Biomarkers, 2008; 13(6): 618–636

informa
healthcare

The discovery and early validation of novel plasma biomarkers in mild-to-moderate Alzheimer's disease patients responding to treatment with rosiglitazone

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Abstract

Recent advances in clinical, pathological and neuroscience studies have identified disease-modifying therapeutic approaches for Alzheimer's disease that are now in clinical trials. This has highlighted the need for reliable and convenient biomarkers for both early disease diagnosis and a rapid signal of drug efficacy. We describe the identification and assessment of a number of candidate biomarkers in patients with Alzheimer's disease and the correlation of those biomarkers with rosiglitazone therapeutic efficacy, as represented by a change in the Alzheimer's Disease Assessment Scale-Cognitive (ADAS-Cog). Plasma from 41 patients with Alzheimer's disease were analysed by open platform proteomics at baseline and after receiving 8 mg rosiglitazone for 24 weeks. From a comparison of protein expression following treatment with rosiglitazone, 97 proteins were observed to be differentially expressed with a p-value <0.01. From this analysis and comparison to recently published data from our laboratory, a prioritized list of 10 proteins were analysed by immunoassay and/or functional assay in a wider set of samples from the same clinical study, representing a rosiglitazone dose response, in order to verify the changes observed. A number of these proteins appeared to show a correlation with change in ADAS-Cog at the higher treatment doses compared with the placebo. Alpha-2-macroglobulin, complement C1 inhibitor, complement factor H and apolipoprotein E expression showed a correlation with ADAS-Cog score at the higher doses (4 mg and 8 mg). These results are discussed in light of the pathology and other recently published data.

Keywords: *Alzheimer's disease, apolipoprotein E, biomarkers, complement C1 inhibitor, complement factor H, plasma*

(Accepted 2 September 2008)

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500802445199

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by cognitive deficits including loss of memory, judgement, comprehension and deterioration in global function. The incidence of AD is projected to increase significantly as the population ages and is estimated to reach >41 million sufferers by the year 2020. It is believed that disease-modifying therapies will be more beneficial if they can be applied very early in the progress of the disease, before clinical signs and symptoms are recognized (Lovestone et al. 2006, Vardy et al. 2006). Currently acetylcholinesterase inhibitors are widely used in mild to moderate patients; however they are not efficacious in all patients and where they do show therapeutic value they lose efficacy over time. Additional new therapies are therefore required to satisfy the unmet medical need. Recently, the results of a phase IIb clinical trial (GSK Study Protocol AVA100193) of the peroxisomal profilerator-activated receptor γ (PPAR γ) agonist rosiglitazone were published (Risner et al. 2006). Mild to moderate AD patients received either placebo, or 2, 4 or 8 mg rosiglitazone for 24 weeks. The primary efficacy endpoints included the AD Assessment Scale-Cognitive (ADAS-Cog). Although no statistically significant difference was observed in change from baseline in ADAS-Cog at 24 weeks between the placebo and the rosiglitazone groups, there was a significant interaction between ADAS-Cog and apolipoprotein E (APOE) ε4 allele status. The APOE gene has been associated with AD for a number of years, with the presence of the APOE ε4 allele a significant risk factor for developing the disease (Roses 1996). Exploratory analysis of study AVA100193 indicated that patients without the APOE ε4 allele showed a significant improvement in ADAS-Cog at the higher (8 mg) dose of rosiglitazone, whereas patients who carried an APOE ε4 allele showed no improvement and some decline was noted.

At the same time that this clinical study was underway, the authors completed a cross-sectional proteomic analysis of plasma from patients with mild to moderate AD and matched controls, identifying a number of proteins which may be potential biomarkers of AD (Cutler et al. 2008). The aim of the present study was to expand these initial findings, by performing a proteomic analysis of plasma from the patients samples in the phase IIb clinical trial of rosiglitazone, cross-compare the data with existing information on AD biomarkers, and subsequently employ specific assays to identify novel markers of rosiglitazone drug efficacy in an AD patient population.

Materials and methods

Subjects and samples

The study population has been described previously in detail (Risner et al. 2006). Briefly, 687 patients were screened at 67 centres in Europe and New Zealand, and 511 patients were in the intention-to-treat population. All subjects were Caucasian, two-thirds were female, the mean age was 70.7 (SD 8.55) years and the mean MMSE score at baseline was 21.2 (SD 3.15). Prior to patient participation both formal approval of the study and informed consent were obtained from the patient and/or caregiver. Clinical information was archived and anonymized for the progression of the proteomic analysis and follow-up assays.

EDTA plasma was obtained from Alzheimer's patients in a 24-week double-blind, randomized, placebo-controlled dose-ranging phase IIb study designed to investigate the effects of rosiglitazone (extended release tablets) on cognition in subjects with mild

to moderate AD. For the proteomics study a subset of patients from the original phase IIb study were analysed, based on provision of appropriate consent. Table I highlights the samples obtained for proteomics. Forty-one patients were analysed in the initial proteomics study, representing the 8-mg treatment group for whom the APOE genotype was determined (Table I). Analysis of APOE genotypes indicated 17 carried at least one copy of the APOE ε4 allele, whereas 24 carried no copies of the APOE ε4 allele. Each of the 41 patient samples was analysed at baseline (week 0) and week 24.

Proteomics

EDTA plasma samples were thawed at 37°C and centrifuged at 14 000*g* for 10 min at room temperature. Albumin depletion of the supernatants was immediately carried out using a 96-well plate format packed with 0.25 g settled Purabead®6XL Blue SA High Load gel (Prometic BioSciences Inc., Cambridge, UK), and washed with 10 column volumes of 50 mM phosphate-buffered saline, pH 7.4. The depleted material, contained in the flow through and first four phosphate-buffered saline washes, were concentrated using a 5 kDa MW cut-off Polyethersulphone Vivaspin 6 centrifugal filtration device (Vivascience, Epsom, UK). Albumin-bound protein was eluted using four column volumes of 8 M urea and concentrated as described for albumin-depleted protein. The protein concentration was then measured using a bicinchoninic acid assay (Sigma, Poole, UK) with a bovine serum albumin standard. Albumin depletion was confirmed using a bromocresol purple assay (Pinnell & Northam 1978).

Samples containing a total of 400 μg protein from albumin-depleted plasma were diluted into isoelectric focusing sample buffer containing 8 M urea, 4% (w/v) CHAPS, 65 mM DTT, 0.5% pH 3-10NL IPG buffer (GE Healthcare, Little Chalfont, UK) and subjected to isoelectric focusing on 24-cm, pH 3-10NL immobilized pH gradient strips (Immobiline™ DryStrip; GE Healthcare) for 84 000 Vh using a Multiphor II flatbed electrophoresis system (GE Healthcare) according to the manufacturer's instructions. Focused strips were subjected to reduction and alkylation, in a buffer comprising 0.375 M Tris–HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (w/v) glycerol, 2% (w/v) dithiothreitol, for 15 min at 37°C with shaking, followed by 15 min at 37°C, with shaking, in the same buffer with 2.5% (w/v) iodoacetamide replacing the

Table I. Subject characteristics. A total of 193 patients provided 386 samples representing samples at baseline (week 0) and 24 weeks of treatment. Patients received either placebo, 2 mg, 4 mg or 8 mg rosiglitazone. Appropriate consent was obtained for genetic testing and APOE genotype was determined to identify patients who possess at least one APOE ε4 allele (APOE ε4+) and those in which the allele is absent (APOE ε4 −). Those patients for which genotyping consent was not obtained are classified as 'unknown'. A total of 384 samples were tested during the specific assay phase. For the proteomics (2DE) analysis 41 patients were analysed from the 8 mg dose group at both baseline and 24 weeks of treatment.

	Week 0			Week 24		
Dose	ApoE ε4+	ApoE ε4 −	Unknown	ApoE ε4+	ApoE ε4 −	Unknown
Placebo	17	16	6	17	16	6
2 mg	14	24	10	14	24	10
4 mg	17	24	12	17	24	12
8 mg	17	24	12	17	24	12

dithiothreitol. Reduced and alkylated strips were subjected to SDS-PAGE in the second dimension on 12% (w/v) acrylamide slab gels ($20 \times 25 \times 0.15$ cm), essentially as described by Bjellqvist et al. (1993) and detailed on the Swiss 2D-PAGE database (http://expasy.ch/ch2d). Proteins were stained with SYPRO Ruby (Invitrogen Ltd., Paisley, UK) and destained in 10% (v/v) ethanol, 7% (v/v) acetic acid. Digital images (16-bit) were acquired on Fuji FLA5000 imaging systems, employing excitation at 473 nm with a 575 nm LP filter. Individual protein features were detected, matched and quantified using Progenesis Discovery software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Gel cores of spots were excised using a Kcore spot-cutting robot (KBiosystems, Basildon, Essex, UK).

Protein identifications were carried out essentially as described previously (White et al. 2003). Briefly, polyacrylamide gel cores of protein spots with altered expression profiles were subjected to *in situ* tryptic digestion using a MassPrep liquid handling system (Waters, Manchester, UK). The digests were analysed by nanoscale capillary liquid chromatography tandem mass spectrometry (LC/MS/MS) using a Waters CapLC and Stream Select Module to deliver a flow of 5 μl min^{-1}, split to approximately 200 nl min^{-1}. An LC Packings μ-Precolumn, C18 PepMap 100 (Dionex, The Netherlands) guard column trapped the peptides prior to separation on a NAN75-15-03-C18-PM, PepMap 100 column, 75 μm ID 15 cm. Peptides were back-flushed from the guard column onto the analytical column at 200 nl min^{-1} and eluted with a gradient of acetonitrile. Mass spectrometric information was obtained using an orthogonal acceleration quadrupole-Tof mass spectrometer (Q-Tof Ultima API; Waters, Manchester, UK) equipped with a Z-spray source for nanoflow analysis and operated in V-mode at 7500–8000 resolution at FWHM (full width half maximum). The instrument was calibrated externally with Glu-Fib peptide having a lock mass of 785.8426 Da to achieve a typical mass accuracy of 5 ppm. Chromatographic peak width at baseline was maintained at 20–30 s. Data-directed analysis was carried out, using MassLynx data acquisition software (Waters, Manchester, UK) where automatic MS/MS was acquired on the eight most intense, multiply-charged precursor ions in the m/z range 400–1500. MS/MS data were acquired over the m/z range 50–1975 using a scan duration of 0.92 s with an interscan delay of 0.08 s. Raw data were smoothed (Savitzky-Golay, 3 channel, 2 smooths) and centroided (4–6 channels, 80 centroid top%, using areas). Peak lists generated from LC/MS/MS fragmentation data were then searched against an in-house non-redundant protein database using the Mascot search engine program (Matrix Science, UK). Mass tolerance was set at 0.25 Da in the MS mode and 0.1 Da in the MS/MS mode and searches incorporated variable modifications including phosphorylation, carbamidyl-methylation, and methionine oxidation. Outputs from Mascot were monitored and evaluated by the operator.

Enzyme-linked immunoassays, Western blots and functional assays

Separate aliquots of plasma samples for all doses in Table I (384 samples) were obtained and stored in aliquots at -80^{0}C until analysis. Aliquots were thawed at room temperature and stored for a maximum of 1 h before assay. The appropriate dilution was prepared after gentle mixing of the sample and then assayed by enzyme-linked immunoassays (ELISA), Western blot or functional assay according to

manufacturer's instructions and/or in-house evaluation. The following were determined by commercial ELISA kits. Alpha-2-macroglobulin (AssayPro, MO, USA), Angiotensin 1 (Phoenix Pharmaceuticals, Burlingame, CA, USA), ApoE (MBL, Naka-ku Nagoya, Japan), apolipoprotein J/Clusterin (Science Equip Ltd, York, UK), complement factor 1 inhibitor (Technoclone, Surrey, UK), serpin F1/pigment epithelium-derived factor (PEDF; Bioproducts MD, Middletown, MD, USA) and transthyretin/pre-albumin (Immunodiagnostik, Bensheim, Germany).

The following commercial antibodies were used to develop sandwich ELISAs. Lumican (R&D Systems, Europe, Abingdon, UK, goat polyclonal unconjugated and biotinylated antibodies). The following proteins were assayed by Western blot using commercial antibodies. Complement factor C1 inhibitor (sheep polyclonal antibody from Biodesign) and serpin F1/PEDF (mouse monoclonal from Chemicon, Berkeley, CA, USA).

A functional (activity) assay for serum paraoxonase 1 (Enzcheck Paraoxonase fluorescent assay) was obtained from Invitrogen

In all cases, the evaluated assays were used to determine the plasma concentration of the protein of interest for five normal male and five normal female plasma samples in order to determine baseline values. The parameters that were assessed in terms of assay performance included specificity, linearity, reproducibility (interassay and intra-assay). A coefficient of variation of less than 30% was considered acceptable.

Statistics

Prior to statistical analysis, spot volume data from 2DE were mean normalized and then $\log_{10}$ transformed. Employing a general linear model, using $\log_{10}$ spot volume and accounting for patient, time and treatment response effects (improvement in ADAS-Cog), the change in 2DE gel spot level in relation to rosiglitazone treatment time and associated p-value were calculated. In addition, to identify spots whose expression were differentially regulated dependent upon patient treatment response, time versus treatment response interaction effects were also determined. All statistical analyses were performed using SAS v9.

For individual assays using microtitre plates, the patient samples were randomly allocated to each plate following input from the statistician. The total number of patients from each treatment group was divided between the plates to ensure there was a similar representation of each treatment group across the plates. Then the patients from each treatment group were randomly allocated to a plate. Baseline and week 24 samples from each patient were placed on the same plate to ensure the within patient variability was not confused with the variability between plates.

Prior to the statistical analysis, the read-out values from the protein assays were normalized by dividing by the plate median to correct for variability between plates. Then for each protein measured, and each treatment group in turn, the change in protein level (week 24 minus baseline) was calculated and modelled against the change in ADAS-Cog score to explore the correlation between both variables. Each correlation was performed using a linear regression analysis and the r^2 value along with its p-value was used to assess the strength of the correlation. A more comprehensive analysis was also performed adjusting for background factors such as genotype (presence or absence of an ApoE ε4 allele) status, age and gender. The baseline ADAS-Cog score was also considered as an extra covariate in the analysis to correct for differences between

patients with varying levels of disease severity. The assumptions of these analyses were tested using residual plots. From the outset, it was recognized that finding a potential correlation would be difficult due to the complex nature in which the data were generated and the limited sample size. With this in mind, levels of significance were set at 0.10. The statistical analyses were performed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and Statistica 6.1.409 (StatSoft Inc. 1983–2003).

Results

Proteomics analysis was performed on plasma from the highest (8 mg) dose group from a phase IIb clinical study designed to assess the efficacy of rosiglitazone in mild-to-moderate AD (Risner et al. 2006). Forty-one patients with appropriate consent were selected for proteomic analysis, providing plasma at baseline ($t=0$) and at 24 weeks. Eighty-two plasma samples were therefore depleted of albumin as described in materials and methods. The albumin-depleted fractions were analysed by 2DE.

Statistical analysis was performed to identify proteins which showed a potential correlation with rosiglitazone treatment as defined by an intrapatient analysis of protein expression at baseline and 24 weeks post-treatment. Ninety-seven spots were seen to show a change in expression with $p<0.01$, which was notably larger than the 16 calculated to appear by statistical chance. These spots were identified by mass spectrometry giving 55 proteins. The identities of these proteins with fold changes and peptide sequences are given in a Supplementary Table. In some cases the same protein was identified in multiple spots. An example is given in Figure 1 where a number of different forms of ApoE were identified, some showing a decrease in expression and others an increased expression. These included lower molecular weight forms (e.g. spot 1695) believed to be truncated forms of ApoE. The truncation of ApoE in spot 1695 was consistent with the sequence coverage as illustrated in Figure 1. Other proteins observed to display altered expression included nine additional apolipoproteins and 13 proteins associated with the complement cascade. Of the 55 proteins identified in this study 38 had been previously identified in our laboratory as being associated with AD in a cross-sectional analysis of plasma from mild-to-moderate Alzheimer's patients compared with age-matched controls (Cutler et al. 2008).

In addition, an analysis of potential correlation with changes from baseline in ADAS-Cog score was undertaken. Five spots relating to four proteins (alpha 2 antiplasmin, alpha-1-antichymotrypsin, apolipoprotein J and ApoE) showed a correlation, with a p-value <0.01. This was less than the number which may have been expected due to statistical chance, possibly reflecting the relatively small number of samples within a phase IIb clinical study of this nature. As the effects relating to rosiglitazone efficacy had been previously shown to be related to APOE ε4 allele status a further analysis was undertaken to define any proteins whose expression may be dependent upon the APOE ε4 allele. Although five spots showed an APOE ε4-related effect as defined by p-value <0.01 (most notably ApoE itself) this was again less than the number of changes which may have been expected by chance.

Ten proteins were selected for further assessment by either immunoassay or functional assay based on statistical significance and comparison to previous studies (Table II). For each assay the change in protein expression or activity was correlated with the change from baseline in ADAS-Cog score over the 24-week period. Analysis was performed on all 384 proteomic samples available in the placebo, 2 mg, 4 mg or

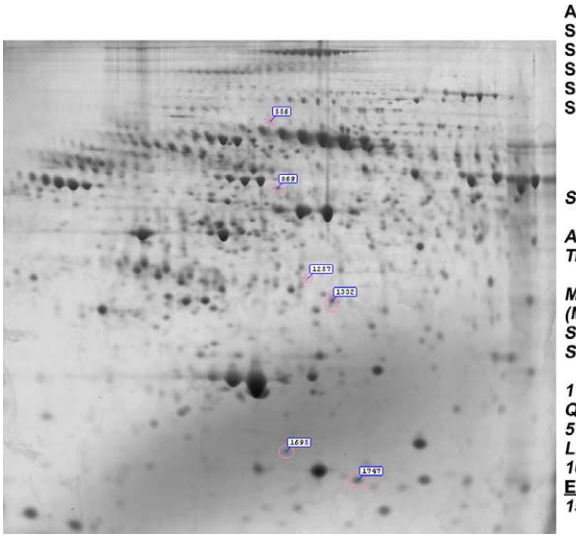

Figure 1. Image of plasma analysed by 2DE on pI 3-10 gel. Proteins are separated by pI in the horizontal dimension with the acidic pI to the left and the basic pI to the right. Proteins are separated by molecular weight in the vertical dimension with high-molecular weight proteins at the top and low-molecular weight proteins towards the bottom. The gel has been annotated with ApoE for illustrative purposes. Six spots were found to contain ApoE as illustrated (spots 1332, 1257, 869, 556, 1747 and 1695) with fold change relative to baseline (t = 0). The sequence coverage is given for the low-molecular weight, 'truncated' ApoE in spot 1695. Peptides identified are shown in underlined bold type.

8 mg dose group (Table I). For this analysis $p < 0.10$ was considered to be statistically significant. Interestingly four proteins showed a correlation with ADAS-Cog score in at least one of the higher dose groups (4 mg or 8 mg) (Figure 2). These proteins were complement C1 inhibitor, alpha-2 macroglobulin, ApoE and complement factor H assayed by Western blotting. While the clinical efficacy of rosiglitazone was noted to be dependent on APOE allele status, with only the ApoE ε4-negative subjects showing a significant improvement on ADAS-Cog, our data suggest that the plasma proteomic biomarker correlations are not dependent upon gender or APOE allelic status, with statistical correction for these factors having negligible impact upon the results. Interestingly, although a correlation was noted between protein expression and change in ADAS-Cog for the specific assays of the four proteins above, a pair-wise comparison of the protein levels before and after treatment did not show a significant *p*-value by Student's *t*-test (data not shown). While such a drug-specific effect may be seen in larger studies these data may support the conclusion that the observed correlations are due to a link between the biomarkers and cognitive improvement rather than with drug treatment.

Figure 3 illustrates a representative gel from the Western blot analysis of complement C1 inhibitor. Band 1 was found to show a correlation with ADAS-Cog at the 4 mg dose, whereas the lower molecular weight band (band 3) showed a correlation with ADAS-Cog at the 8 mg dose. The association of a higher molecular weight form with AD is consistent with previous data from the cross-sectional analysis (Cutler et al. 2008). The mass spectrometry identifications of the 2DE spots in the current study indicate the presence of complement C1 inhibitor and complement C1r.

Table II. ELISA and Western based analysis of specific plasma proteins. Nine proteins were analysed at each dose group by either ELISA and/or Western blot. Where more than one band was identified by Western blot these are listed separately. For example, three bands were quantified for complement C1 inhibitor as shown in Figure 3. Serum paraoxonase 1 was assayed for functional activity. Each protein was statistically analysed for correlation with drug efficacy as defined by change in ADAS-Cog score and recorded as both r^2 and p-value. A p-value <0.10 was considered significant and these data are shown in bold italics.

		Dose							
		Control		2 mg		4 mg		8 mg	
Protein	Assay platform	r^2	p-Value	r^2	p-Value	r^2	p-Value	r^2	p-Value
Alpha-2-macroglobulin	Western band 1	−0.012	0.933	0.079	0.616	0.140	0.356	0.22	0.133
	Western band 2	−0.166	0.257	0.006	0.970	***0.312***	***0.039***	−0.02	0.923
	Western band 3	−0.137	0.355	0.015	0.920	***0.281***	***0.058***	0.04	0.785
	ELISA	−0.072	0.676	−0.032	0.847	0.172	0.247	−0.20	0.187
Apolipoprotein E	Western	−0.054	0.717	−0.113	0.466	***0.261***	***0.070***	0.073	0.613
Apolipoprotein J	Western	−0.043	0.800	−0.115	0.447	0.009	0.949	−0.141	0.325
Complement C1 inhibitor	Western band 1	−0.025	0.887	0.061	0.685	***−0.358***	***0.012***	0.130	0.362
Complement C1 inhibitor	Western band 2	0.084	0.608	0.076	0.616	−0.203	0.158	0.166	0.245
Complement C1 inhibitor	Western band 3	0.084	0.610	0.097	0.518	−0.078	0.891	***0.235***	***0.098***
Complement C1 inhibitor	ELISA	−0.061	0.716	***0.275***	***0.062***	−0.018	0.902	−0.133	0.357
Complement factor H	Western	−0.195	0.248	−0.032	0.843	0.014	0.345	***−0.278***	***0.061***
Pigment epithelium derived factor	Western	−0.169	0.300	−0.014	0.920	−0.122	0.403	0.134	0.354
Pigment epithelium derived factor	ELISA	0.164	0.318	0.064	0.668	−0.163	0.258	−0.077	0.589
Serum paraoxonase 1	Functional	−0.211	0.150	0.052	0.735	0.210	0.147	−0.085	0.558
Lumican	ELISA	−0.281	0.081	−0.049	0.740	0.1603	0.256	0.063	0.633
Transthyretin	ELISA	−0.154	0.349	0.118	0.428	0.153	0.280	0.190	0.180
Alpha-1-anti-chymotrypsin	ELISA	0.193	0.239	0.152	0.303	0.080	0.582	0.002	0.883

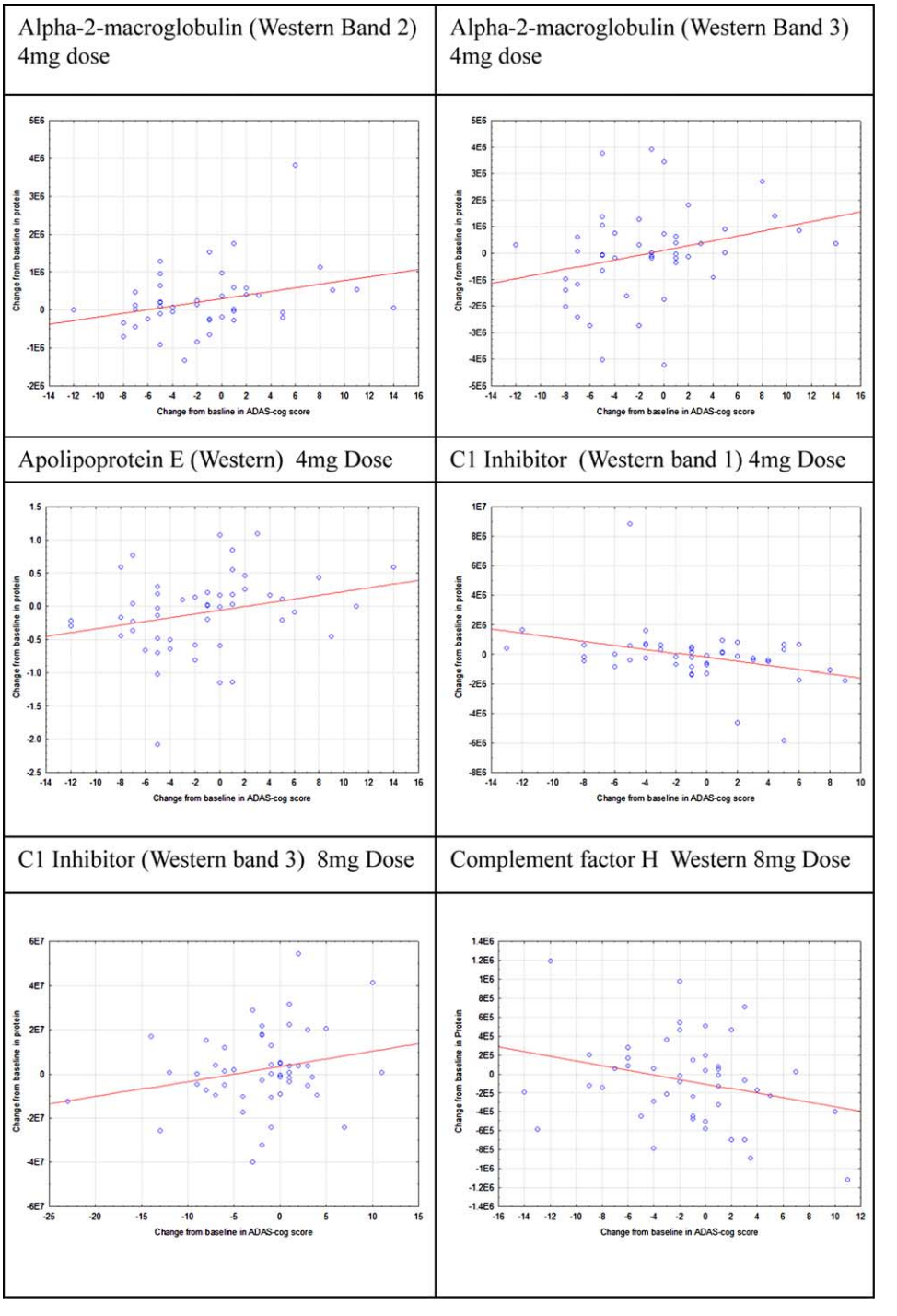

Figure 2. Statistical analysis of the correlation of individual proteins with change in baseline ADAS-Cog. Correlations are shown for those proteins showing significant correlations in Table II.

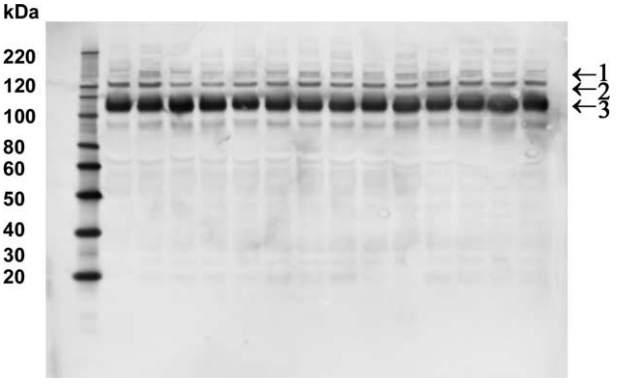

Figure 3. Western blot analysis of complement C1 inhibitor. The first lane (left) represents molecular weight markers as labelled. Subsequent lanes represent statistically randomized plasma samples from patients separated by molecular weight on SDS PAGE and immunoblotted for complement C1 inhibitor as described in Materials and methods.

It is therefore possible that this higher molecular weight band represents a complex of the two proteins, which is known to form in plasma (Davis et al. 2007). A lowering in such a complex, seen in AD would be consistent with reduced inhibition of the classical complement pathway. This is being investigated further in our laboratory.

While the correlations observed did not show the ideal dose response and effects in most cases did not replicate, in both the 4 mg and 8 mg dose groups, it is noteworthy that the significant correlations were observed largely in the 4 mg and 8 mg dose groups and no correlation was seen in the placebo group. When considering the nature of a phase IIb trial and the finite statistical power which is available, especially in a heterogeneous human population, the data obtained seem to indicate a potential correlation between the levels of these proteins and the drug efficacy.

Discussion

Complement C1 inhibitor is a regulator of the complement cascade system not only regulating key members of the cascade including complement C1r and complement C1s but also regulating other critical peripheral systems such as Factor XIIa, kallikrein and plasmin (Davis et al. 2007). The levels of complement C1 inhibitor have previously been noted to be stimulated in AD brains, with increased inactivated levels noted in activated astrocytes and dystrophic neurites in areas surrounding AD plaque (Veerhuis et al. 1996, 1998). Our laboratory recently identified a high-molecular weight form of complement C1 inhibitor, consistent with a complex of complement C1 inhibitor and complement C1r, which was lowered in AD compared with age-matched controls (Cutler et al. 2008). This molecular weight form is coincident with band 1 in the current study (Figure 3). Western blot analysis of the SDS PAGE was also consistent with the presence of both complement C1 inhibitor and complement C1r in Band 1 (data not shown). It is noteworthy that in our current study band 1 shows a negative correlation with ADAS-Cog at the 4 mg dose ($p = 0.012$; Table II). A negative change from baseline ADAS-Cog represents an improvement in cognition and as such a negative correlation with change in protein expression would represent an improvement in cognition with increasing protein. Additionally, the spot on the

2DE gel which was consistent with this higher molecular weight form was seen to increase on treatment with rosiglitazone with a fold change of 1.3. These data together are consistent with an increase in the higher molecular weight form of complement C1 inhibitor following treatment with rosiglitazone. The observation that band 3 on the Western blot appears to decrease with clinical improvement may suggest that on treatment with rosiglitazone there is a shift from a free form of complement C1 inhibitor (band 3) to a complex (band 1). Such a lowering of the free form of complement C1 inhibitor and an increase in the complexation with complement C1r following treatment may represent a move towards restoration of the control of the classical complement activation. While further work would be needed to conclusively establish this, our data are consistent with a decrease in complexation and therefore an increase in complement activation during AD and a reversal on therapy. Other studies have shown that the levels of complement C1 inhibitor are lowered in the Alzheimer brain (Walker et al. 1995) and while several complement factors are elevated in the Alzheimer brain in response to proinflammatory cytokines, there is no concomitant increase in complement C1 inhibitor (Verrhuis et al. 1999).

Alpha-2-macroglobulin is a protease inhibitor known to form a complex with β-amyloid (Kovacs 2000). It may be that alpha-2-macroglobulin plays a role in β-amyloid clearance via the low-density lipoprotein receptor (Tooyama 2001). Alpha-2-macroglobulin polymorphism has been linked to AD although the calculated risk factor is significantly less than that for ApoE (McGeer & McGeer 2001). Complement factor H is regulatory protein with a role in the alternative complement pathway and has also been found to be present in association with β-amyloid in the plaques associated with AD (Strohmeyer et al. 2002, Honda et al. 2000). Alpha-2-macroglobulin and complement factor H were both recently reported as potential plasma biomarkers for AD (Hye et al. 2006). Both proteins were identified in our recent cross-sectional analysis but failed to replicate in the subsequent assays (Cutler et al. 2008). It is interesting to note that in the current study not only were these proteins again highlighted as showing an expression change, in this case in response to rosiglitazone, but they both showed a correlation with ADAS-Cog score in response to at least one of the higher dosing regimes (4 mg or 8 mg). Complement factor H inhibits the alternate complement cascade in a similar manner to complement C1 inhibitor controlling the classical cascade. Complement factor H is known to bind to complement C3. On investigation of the 2DE data, we noted that in the spot representing the change in expression of complement factor H there was evidence of complement C3 being present in the same spot. Our data are therefore consistent with a reduction in complexes associated with inhibition of both the classical and alternate complement cascades in AD patients and a reversal of this (i.e. increased modulation of the complement cascades) on treatment with rosiglitazone.

A single note of caution should be raised however. Unlike the other three proteins identified in this study, alpha-2-macrogloubulin was also seen to show a correlation with peripheral adiponectin levels in higher dose groups (data not shown). Adiponectin is a marker of the peripheral activity of rosiglitazone and the other members of this class of drug. As such we cannot rule out the possibility that change in alpha-2-macroglobulin levels may reflect the peripheral rather than the central activity of rosiglitazone. It is noteworthy that the other three proteins did not show a correlation with adiponectin levels and therefore are likely to reflect the central effect, as noted by a change in ADAS-Cog score, rather than a peripherally mediated effect.

As illustrated in Figure 1 we observed six ApoE protein-related spots showing differential expression with spots (1332 and 1695), showing an increase on rosiglitazone treatment and four spots (1257, 869, 556 and 1747) showing a decrease on treatment. In specific assay ApoE showed a correlation at the 4 mg dose ($p = 0.07$). Further work would be needed to establish the absolute forms of the various ApoE spots and establish the relationship which may exist between them in terms of post-translational modification and proteolytic processing. In a similar manner to that seen for complement C1 inhibitor, this may generate important information leading to a more specific, quantitative and higher throughput assay and may ultimately form part of a panel of biomarkers including complement C1 inhibitor.

Three of the four proteins identified in this study play a role in regulating the complement cascade (Davis et al. 2007). Neuroinflammation is known to play a significant role in pathology of AD and as such it is possible that the changes in these proteins/complexes may directly or indirectly reflect the disease process (Bonifati & Kishore 2007). In a recent paper, Stevens et al. (2007) described how in early development the complement system may be activated to facilitate the synaptic pruning essential for development of the CNS and speculated that during neurodegenerative diseases such a process may be reactivated leading to aberrant removal of neurones. Further work will shed additional light on the role of the classical and alternate complement cascade and its regulation in AD pathology and therapy.

The finding of altered expression of several proteins in plasma during treatment of AD patients with rosiglitazone is an interesting one. The findings reported here, including the changes in the complement cascade, are in line with theories that the actions of PPAR agonists in the CNS may be in part due to anti-inflammatory effects (Stahel et al. 2008). Moreover the fact that these proteins have already been proposed as AD biomarkers in other analyses in different patient populations reinforces the validity of the previous data and the fact that discovery and validation of disease biomarkers requires several stages with large numbers of samples from different populations (Cutler et al. 2008, Hye et al. 2006). Previously we have identified a number of potential biomarkers of AD. This study extends those findings into a drug treatment group and indicates that a number of those proteins previously identified may have value as markers of therapeutic effect. While one of the markers (alpha-2-macroglobulin) shows a correlation with an established marker of the peripheral activity of PPARγ agonists, further work in additional larger populations will be needed to establish the value of these proteins as pharmacodynamic markers of the CNS-based activity. The data presented here represent another contribution to the postulation that complement C1 inhibitor, complement factor H, alpha-2-macroglobulin and ApoE are AD plasma biomarkers and one or more of these may represent reliable markers of drug efficacy.

Acknowledgements

We would like to thank Arthur Moseley (current address, Duke University, NC, USA), Mary Moyer (GlaxoSmithKline, Research Triangle Park, NC, USA), David Bell, Mike Lee, Sally Wetten and David Wille (GlaxoSmithKline, Harlow, UK).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary Table . Proteins identified as changing in the proteomic analysis of the rosiglitazone study. Protein identifications are given with sequence coverage of peptides used to identify the protein and fold change of the relevant spots. In a number of cases more than one spot was seen to display altered expression for a given protein and these are listed with the downregulation first and upregulation following. Proteins previously identified as being associated with Alzheimer's disease in the recent paper are also denoted by a 'Y' in the appropriate column.

Protein	HUGO symbol	Cutler et al., 2007	Fold change	Sequence information
Alpha-1-antichymotrypsin	SERPIN A3	Y	−2.0, −1.2, −1.2	ADLSGITGAR EQLSLLDR ITLLSALVETR LINDYVK MEEVEAMLLPETLK+2 Oxidation (M) EIGELYLPK
Alpha-1-B-glycoprotein	A1BG	Y	−1.3, −1.2, −1.2, −1.2, −1.2	LLELTGPK LETPDFQLFK CLAPLEGAR+Carbamidomethyl (C) GVTFLLR
Alpha-2-antiplasmin	SERPIN F2	Y	−2.0, −1.4, −1.2, 1.5	FDPSLTQR LGNQEPGGQTALK
Alpha-2-HS glycoprotein	AHSG	Y	−1.2	CDSSPDSAEDVR+Carbamidomethyl (C); Phospho (STY) CDSSPDSAEDVRK+Carbamidomethyl (C); Phospho (STY) CNLLAEK+Carbamidomethyl (C) HTLNQIDEVK
Alpha-2-macroglobulin	A2M	Y	−5.6, −1.2, −1.2, −1.2, 1.3, 3.3	LPPNVVEESAR SASNMAIVDVK+Oxidation (M) NEDSLVFVQTDK FEVQVTVPK SSSNEEVMFLTVQVK+Oxidation (M) SSGSLLNNAIK SDIAPVAR VGFYESDVMGR+Oxidation (M)
Angiotensinogen	AGT	Y	−1.3, −1.3, −1.2, 1.5	ALQDQLVLVAAK SLDFTELDVAAEK VANPLSTA
Antithrombin-III			−1.3, −1.3, −1.3, −1.2, −1.2, −1.2	ATEDEGSEQKIPEATNR FSPENTR LQPLDFK EVPLNTIIFMGR+Oxidation (M) VAEGTQVLELPFK IEDGFSLK LPGIVAEGR

Supplementary Table (*Continued*)

Protein	HUGO symbol	Cutler et al., 2007	Fold change	Sequence information
Apolipoprotein A1	APOA1	Y	−1.6, −1.4, −1.3, −1.2, −1.3, 1.2	DYVSQFEGSALGK QGLLPVLESFK AELQEGAR AKPALEDLR DLATVYVDVLK VQPYLDDFQK VSFLSALEEYTK QKVEPLR
Apolipoprotein AII	APOA2		−1.3	SPELQAEAK
Apolipoprotein AIV	APOA4	Y	−1.2, −1.2, −1.2, −1.2, −1.2, 1.2	ALVQQMEQLR+Oxidation (M) ISASAEELR LAPLAEDVR LEPYADQLR
Apolipoprotein CIII	APOC3	Y	−1.8	SEAEDASLLSFMQGYMK+2 Oxidation (M) DALSSVQESQVAQQAR TAKDALSSVQESQVAQQAR
Apolipoprotein D	APOD		−1.6, −1.4, −1.3, −1.3	IPTTFENGR MTVTDQVNCPK.L+2 Deamidation (NQ); Oxidation (M); Propionamide (C) NPNLPPETVDSLK IPTTFENGR.C+Deamidation (NQ)
Apolipoprotein E	APOE	Y	−2.2, −1.7, −1.3, −1.3, −1.3, −1.2, −1.2, 1.3, 1.3, 1.7	VLNQELR LEEQAQQIR VQAAVGTSAAPVPSDNH AATVGSLAGQPLQER LQAEAFQAR LGADFEDVCGR.L+Carbamidomethyl (C) LGPLVEQGR KVEQAVETEPEPELR ELQAAQAR SELEEQLTPVAEETR VEQAVETEPEPELR GEVQACLGQSTEELR+Deamidation (NQ); Propionamide (C) GEVQAFLGQSTEELR EQVAEVR AKLEEQAQQIR LDEVKEQVAEVR
Apolipoprotein J	CLU	Y	−1.7, −1.3, −1.3, −1.3, −1.2, −1.2	ELDESLQVAER IDSLLENDR ASSIIDELFQDR.
Apolipoprotein M	APOM		1.2	AFLLTPR FLLYNR
Apolipoprotein L1	AOPOL1	Y	−1.3	VNEPSILEMSR+Oxidation (M) VTEPISAESGEQVER
Apolipoprotein H (beta-2-glycoprotein)	APOH	Y	1.3	ATVVYQGER

Supplementary Table (*Continued*)

Protein	HUGO symbol	Cutler et al., 2007	Fold change	Sequence information
Complement C3	C3	Y	−1.6, −1.4, −1.4, −1.3, −1.3, −1.2, −1.2, −1.2, −1.2, −1.2, −1.2, −1.2, −1.2, 1.2, 1.2, 1.2, 1.3, 1.5, 1.5, 1.5, 3.3	QELSEAEQATR IPIEDGSGEVVLSR VLLDGVQNPR LVAYYTLIGASGQR QPVPGQQMTLK ISLPESLK TVMVNIENPEGIPVK+Oxidation (M) ENEGFTVTAEGK SGSDEVQVGQQR QLANGVDR+Deamidation (NQ) NTMILEICTR.Y+Carbamidomethyl (C); Oxidation (M)
Complement component 1 inhibitor	SERPING1	Y	1.3	VTTSQDMLSIMEK+2 Oxidation (M) LLDSLPSDTR
Complement component 1 r subcomponent	C1R	Y	1.3	QDACQGDSGGVFAVR+2 Deamidation (NQ); Propionamide (C) YTTTMGVNTYK+Oxidation (M)
Complement component 4A	C4A	Y	−1.2, −1.2	AEFQDALEK EMSGSPASGIPVK+Oxidation (M) CSVFYGAPSK.S+Carbamidomethyl (C) GLQDEDGYR
Complement component 4B	C4B	Y	−1.2, −1.2, −1.2, −1.2, −1.2, 1.2	AEFQDALEK EMSGSPASGIPVK+Oxidation (M) GQIVFMNR+Oxidation (M) CSVFYGAPSK+Carbamidomethyl (C)
Complement component C7	C7		−1.2, 1.2, 1.2	AASGTQNNVLR DGFVQDEGPMFPVGK+Oxidation (M) QNDFNSVEEK VLFYVDSEK EQTMSECEAGALR+Carbamidomethyl (C); Oxidation (M) KVFSGDGK
Complement component C6	C6	Y	1.1	IGESIELTCPK+Carbamidomethyl (C) ALQEYAAK KALQEYAAK
Complement component C8	C8		−1.2, 1.1	MESLGITSR.D+Oxidation (M) AIDEDCSQYEPIPGSQK+Carbamidomethyl (C) SLLQPNK
Complement component C9	C9	Y	−1.4	VVEESELAR
Complement cytolysis inhibitor	CLI		−1.4	EIQNAVNGVK EIQNAVNGVK+Deamidation (NQ) ELDESLQVAER IDSLLENDR TLLSNLEEAK ASSIIDELFQDR EILSVDCSTNNPSQAK+Carbamidomethyl (C)

Supplementary Table (*Continued*)

Protein	HUGO symbol	Cutler et al., 2007	Fold change	Sequence information
Complement factor B	CFB	Y	1.2, 1.3. 1.3	DISEVVTPR EELLPAQDIK
Complement factor H	CFH	Y	1.2	EIMENYNIALR+Oxidation (M) LSYTCEGGFR+Carbamidomethyl (C) TGESVEFVCK+Carbamidomethyl (C) CFEGFGIDGPAIAK+Carbamidomethyl (C) EQVQSCGPPPELLNGNVK+2 Deamidation (NQ); Propionamide (C) IVSSAMEPDR+Oxidation (M) SPDVINGSPISQK DTSCVNPPTVQNAYIVSR+2 Deamidation (NQ); Propionamide (C) EIMENYNIALR+Oxidation (M) NGFYPATR+ Deamidation (NQ)
Complement factor I	IF	Y	1.2	VTYTSQEDLVEK
Cytokeratin 10.			−1.3	VTMQNLNDR+Oxidation (M) ALEESNYELEGK SQYEQLAEQNR
Dermcidin precursor (preproteolysin)			−1.4	ENAGEDPGLAR
Fetuin-B			−1.3	AIFYMNNPSR+Oxidation (M) GSVQYLPDLDDK IFFESVYGQCK+Carbamidomethyl (C) VNDAQEYR
Fibrinogen alpha chain	FGA	Y	−1.7, −1.3, −1.3, −1.2, −1.2, 1.2, 1.2	QFTSSTSYNR GSESGIFTNTK NSLFEYQK QLEQVIAK GGSTSYGTGSETESPR
Fibrinogen beta chain	FGB	Y	−1.2, −1.2, 1.1, 1.2, 1.3	QDGSVDFGR DNENVVNEYSSELEK QGFGNVATNTDGK QGFGNVATNTDGK+Deamidation (NQ) YQISVNK
Fibrinogen gamma	FGG	Y	−1.3, −1.2, −1.2, −1.2	YLQEIYNSNNQK QSGLYFIKPLK RLDGSVDFK VGPEADKYR YEASILTHDSSIR TSTADYAMFK+Oxidation (M)

Supplementary Table (*Continued*)

Protein	HUGO symbol	Cutler et al., 2007	Fold change	Sequence information
Fibronectin			−1.2	FTNIGPDTMR+Oxidation (M) QYNVGPSVSK
Gelsolin	GSN	Y	−1.2, 1.2, 1.3, 1.3	AGALNSNDAFVLK TASDFITK YIETDPANR DSQEEEKTEALTSAK QTQVSVLPEGGETPLFK TGAQELLR LFACSNK+Carbamidomethyl (C) RTPITVVK
Hemopexin	HPX	Y	−5.6, −1.4, −1.4, −1.2, 1.1, 1.2, 1.2, 1.2, 1.3	GGYTLVSGYPK NFPSPVDAAFR VDGALCMEK.+Carbamidomethyl (C); Oxidation (M)
Histidine-rich glycoprotein precursor			−1.3	ALDLINKR EENDDFASFR DGYLFQLLR
Insulin-like growth factor-binding protein complex acid labile chain	IGFALS	Y	1.3	ANVFVQLPR LEYLLLSR DFALQNPSAVPR ELVLAGNR LAELPADALGPLQR TFTPQPPGLER
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	Y	−2.1, −1.3, 1.3, 2.3	ILDDLSPR LALDNGGLAR
Kininogen	KNG	Y	−1.4, −1.3, −1.3, −1.2, 1.5	AVDAALKK DFVQPPTK ENFLFLTPDCK+Carbamidomethyl (C) TVGSDTFYSFK YFIDFVAR QVVAGLNFR YNSQNQSNNQFVLYR IASFSQNCDIYPGK+Carbamidomethyl (C)
Leucine-rich alpha-2-glycoprotein	LRG	Y	−1.4, −1.2	DLLLPQPDLR GQTLLAVAK GPLQLER VAAGAFQGLR YLFLNGNK
Lumican	LUM	Y	−1.4, 1.5	ILGPLSYSK ISNIPDEYFK LKEDAVSAAFK EDAVSAAFK FNALQYLR

Supplementary Table (*Continued*)

Protein	HUGO symbol	Cutler et al., 2007	Fold change	Sequence information
Pigment epithelium-differentiation factor	SERPIN F1	Y	−1.2, 1.2, 1.3	LQSLFDSPDFSK DTDTGALLFIGK TVQAVLTVPK
Prothrombin			−1.4, −1.3, 1.5, 2.3	ELLESYIDGR TATSEYQTFFNPR ETAASLLQAGYK
Serum amyloid A2			−1.5	EANYIGSDK
Serum amyloid P-component			−1.5, 1.2	IVLGQEQDSYGGK AYSDLSR DNELLVYK
Serum paraoxonase	PON1		−1.3, −1.2	QGYFVEAQPK SFNPNSPGK IQNILTEEPK
Sex hormone-binding globulin	SHBG		−1.3, −1.2	QVSGPLTSK QAEISASAPTSLR VVLSQGSK
Transferrin	TF	Y	2.0, 2.1	DGAGDVAFVK DSGFQMNQLR+Oxidation (M) EGYYGYTGAFR HQTVPQNTGGK SVIPSDGPSVACVK.K+Carbamidomethyl (C)
Transthyretin	TTR	Y	−1.1	GSPAINVAMHVFR
Vitamin D-binding protein	GC	Y	−1.7, −.1.6, −1.3, −1.2, −1.2, −1.2, 1.2	ELSSFIDK LPDATPTELAK VLEPTLK YTFELSR LCDNLSTK.N+Carbamidomethyl (C) FPSGTFEQVSQLVK KFPSGTFEQVSQLVK KELSSFIDK LPEATPTELAK FEDCCQEK+2 Carbamidomethyl (C)
Vitronectin	VTN	Y	−1.2	CTEGFNVDK+Carbamidomethyl (C) CTEGFNVDKK+Carbamidomethyl (C) GQYCYELDEK+Carbamidomethyl (C) QPQFISR VDTVDPPYPR.