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The correlation between inflammatory biomarkers and polygenic risk score in Alzheimer's Disease

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Abstract

Plasma biomarkers to aid the early diagnosis of Alzheimer's disease (AD) or to monitor disease progression have long been sought and continue to be widely studied. Biomarkers that correlate with AD polygenic risk score, a measure of the polygenic architecture of the disease and highly predictive of AD status, would be excellent candidates. Therefore, we undertook a preliminary study to assess the association of plasma inflammatory biomarkers with an overall AD polygenic risk score as well as with an inflammation-specific AD polygenic risk score in a sample set of 93 AD cases. We measured five complement biomarkers (complement receptor 1 (CR1), clusterin, complement component 9 (C9), C1 inhibitor (C1inh), terminal complement complex (TCC)) and the benchmark inflammatory marker C-reactive protein (CRP). Plasma clusterin level showed an association with overall AD polygenic risk score, whilst clusterin, C1inh and CRP levels each displayed some association with the inflammatory-specific AD polygenic risk score. The results suggest that elevated plasma levels of inflammatory biomarkers, including complement proteins, associate with polygenic risk scores in AD, further strengthening the link between genetic and biomarker disease predictors and indicating a potential role for these markers in disease prediction and patient stratification in AD.

Keywords: Alzheimer's disease; Inflammation; Complement; Biomarker; Polygenic risk score

Introduction

There are 850,000 people with dementia in the UK, with numbers set to rise to over 1 million by 2025 and to 2 million by 2051 (Alzheimer's Society). Alzheimer's disease (AD) is the most common type representing 62% of dementia cases. There is substantial evidence supporting the involvement of inflammation in the pathogenesis of AD. History of serious head injury, which typically causes brain inflammation, is known to be a risk factor for AD (Mortimer et al., 1991; Fleminger et al., 2003; Barnes et al., 2014) and systemic infections, another cause of inflammation, also accelerate the disease (Licastro et al., 2104; Bu et al., 2015; Maheshwari et al., 2015). Epidemiological studies have suggested that anti-inflammatory drugs like indomethacin and ibuprofen reduce the risk of AD (Rogers et al., 1993; Breitner et al., 1994, Rich et al., 1995; McGeer et al., 1996; Vlad et al., 2008). Evidence of inflammation, including activated microglia and astrocytes, as well as various cytokines and complement activation products, have been found around amyloid plaques and dystrophic neurites in AD brain (Eikelenboom and Stam, 1982; Yasojima et al., 1999). All these findings support the involvement of inflammation in AD but do not indicate whether it is a primary or secondary event. However, recent genetic evidence from genome wide association studies (GWAS), including pathway analysis, has highlighted a significant aetiological role for immune-related processes and inflammation in AD (Harold et al., 2009; Jones et al., 2010; Lambert et al., 2009; Lambert et al., 2010; Lambert et al., 2013; Jones et al, 2015).

The complement system is a pivotal part of the innate immune system and a key driver of inflammatory processes. Complement consists of more than 30 component proteins, regulators and receptors, which work together to provide defence against infection and to clear toxic material (Morgan 2015). Dysregulation of the balance between complement activation and inhibition may contribute to neuroinflammation and disease. Complement activation has been shown to occur in the AD brain, even at very early stages of the disease (Loeffler et al 2008) and discovery/panel-based studies investigating blood protein markers have reported significant findings with complement proteins (Hye et al., 2006; Thambisetty et al., 2011; Kiddle et al., 2014; Muenchhoff et al., 2015; Hakobyan et al., 2016).

Genetic studies have identified AD-associated variants in complement pathway genes. Associations between disease status and common single nucleotide polymorphisms (SNPs) in clusterin (*CLU*) were identified in a two-stage study GWAS involving over 16,000 individuals (Harold et al., 2009). A second GWAS study of over 2000 AD and 5000 control individuals replicated the *CLU* finding and also identified an association with a SNP in complement receptor 1 (*CR1*) (Lambert et al., 2009). The association of these loci has since been robustly replicated (Carrasquillo et al., 2010; Corneveaux et al., 2010; Jun et al., 2010; Seshadri et al., 2010; Lambert et al., 2013). GWAS results such as these, even

though a huge success and of great importance to the field, still only explain a very small amount of the genetic risk in AD. The residual genetic risk is likely to reside both in rare genetic variation with larger effect sizes like that of *TREM2* variants for example (Guerreiro et al., 2013; Jonsson et al., 2013; Jin et al., 2014), and in multiple small effects implicated by polygenic risk score analyses (Escott-Price et al., 2015). A polygenic risk score (PS) encompasses more of the causal variance because it is calculated based not solely on genome-wide significant polymorphisms, but on all nominally associated variants at a defined significance threshold (typically thousands of variants). We have investigated the polygenic architecture of AD using the powerful International Genomics of Alzheimer's Project (IGAP) GWAS dataset (Lambert et al., 2013) and demonstrated that PS could predict AD status by over 78% (Escott-Price et al., 2015). Here we describe a pilot study to test whether plasma biomarkers correlate with PS. We analysed a panel of complement and inflammatory biomarkers, selected based on literature and in-house evidence of relevance to AD (CR1, clusterin, C9, C1inh, TCC and CRP), in a subset of the Genetic and Environmental Risk for Alzheimer's disease (GERAD1) cohort (Harold et al., 2009) (N=93). PS profiles were calculated for these patients, using the full PS model (Escott-Price et al. 2015) and an immune specific PS that includes only those genes relevant to immunity and inflammation. The plasma biomarker measurements were tested for correlation with the 'full' and immune-specific AD PS profiles.

Methods

Samples

Blood for plasma separation was collected in 6 ml volume using EDTA anticoagulant tubes. Plasma samples were separated (1600 g/15 mins) within 24 h of collection and stored in aliquots at -80°C until analysis.

This study utilised plasma samples from 93 AD cases (57 females/36 males) with data available for polygenic risk score calculation. The scores were calculated and normalised as previously described (Escott-Price et al. 2015), utilising the complete IGAP discovery dataset (Lambert et al. 2013), a p-value significance threshold of 0.5 and including *APOE* genotype, age and gender. The full PS model included 87,605 single nucleotide polymorphisms (SNPs). The immune specific PS (IPS) was generated using 2,177 SNPs identified from the immune-specific AD enriched pathways described in the IGAP study (Jones et al., 2015).

Quantifying the levels of clusterin, C-reactive protein, complement receptor-1, C1 inhibitor, C9 and terminal complement complex in plasma

The plasma levels of clusterin and C-reactive protein (CRP) were determined using commercially available human clusterin and CRP DuoSet ELISAs (R&D systems) and the protocols followed as described by the manufacturer.

The plasma levels of complement receptor-1 (CR1), C1 inhibitor (C1inh), C9 and terminal complement complex (TCC) were determined using sandwich ELISAs developed in-house with optimised antibody pairs developed in-house. Maxisorp (Nunc Life Technologies) plates were coated with 50 µl/well of capture antibody (1-5 µg/ml in 0.1 M carbonate buffer pH9.6), and incubated for 1 hour at 37ºC. The plates were washed 3x in PBS + 0.1%Tween-20 (PBST) and then blocked with 100 µl/well of 2% BSA-PBST for 1 hour at 37ºC. After washing the plates 3x in PBST, 50 µl/well of an 8-point serial dilution of standard protein in 1% BSA-PBST was added in duplicate to individual wells followed by addition of plasma samples in duplicate to separate wells (diluted as necessary in 1%BSA-PBST). The plate was incubated for 1.5 hours at 37ºC. After washing 3x in PBST, 50 µl/well of 1-2 µg/ml HRP labelled detection antibody diluted in 1% BSA-PBST was added and the plates were incubated 1hr at 37ºC. Wells were washed 3x in PBST and bound antibody was visualised with orthophenylenediamine (SIGMAFAST™ OPD). Colour development was stopped by the addition of 10% sulphuric acid, and absorbance was measured at 492 nm. See table 1 for individual details for each assay.

All standards and samples for all ELISAs were tested in duplicate. The intra-assay coefficients of variation (CV) % were 5.55 for Clusterin, 9.47 for CRP, 8.45 for CR1, 5.21 for C1inh, 15.77 for C9 and 12.05 for TCC. The inter-assay CV's were 5.16 for Clusterin, 7.88 for CRP, 21.95 for CR1, 21.79 for C1inh, 21.97 for C9 and 9.71 for TCC.

Statistical analysis

Protein concentrations were determined automatically from standard curves plotted using GraphPad Prism5 and data analysis was performed using statistical software R version 3.2.3. Spearman correlation tests were used to identify correlations between protein analyte levels and PS or IPS. Correlation coefficients were calculated for any analyte with a p value less than 0.1. The Mann-Whitney test was used to look for differences in protein levels between cases with high and low PS or IPS. Effect sizes were computed for the analytes that showed a significant difference.

Results

The concentrations of the 6 different biomarkers measured in the AD samples are shown in table 2. In the selected sample, the PS (normalised) ranged from -2.12 to 2.53 and the IPS (normalised) ranged from -2.34 to 3.11 (high scores are associated with increased AD risk). There was no correlation between PS and IPS (the Spearman correlation coefficient between the scores was 0.06, with a p-value of 0.56).

The cohort was tested for correlations between individual biomarker levels and PS (figure 1). Of the six analytes measured, only one, clusterin, was significantly positively correlated with PS (correlation coefficient 0.2, p=0.05) in that as the level of clusterin increased so did the PS. None of the other 5 measured proteins were significantly correlated with PS. To further explore the relationship between PS and analyte concentrations, cases at the high and low extremes of PS (defined as those with a PS more than 1 standard deviation above or below the mean) were compared for individual biomarker levels (figure 2 and table 3). Clusterin was the only biomarker to show a statistically significant difference between cases with a high and low PS (clusterin concentration: high PS, 264 μg/ml; low PS, 314 μg/ml; effect size 1.24, p=0.03).

The data were also examined for correlations between biomarker levels and IPS (figure 3). Two of the analytes, C1inh and clusterin, were significantly positively correlated with IPS (C1inh correlation coefficient 0.22, p=0.05; clusterin correlation coefficient 0.25, p=0.02). C9 and CRP trended towards a positive correlation with IPS but the correlations were not statistically significant (C9 correlation coefficient 0.19, p=0.08; CRP correlation coefficient 0.16, p=0.13), and CR1 and TCC showed no correlation with IPS. Comparisons of individual biomarker levels between those with the highest IPS and those with the lowest IPS (figure 4 and table 4) showed that C1inh levels were significantly higher in cases with a high IPS than in cases with a low IPS (212μg/ml versus 154 μg/ml; effect size 1.55, p-value 0.008). CRP levels were also significantly different between high and low IPS (4.99 μg/ml versus 0.75 μg/ml; effect size 14.2; p-value 0.02). CR1, C9, clusterin and TCC showed no significant difference in concentration between high and low IPS.

Note that as the study was a small scale hypothesis-driven pilot study multiple testing corrections were not applied and all p-values presented are uncorrected.

Discussion

The AD field is pursuing the identification of plasma biomarkers, or biomarker sets, that are sensitive detectors of early disease and/or highly predictive of disease progression. Biomarkers that correlate with AD PS would be excellent candidates. Therefore, we undertook the described study to assess the association of plasma inflammatory biomarkers with an overall AD PS as well as with an inflammatory specific AD PS in a sample set of AD cases.

From the six analytes measured in this sample set we only observed a correlation with PS for one of the analytes – clusterin. Several published studies have reported elevated plasma levels of clusterin in AD compared to controls (Thambisetty et al., 2010; Thambisetty et al., 2012; Schrijvers et al., 2011; Jongbloed et al., 2015; Hakobyan et al, 2016). Taken together with our findings, these data suggest that elevated plasma clusterin level is a valid marker for AD.

When focussing in on the IPS more of the markers were associated with this outcome. Clusterin and C1inh demonstrated a statistically significant correlation with IPS, and C1inh and CRP showed a statistically significant difference between those with high and low IPS. It is, perhaps, not surprising that more of the selected analytes were correlated with IPS than PS as these markers were specifically chosen for their roles in inflammation. Both clusterin and C1inh are inhibitors of complement activation; clusterin is a fluid-phase inhibitor of the membrane attack complex, while C1inh inhibits the C1 complex of the classical pathway of complement activation and the MBL/MASP complex of the lectin pathway. Both are suicide inhibitors, consumed in the act of inhibition and both are acute phase reactants; plasma levels in inflammatory disease thus represent a balance between consumption and increased synthesis. CRP is a major acute-phase reactant that can increase 1000-fold or more in plasma concentration in response to inflammation. The finding that CRP levels were only significantly increased when the extremes of the IPS were compared suggests that the observed changes in the complement biomarkers were not solely driven by the acute phase response but reflected other disease processes. The finding that more of the markers measured in this study correlated with IPS than PS is further evidence of their functional relevance and highlights the need for focused/targeted approaches to AD research by stratifying patients using biomarkers and other measurables in order to identify disease subtypes rather than looking at the disease as a whole.

We stress that the study reported here is preliminary and utilises a relatively small sample set; we recognise that only one of the associations observed with these biomarkers survives adjustment for multiple testing (C1inhibitor levels in samples with low IPS versus high IPS) and the other reported findings could be false positives. However, the study presented here does flag the potential usefulness

of testing the association of plasma inflammatory biomarkers with polygenic scores in AD. This study cohort comprised patients with established AD; however, it might be more relevant and informative to test associations of PS and IPS with plasma biomarkers in patients with mild cognitive impairment or early AD, as well as in cognitively normal controls.

To summarize, we have identified associations between plasma inflammatory biomarkers and polygenic scores in AD that further strengthens the link between genetic and biomarker disease predictors. While noting that replication in independent sample sets is essential, our data provide the first evidence that several inflammatory biomarkers, including complement proteins, associate with high polygenic risk scores, increasing confidence that these markers will help disease prediction and patient stratification in AD.

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Plasma Samples

Plasma samples, N=93, from Alzheimer's disease (AD) cases, were obtained from the Genetic and Environmental Risk for Alzheimer's disease (GERAD1) cohort (Harold et al., 2009), recruited by the Medical Research Council (MRC) Genetic Resource for AD (Cardiff University; Kings College London; Cambridge University; Trinity College Dublin). All cases met criteria for probable (NINCDS-ADRDA, DSM-IV) or definite (CERAD) AD. Sample collection was supported by the Medical Research Council (MRC), Wellcome Trust, Alzheimer's Research UK (ARUK), Welsh Assembly Government and Mercer's Institute for Research on Ageing.

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Polygenic Risk Score Calculations

Training Data Set (IGAP):

We thank the International Genomics of Alzheimer's Project (IGAP) for providing summary results data for these analyses. The investigators within IGAP contributed to the design and implementation of IGAP and/or provided data but did not participate in analysis or writing of this report. IGAP was made possible by the generous participation of the control subjects, the patients, and their families. The i–Select chips were funded by the French National Foundation on Alzheimer's disease and Related Disorders. EADI

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Test Data Set (GERAD):

Genetic and Environmental Risk for Alzheimer's disease (GERAD1) Consortium data (Harold et al. 2009) was used as a test data set for polygenic score analysis. GERAD1 consists of 3,333 cases and 1,225 elderly screened controls genotyped at the Sanger Institute on the Illumina 610-quad chip. Study recruitment was via the Medical Research Council (MRC) Genetic Resource for AD, as described for plasma samples above, the Alzheimer's Research UK (ARUK) Collaboration (University of Nottingham; University of Manchester; University of Southampton; University of Bristol; Queen's University Belfast; the Oxford Project to Investigate Memory and Ageing (OPTIMA), Oxford University); Washington University, St Louis, United States; MRC PRION Unit, University College London; London and the South East Region AD project (LASER-AD), University College London; Competence Network of Dementia (CND) and Department of Psychiatry, University of Bonn, Germany and the National Institute of Mental Health (NIMH) AD Genetics Initiative. Data was combined with Illumina HumanHap300 BeadChip data from 608 AD cases and 853 elderly screened controls ascertained by the Mayo Clinics in Jacksonville, Florida and Rochester, Minnesota as well as the Mayo Brain Bank. All AD cases met criteria for either probable (NINCDS-ADRDA, DSM-IV) or definite (CERAD) AD. All elderly controls were screened for dementia using the MMSE or ADAS-cog, were determined to be free from dementia at neuropathological examination or had a Braak score of 2.5 or lower. A total of 5770 population controls were included in GERAD GWAS including the 1958 British Birth Cohort (1958BC) (http://www.b58cgene.sgul.ac.uk), NINDS funded neurogenetics collection at Coriell Cell Repositories (Coriell) (see http://ccr.coriell.org/), the KORA F4 Study, Heinz Nixdorf Recall Study and ALS Controls. The ALS Controls were genotyped using the Illumina HumanHap300 BeadChip. All other population controls were genotyped using the Illumina HumanHap550 Beadchip.

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Tables and Figures

Table 1: In-house ELISAs

Analyte	Capture antibody	Detection antibody	Standard curve	Plasma dilution
CR1	1 µg/ml RP anti-CR1	1 µg/ml MM HRP labelled anti-human CR1	50, 25, 12.5, 6.25, 3.125, 1.565, 0.78, 0 ng/ml	1:2
C9	1 µg/ml MM anti-C9	1 µg/ml RP HRP labelled anti-human C9	200, 100, 50, 25, 12.5, 6.25, 3.125, 0 ng/ml	1:2000
C1 inhibitor	1 µg/ml MM anti-C1 inhibitor	1 µg/ml RP HRP labelled anti-human C1 inhibitor	values 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0 ng/ml	1:16,000
TCC	5 µg/ml MM anti-TCC	2 µg/ml MM HRP labelled anti-human TCC	15000, 7500, 3750, 1875, 937.5, 468.75, 234.375, 0 ng/ml,	1:32

Abbreviations: *MM, mouse monoclonal antibody; RP, rabbit polyclonal antibody*

Table 2: Means and range of protein levels in AD cases

analyte	n	Range	mean	SD
CR1	93	6.74 – 32.16 ng/ml	15.97	5.26
C9	90	31.78 – 158.38 µg/ml	78.10	28.54
C1inh	91	74.23 – 340.09 µg/ml	188.85	58.46
CLU	91	160.15 – 414.49 µg/ml	279.80	55.06
CRP	93	0.18 – 40.61 µg/ml	2.89	5.23
TCC	91	63.35 – 234.14 ng/ml	137.04	34.29

Table 3: Comparison of biomarker levels between cases with highest PS and cases with lowest PS
Boldface indicates statistically significant p values (p ≤0.05)

	PS<mean-1SD			PS>mean+1SD			
analyte	Score cut off	N cases	Analyte mean	Score cut off	N cases	Analyte mean	p (effect)
CR1	-0.34	14	15.10	1.55	12	18.16	0.19
C9	-0.34	13	83.65	1.55	11	73.88	0.49
C1inh	-0.33	13	196.03	1.55	12	206.23	0.82
CLU	**-0.34**	**14**	**263.73**	**1.55**	**11**	**314.08**	**0.03 (1.24)**
CRP	-0.34	14	7.91	1.55	12	1.48	0.13
TCC	-0.36	14	135.26	1.55	12	142.27	0.37

Table 4: Comparison of biomarker levels between cases with highest IPS and cases with lowest IPS
Boldface indicates statistically significant p values (p ≤0.05)

	IPS<mean-1SD			IPS>mean+1SD			
analyte	Score cut off	N cases	Analyte mean	Score cut off	N cases	Analyte mean	P (effect)
CR1	-0.63	10	15.8	1.28	14	15.02	0.71
C9	-0.64	10	66.55	1.21	14	83.11	0.21
C1inh	**-0.63**	**10**	**154.44**	**1.24**	**15**	**211.8**	**0.008 (1.55)**
CLU	-0.64	10	248.83	1.2	14	286.47	0.08
CRP	**-0.63**	**10**	**0.75**	**1.29**	**14**	**4.99**	**0.02 (14.2)**
TCC	-0.63	10	130.85	1.3	14	135.84	0.93

Figure 1: correlations between biomarkers and AD PS

Scatter plots of each biomarker against the normalised AD PS. The red line is the linear regression line. P-values for test of correlation: CR1 p=0.12, C9 p=0.30, C1 inh p=0.58, CLU p=0.05, CRP p=0.23, TCC p=0.63. The correlation coefficient for CLU is 0.20.

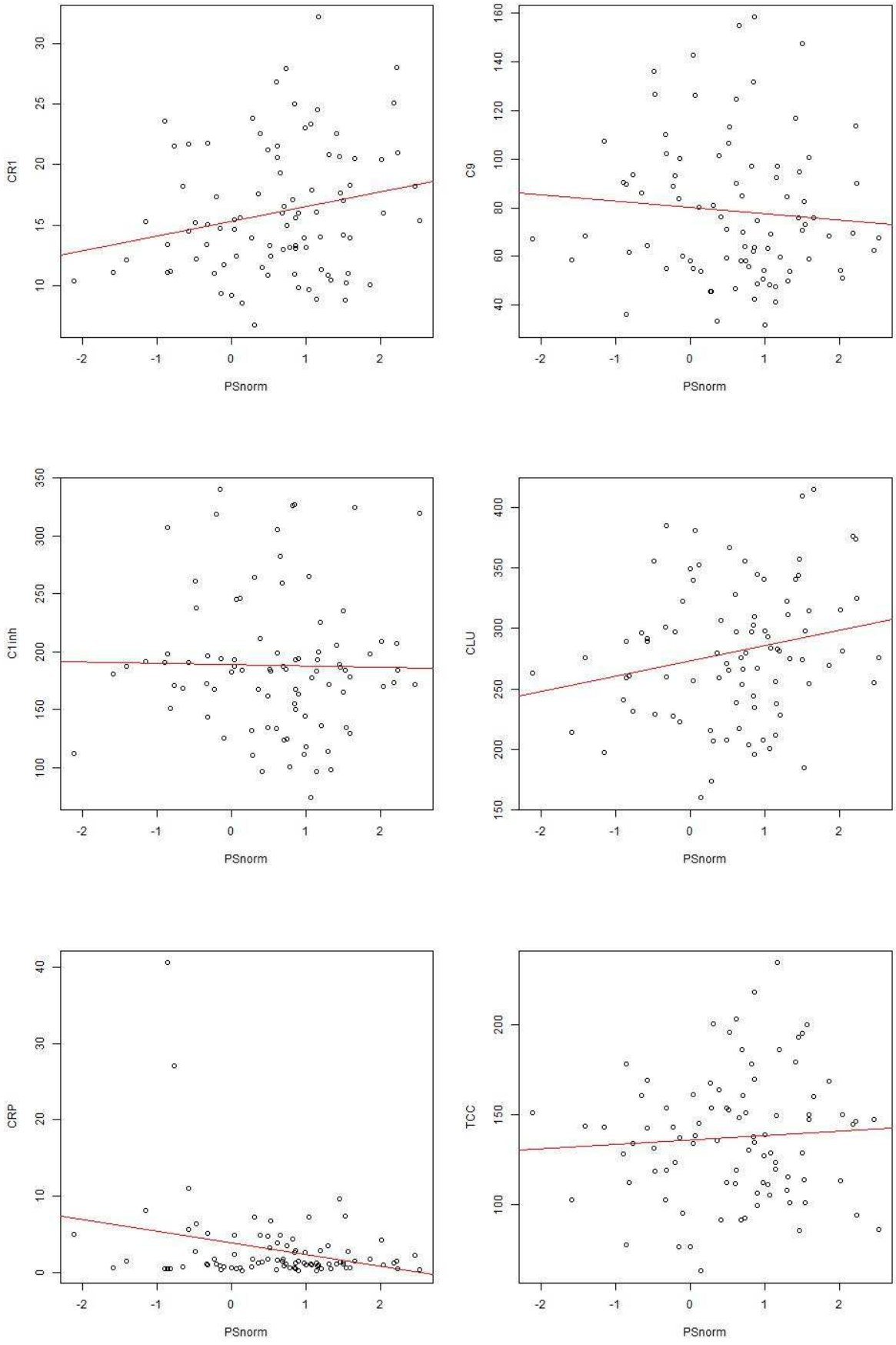

Figure 2: Box plots comparing biomarker levels in AD cases with low and high PS scores

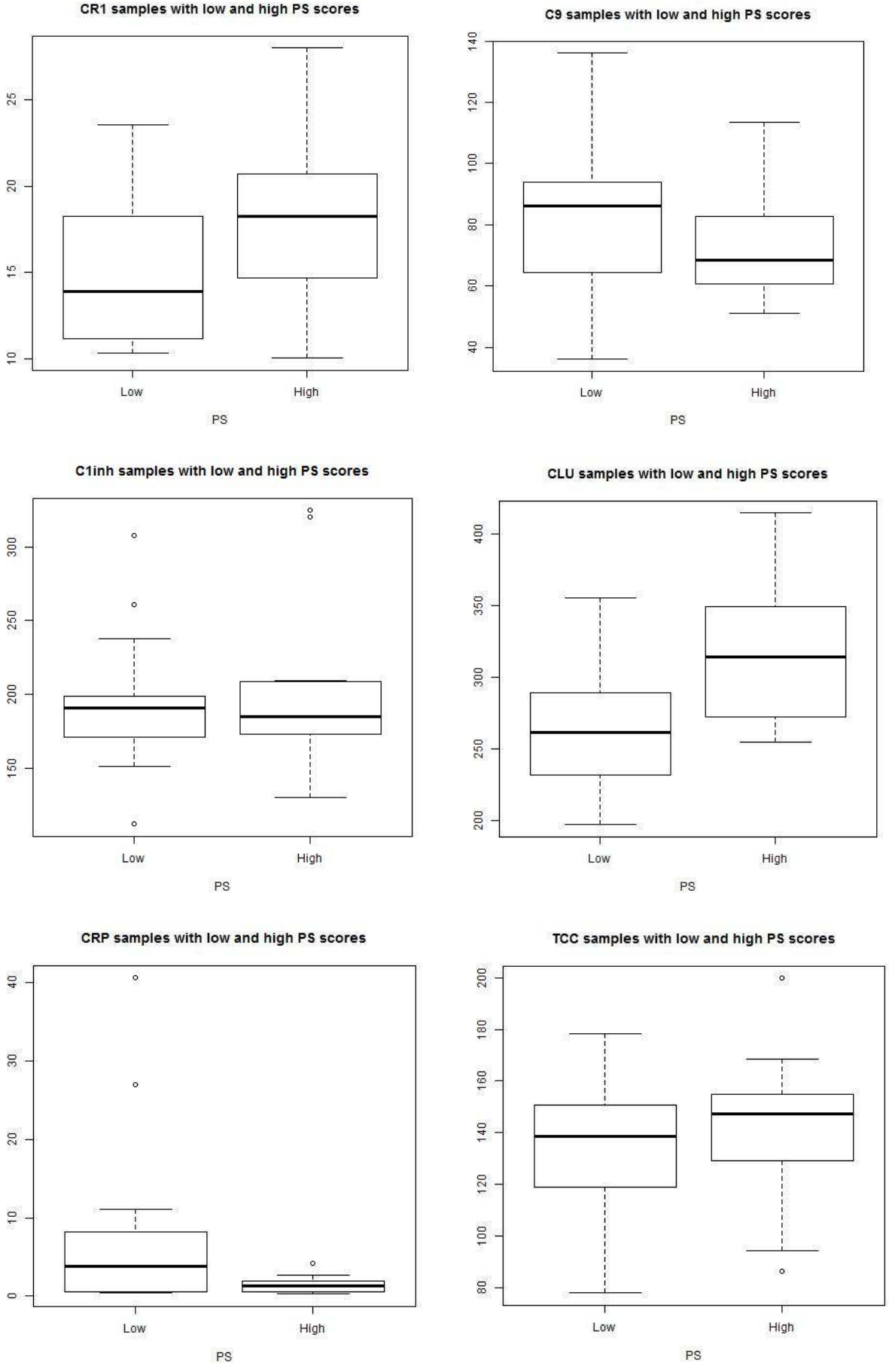

Figure 3: correlations between biomarkers and AD IPS

Scatter plots of each biomarker against the normalised AD IPS. The red line is the linear regression line. . P-values for test of correlation: CR1 p=0.84, C9 p=0.308, C1 inh p=0.05, CLU p=0.02, CRP p=0.13, TCC p=0.65. The correlation coefficient for C1 inh is 0.22 and for CLU is 0.25.

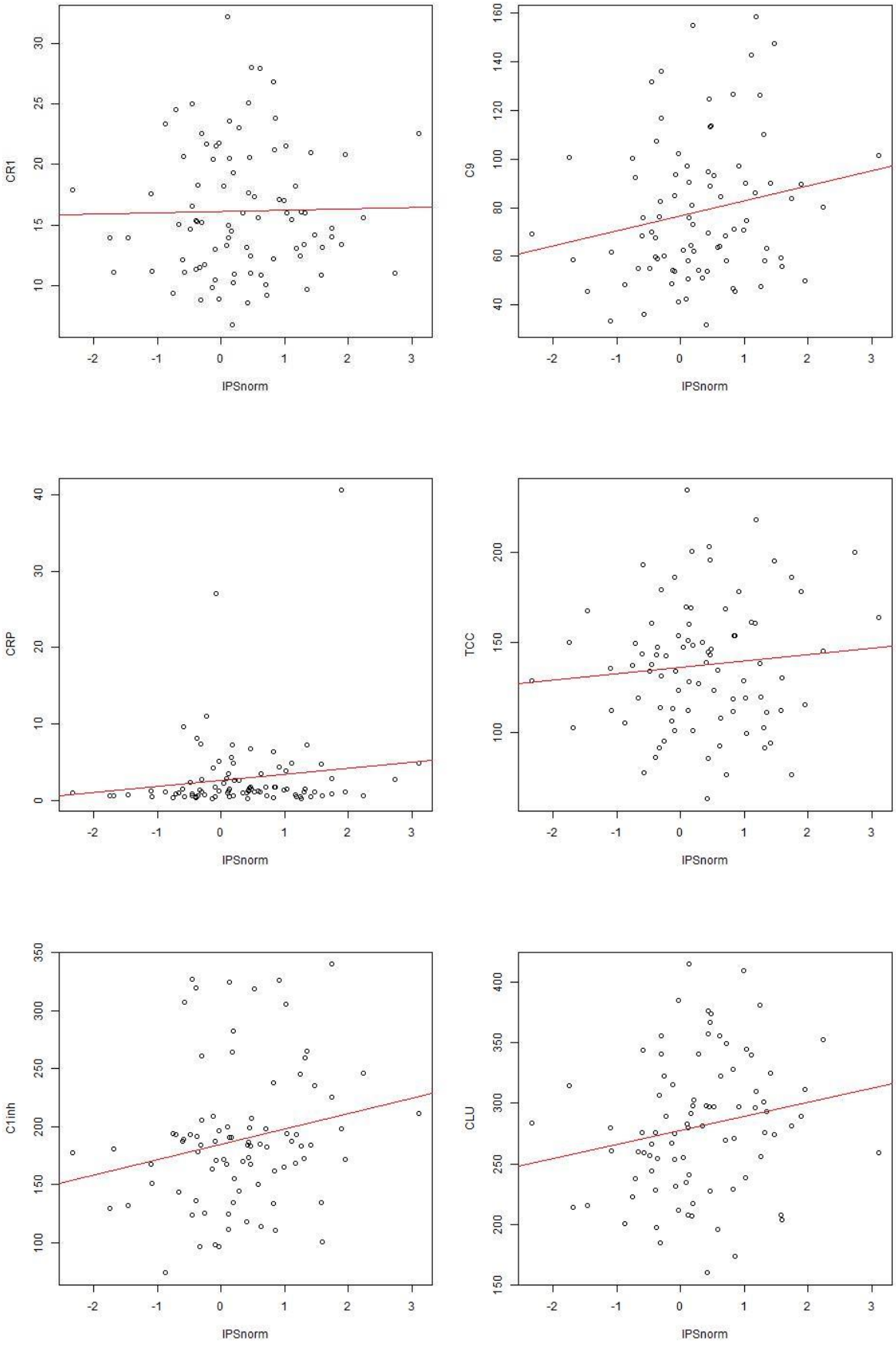
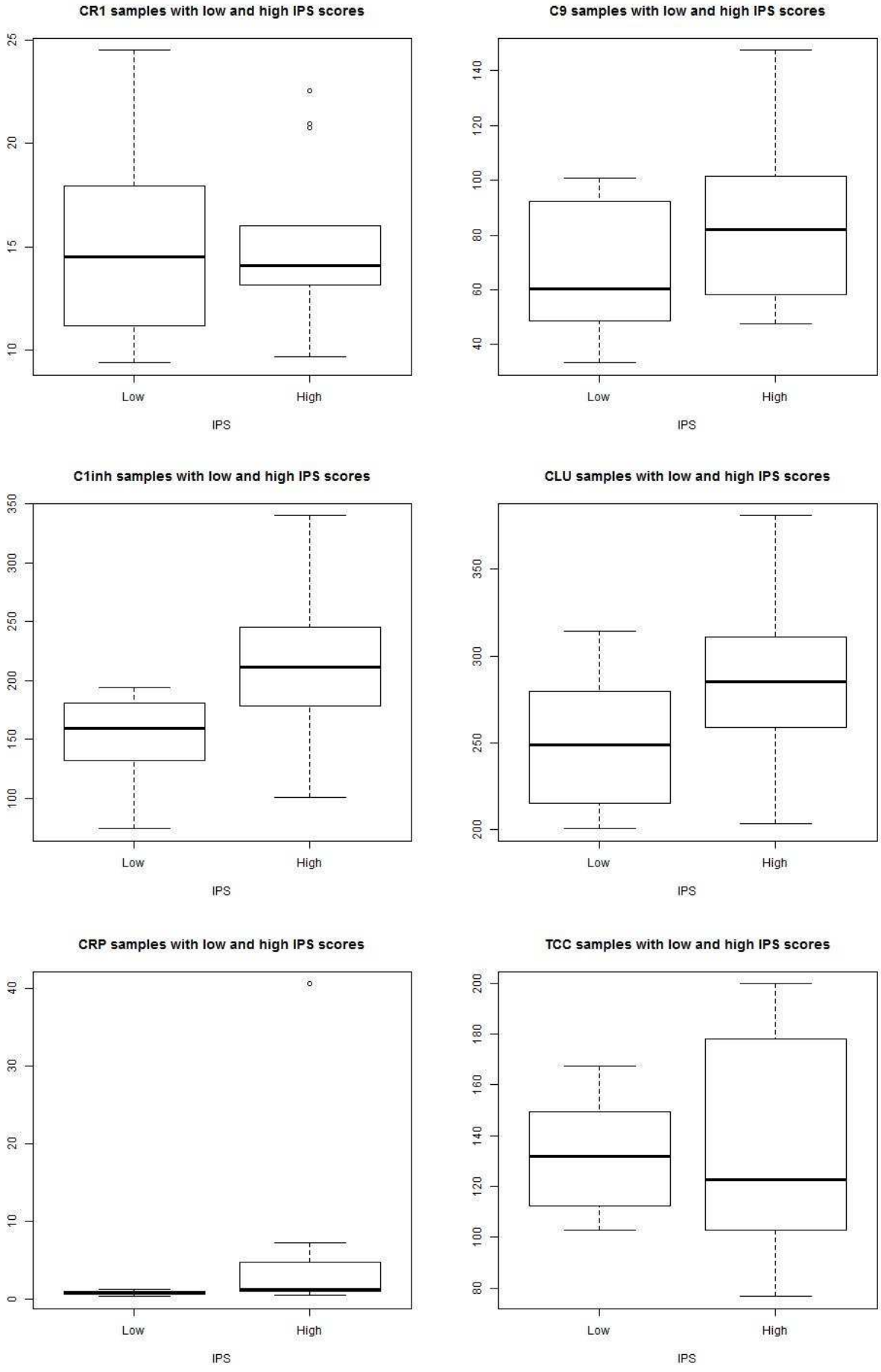
Figure 4: Box plots comparing biomarker levels in AD cases with low and high IPS scores