0.006	0.911
Trypsin		P07477	4.9E-4	0.006	0.911
Interleukin-6 receptor subunit alpha	IL-6 sRα	P08887	3.4E-4	0.007	0.911
Albumin		P02768	2.6E-4	0.010	0.999

Figure 1. Scatter plots showing the four most significant protein level changes associated with the rate of decline. All proteins four protein show a greater increase in faster declining patients than in slow declining patients. Complement C2, Complement C9 and Mannose-binding proteins are members of the complement cascade.
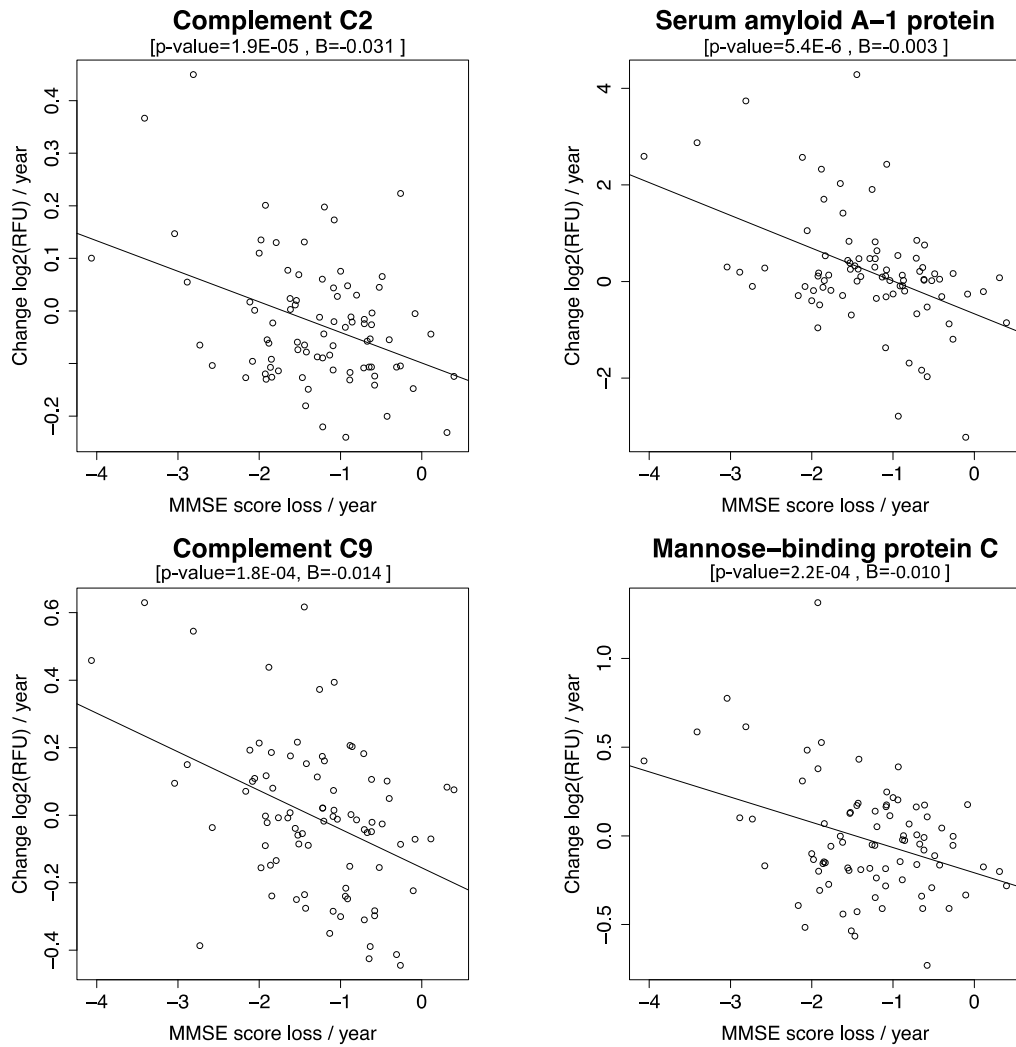
Figure 2. Changes in plasma levels for the four most significantly changing proteins for each of the diagnostic groups compared to normal aging controls (CTL). All four proteins appear to slowly increase in abundance in healthy aging, which is reduced and even reversed in AD.

DC−SIGN−related protein
[p-value=3.2E-8, B=-5.9E-4]

Aurora kinase A
[p-value=3.8E-6 , B=-2.8E-4]

C−C motif chemokine 3
[p-value=9.9E-6, B=-3.4E-4]

Repulsive guidance molecule A
[p-value=1.3E-5, B=-3.2E-4]