# No evidence to suggest that the use of acetylcholinesterase inhibitors confounds the results of two blood-based biomarker studies in Alzheimer's disease

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# Abstract

**Background:** There is an urgent need to discover Alzheimer's disease (AD) biomarkers that are both easily measured and reliable. Research into blood-based biomarkers for AD using transcriptomics and proteomics has been an attractive and promising area of research. However, to date researchers have not looked into the possibility of AD medication being a confounding factor in these studies.

**Objective:** This study explored whether acetylcholinesterase inhibitors (AchEIs), the main class of AD medication, are a confounding factor in AD blood biomarker studies.

**Methods:** The most promising blood transcriptomic and proteomic biomarkers from two recent studies were analyzed to determine if they were differentially expressed between AD subjects on AchEIs and subjects that were not.

**Results:** None of the gene or protein biomarkers analyzed were found to be significantly altered between subjects in either group.

**Conclusion:** This study found no evidence that AchEIs are a confounding factor in these published AD blood biomarker studies. Further work is needed to confirm that this is also the case for other proposed biomarkers.

## 1. Introduction

Alzheimer's disease (AD) is a common, costly and fatal neurodegenerative disorder. It manifests in the form of progressive cognitive decline, including memory loss, executive dysfunction, psychiatric symptoms and behavioral disturbances [1]. Currently, a definitive diagnosis of AD can only be obtained after post-mortem dissection of brain tissue. AD diagnosis therefore relies on robust clinical evaluation, sometimes including assessment of pathology using cerebrospinal fluid (CSF) biomarkers [2], or pathology measures from brain scans; usually Magnetic Resonance Imaging (MRI) or Positron Emission Tomography (PET) [3,4].

Despite these efforts, diagnosis of AD remains difficult, especially in areas where access to advanced neuroimaging equipment remains limited. A lumbar puncture to acquire CSF for analysis is also relatively invasive and inappropriate in certain clinical environments. It has been suggested that up to two-thirds of dementia patients go un-diagnosed [5], and that by the time an AD diagnosis is made, the underlying pathological processes have been developing for around 20 years [6]. There is therefore an urgent need to develop investigative techniques that are cost effective, easy to administer, and capable of aiding the diagnosis of AD in its early stages. Though undoubtedly challenging, this may prove useful for enriching clinical trials for subjects whose pathology is less advanced.

Recently, the analysis of blood samples to develop a blood-based diagnostic test has been an attractive area in AD biomarker research. Blood samples of AD

subjects can be collected with relative ease, and analyzed to determine differences in protein or messenger RNA (mRNA) quantity that might elucidate underlying biological changes in the disease state [5]. Studies such as Booij *et al.* [7], Fehlbaum-Beurdeley *et al.* [8] and more recently Lunnon *et al.* 2012 [9] have demonstrated that whole-blood profiling of mRNA can generate evidence of AD associated differences in gene expression. Similarly, studies such as Ray *et al.* [10], Doecke *et al.* [11], and Sattlecker *et al.* [12] have analyzed blood protein quantities to identify proteins with significantly altered blood levels in AD. Zurbig and Jahn [13], Lista *et al.* [14], Kiddle *et al.* [15] and Chiam *et al.* [16] have also recently reviewed the blood-based proteins most commonly associated with AD, finding a modest degree of replication between studies. Given the prospect of a cheap and convenient diagnostic test should success be achieved, it is likely that research into blood-based biomarkers, while not without limitations, will continue to be an appealing avenue in AD biomarker discovery [17,18].

Although there has been a significant amount of research generated in the field, very little has been done to look into the potential effects of medication as a confounding factor in blood-based biomarker discovery. This is important as the medication an individual is receiving depends largely on their diagnosis. Medication could potentially affect the composition of biological molecules in the blood. For example, AD patients are often placed on a wide range of psychotropic drugs and recent evidence suggests that psychotropic drugs affect the expression of AD related genes in blood [19].

There are currently two classes of drugs used to treat the cognitive symptoms of AD, acetylcholinesterase inhibitors (AchEIs) and memantine (an NMDA receptor antagonist). Of these, AchEIs are the most common class of drug used in the treatment of AD [20]. These drugs act by inhibiting the enzyme acetylcholinesterase. This prevents the breakdown of the neurotransmitter acetylcholine at synapses, thus increasing the strength of neural transmission in the brain [21,22]. Treatment with AchEIs has been found to modulate the expression of pro- and anti-inflammatory cytokines in the blood of AD patients [23]. Given the high prevalence of AchEI use among AD subjects, it is therefore pertinent to determine if the potential gene and protein blood biomarkers identified in studies are indeed due to biological changes as a result of AD and not due to the effects of these drugs.

This study seeks to build on the results from Lunnon *et al.* 2012 [9] and Sattlecker *et al.* [12] by analyzing the most promising gene and protein biomarkers identified in AD subjects by both studies and determining if the use of AchEIs confounds the association between these blood-based biomarkers and AD.

## 2. Methods and Materials

### 2.1 Samples and clinical data from the AddNeuroMed cohort

As described in Lunnon *et al.* 2012 [9] and Sattlecker *et al.* [12], blood samples were obtained from subjects participating in the AddNeuroMed (ANM) study

[24-26]. Subjects were located at six different study sites across Europe, namely London, Lodz, Toulouse, Perugia, Kuopio and Thessaloniki. Informed consent was appropriately taken according to the Declaration of Helinski (1991) and ethical approval was obtained at each site. A diagnosis of AD was attained using the NINCDS-ADRDA criteria  [27] and the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV).

All subjects went through a semi-structured interview in order to collect the necessary demographic and medical information. This included an array of neuropsychological assessments such as the Mini Mental State Examination [28], Global Deterioration Scale [29] and the Alzheimer's Disease Assessment Scale-Cognitive subscale [30]. Information regarding the kind of neuropsychiatric medication each subject was currently prescribed was also obtained.

Blood samples were drawn from subjects by venipuncture and collected in PAXgen vacutainer tubes (Qiagen) for RNA analysis and EDTA glass tubes for proteomic analysis.

Relevant demographics and clinical data for these subjects were extracted from the AddNeuroMed database using CohortExplorer  [31].

## 2.2 Gene Expression Study

Blood samples collected from the ANM cohort were analyzed to determine mRNA gene expression profiles. Full details of the data collection and preprocessing procedure can be found in Lunnon *et al.* 2012 [9] and Voyle *et al.* (manuscript in preparation), and is only discussed briefly here.

### 2.2.1 Data Collection

The vacutainer tubes containing blood samples for RNA analysis were inverted 8-10 times and stored at -24°C for 24h before lowering the temperature to -80°C until RNA extraction. RNA was extracted using the PAXgene blood RNA kit (Qiagen), following the manufacturer's protocol. The 2100 Bioanalyser (Agilent Technologies) was then used to evaluate the quality of the extracted RNA. Only RNA samples that exceeded a RNA integrity number (RIN) of 7.0 were used in the analysis.

Microarray processing was conducted at the University of California in Los Angeles. The RNA samples were processed on Illumina Human HT-12 v3 Expression BeadChips (Illumina), each containing 48,803 probes. RNA was amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion) and gene expression values were obtained using the Lumi package within the R Bioconductor project [32].

### 2.2.2 Preprocessing

Raw gene expression data was subject to a model based background correction for bead array [33]. Negative bead expression levels were used to correct for background noise. The data was then log base 2 transformed and robust spline normalized before outlying samples were iteratively identified by fundamental network concepts and removed [32,34]. To remove any batch effects we adjusted for technical categorical variables using ComBat [35]. The first principal component across housekeeping probes was taken and regressed against technical variables and phenotype in order to account for the principal component across housekeeping probes. Variables significantly associated with the first principal component where then regressed against expression for each probe, and the mean adjusted residuals taken forward for all further analyses. Finally, the data was subset to probes that could be reliably detected in at least 80% of samples in each diagnostic group. Subjects were excluded if lab-based investigations highlighted discrepancies between recorded sex and sex determined by the XIST gene.

### 2.2.3 Probe Selection

Probes that had a significant difference in signal between AD subjects and controls were identified from Lunnon *et al.* 2012 [9]. The most significant probes were identified through a dual-criteria. Firstly a Bonferroni correction was applied to the p-values reported by Lunnon *et al.* 2012 [9] ($\alpha=0.05/19,161$).

Any probe that met the corrected threshold of p-value < α and had an absolute fold change of >0.5 was identified for further analysis.

### 2.2.4 Subject Selection

In Lunnon *et al.* 2012 [9], a total of 356 samples (116 control, 127 mild cognitive impairment (MCI), 113 AD) were put through RNA microarray processing. Of this a subset was extracted for analysis in our study, as described below. Of the 113 AD subjects, 100 had complete demographic data for medication, age, gender, MMSE score, and *APOE* status. It was decided that subjects on memantine, another form of AD cognitive drug would be excluded as there were too few subjects to conduct a conclusive analysis. Therefore 5 subjects on memantine and 6 subjects on both AchEI and memantine were excluded. This resulted in a total of 89 AD subjects for gene expression analysis.

## 2.3 Proteomic Study

Similar to the gene expression analysis described above, blood samples collected from the ANM cohort were analyzed to determine protein quantities. A detailed account of the data collection and preprocessing procedure can be found in Sattlecker *et al.* [12] and is described only briefly here.

### 2.3.1 Data Collection

EDTA tubes with the blood samples were centrifuged at 2,000 rpm at 4°C for 10 minutes within approximately 2 hours of collection. The resulting plasma

supernatant was then collected and divided into aliquots before being frozen at -80°C until protein measurement.

Protein quantities were measured using the new Slow Off-rate Modified Aptamer (SOMAmer)-based capture array known as "SOMAscan" (SomaLogic, Inc). Chemically modified nucleotides are used to transform a protein signal into a nucleotide signal [36]. Microarrays were then used to quantify the signal using relative fluorescence. A total of 1,001 human proteins, representing different molecular pathways and gene families, were quantified this way.

### 2.3.2 Preprocessing

Hybridization controls on the microarray were used to monitor sample-by-sample variability in hybridization, while the median signal across all SOMAmers was used to monitor overall technical variability. Using both the resulting hybridization and median scale factors, data across samples was normalized. An acceptance criterion of 0.4-2.5 was used for values based on historic trends. SOMAmer-by-SOMAmer calibration was established through the repeated measurement of calibration samples. A calibration scale factor is then generated using historic values of these calibration samples. The acceptance criterion for calibrator scale factors is that 95% of SOMAmers must have a calibration scale factor within 60.4 of the median [12].

All measurements obtained were log 2 transformed. Seven sample outliers were identified using principal component analysis in R and were thus removed from

downstream processing [12].

### 2.3.3 Probe Selection

Sattlecker *et al.* [12] had previously identified four proteins (prostate-specific antigen complexed to α1-antichymotrypsin, clusterin, pancreatic prohormone and fetuin B) that were found to have significantly altered levels (q-value < 0.05) in AD subjects when compared to healthy elderly controls. These four proteins were selected for the analysis.

### 2.3.4 Subject Selection

In Sattlecker *et al.* [12], a total of 415 ANM samples (110 control, 109 MCI, 196 AD) underwent SOMAscan proteomic analysis. Like in the gene expression portion of the study, a subset of this was extracted for data analysis using the same criteria. Of the 196 AD subjects, 189 had complete demographic data for medication, age, gender, MMSE score, and *APOE* status. After 8 subjects on memantine and 13 subjects on both AchEI and memantine were excluded, a total of 168 AD subjects were selected for the proteomic portion of the study.

### 2.4 Statistical power calculations

Power calculations were performed using the 'pwr.f2.test' in the 'pwr' R package, based on the approach of Cohen 1988 [37]. Based on the recommendation by Cohen (1988) $f^2$ = 0.02,  0.15 and 0.35 were used to indicate a small, medium and large effect size respectively. A statistical significance level of 0.005 was required, to represent a Bonferroni multiple testing correction for 10 markers (midway between number of gene expression and protein markers studied).

All data analysis was performed using R. The AD subjects that were selected for either the gene expression study or the proteomic study were further split into two groups based on the type of cognitive enhancing AD medication they were on. The first group comprised those on AchEIs, while the second group comprised those subjects not on any form of cognitive enhancing AD medication (non-AchEI). The gene expression study had 72 subjects in the AchEI group and 17 subjects in the non-AchEI group. Similarly, the proteomic study had 129 subjects in the AchEI group and 39 subjects in the non-AchEI group. For both studies, an analysis was then conducted between the AchEI group and non-AchEI group to see if there was any significant difference in gene expression and protein quantity.

Firstly the demographic data was analyzed to ensure that confounding variables could be identified. Discrete variables such as gender and *APOE ε4* allele status were analyzed using Fisher's exact test. Continuous variables such as age and MMSE score were analyzed using linear modeling. A threshold of p-value < 0.05 was set to identify if any of the variables differed between both AchEI and non-AchEI groups.

An analysis was then conducted on the genes and proteins that were pre-selected to determine if there was a significant difference in gene expression or protein levels between AD patients on AchEIs and those that were not. This was conducted using linear modeling with study site added as a covariate in the analysis. The p-values obtained were then adjusted for multiple testing by

applying the false discovery rate (FDR). A threshold of q-value < 0.05 was used to identify differentially expressed genes and differences in blood protein quantity. Box plots were created for genes or proteins of interest and for these, 111 controls (non-AD subjects) from the ANM cohort were included to provide visual comparison of any variation between the groups.

## 3. Results

### 3.1 Statistical power

To examine our ability to detect small, medium and large effects of AchEIs on blood marker levels, we performed statistical power calculations using a significance level of $p = 0.005$. Figure 1 shows that the gene expression study (n = 89) has ~2.3%, ~47% or ~96% statistical power to detect a small, medium or large effect of AchEIs on blood gene expression markers. It also shows that the proteomics study (n = 168) has ~5%, ~89% or ~100% statistical power to detect a small, medium or large effect of AchEIs on blood protein markers. It also shows that even a study with a sample size of 500 would only have ~30% power to detect a small effect of medication on a blood marker.

### 3.2 Gene Expression Study

To investigate the effect of AchEIs on blood gene expression markers of AD, we examined gene expression levels in 89 AD subjects with gene expression, demographic and medication data. No significant differences in gender, age, *APOE* status or MMSE scores were seen between the AchEI group and the non-AchEI group ($p < 0.05$; Table 1). As a result, none of the demographic variables

were factored in as covariates in the analysis of probe signal between both groups.

When a Bonferroni correction was applied, 23 probes were identified that passed the dual-criteria that had been set (p-value < α and absolute fold change > 0.5). These 23 probes were thus selected for analysis (Table 2).

After multiple testing correction was applied, none of the 23 probes analyzed showed any significant difference in signal strength between the group on AchEIs and the group without (q-value < 0.05). Six probes (UQCRH, ATP5O, ATP5EP2, C14orf156, ZMAT2 and LOC653658) were found to be nominally significantly (p < 0.05) associated with the use of AchEIs (Table 2). The box plots for these six probes are shown in Figure 2. Only two of these – LOC653658 and C14orf156 – were still nominally associated with medication use when presence or absence of *APOE ε4* (and it's interaction with medication use) was also accounted for in the model. Visually there does not appear to be a significant difference in expression of these genes between groups.

### 3.3 Proteomic Study

For the 168 AD subjects with proteomic, demographic and medication data, no significant differences in gender, age, *APOE* status or MMSE scores were observed between the AchEI group and the non-AchEI group (p < 0.05; Table 3). None of these variables were thus factored in as covariates for the proteomic analysis.

The four proteins analyzed showed no significant difference in quantity between both groups in a linear model, with none passing significance thresholds either at the nominal ($p < 0.05$) or multiple testing corrected ($q < 0.05$) threshold (See Table 4).

## 4. Discussion

The analysis conducted shows that none of the biomarkers studied are differentially expressed in subjects taking AchEIs. Our sample had reasonable statistical power to detect large effects on gene expression markers, and both medium or large effects on protein markers. This implies that AchEIs are not a large confounding factor affecting the most promising gene or protein biomarkers identified in the studies by Lunnon *et al.* 2012  [9] and Sattlecker *et al.* [12]. If AchEIs had been found to be a large confounding factor, it would have undermined the diagnostic/enrichment potential of the biomarkers identified in those studies. The results of this study, though small in scope and by no means comprehensive, are therefore encouraging and strengthen the validity of these studies. As the first study to examine the possibility of AD blood biomarkers being confounded by AchEIs, it also highlights a previously neglected potential confounding variable.

In this study the sample size of both groups tested was notably imbalanced. The number of subjects in the group not on any AD medication was significantly smaller than the group on AchEIs in both the gene expression and proteomic

analysis. This is understandable since most AD patients would be expected to have some sort of cognitive enhancing medication as treatment. It would also have been interesting to include an analysis of subjects using memantine, the other main class of AD cognitive drugs. Yet in this study too few subjects were on memantine to allow a thorough investigation of this. This limitation could be overcome should larger studies be conducted in the future.

While in this study AchEIs were considered as a single class of drug, three separate AchEIs (donepezil, galantamine and rivastigmine) are the mainstay in AD therapy [20]. Though all three drugs work similarly by inhibiting acetylcholinesterase and preventing the breakdown of acetylcholine at the post-synaptic cleft, it is likely that that there are subtle differences in their effects on underlying biological processes. Therefore if an adequate sample size is available in future studies, all three drugs should be studied individually for their effects on AD blood biomarkers. This is pertinent since studies revealing differences in gene expression as a result of AchEI use have studied AchEIs individually. Specifically, Reale *et al.* [23] investigated the effects of donepezil on blood inflammatory markers in AD patients, while Andin *et al.* [38] investigated the effect of rivastigmine on the glutamate transporter rEAAC1 blood mRNA expression in mice models, both revealing significant effects by the drugs on gene expression.

One possible way of improving the sample size in future studies is to ensure the collection of appropriate medication information in any research cohort, as some subjects were excluded from our study due to incomplete demographic data.

Furthermore with hindsight, medication could have been included as a covariate in the discovery stage of biomarker studies, instead of analyzed post-hoc. We have not seen this approach applied in any of the discovery studies to date and this should therefore be considered for future biomarker studies.

Besides drugs that improve cognitive symptoms, many AD patients are also on other medications to manage the non-cognitive symptoms of the disease. People with dementia are far more susceptible to psychiatric conditions such as mood disorders and psychosis [39]. In such cases, drugs such as antidepressants, neuroleptics, sedatives and hypnotics are often required for treatment [19,40]. Citalopram, an antidepressant, has been found to affect gene expression in AD lymphocytes [41]. Thus it is possible that other psychotropic medications could prove to be a confounding factor in blood biomarker studies and should be explored in future studies. Given that each subject could potentially be on multiple medications, it may also be possible to conduct multivariate analyses to determine if a combination of medications would yield a significant difference in gene expression, although larger cohorts would be needed to identify this.

This study considered promising biomarkers individually, looking at whether the expression of single genes or proteins are affected by AchEIs. However, Sattlecker *et al.* [12] and Lunnon *et al.* 2013 [42], as well as other researchers, have also proposed multivariate biomarker models of AD. These multivariate models could also be investigated to determine if AchEIs, or any other medications, affect their potential utility.

It is also important to consider the possible effects of medication on other potential blood biomarkers. For example, many other potential biomarkers of AD have been highlighted in recent reviews of blood gene expression [17] and protein levels [16].

## 5. Conclusion

Overall this exploratory study has found no evidence that AchEIs are a large confounding factor for the most promising AD blood-based biomarkers identified in both studies. This gives an encouraging indication that the use of AchEIs is unlikely to affect the validity of these biomarkers in potential diagnosis or enrichment applications. However more comprehensive studies need to be conducted to explore the full effects of AchEIs on these and other proposed blood-based biomarkers of AD.

## 6. Acknowledgements

## 7. References

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**Table 1:** Demographic information for subjects included in the gene expression analysis.

| | AchEI | Non-AchEI | p-value |
|---|---|---|---|
| **Total Number** | 72 | 17 | - |
| **Males/Females** | 25/47 | 3/14 | 0.25 |
| **Median Age (IQR)** | 76 (9.25) | 75 (9) | 0.89 |
| **Median MMSE (IQR)** | 22 (7) | 22 (6) | 0.76 |
| **APOE ε4 Status (0/1/2)** | 29/29/14 | 9/7/1 | 0.40 |

Abbreviations: AchEI, Group taking acetylecholinesterase inhibitors; Non-AchEI, Group not on any AD cognitive enhancing medication; MMSE, Mini-Mental State Examination.

**Table 2:** Results for the gene expression analysis showing the 23 probes analyzed with details of the gene name, coefficient value, standard error, p-value and q-value.

| Probe ID | Gene Name | Coefficient | Standard Error | p-value | q-value |
|---|---|---|---|---|---|
| ILMN_2097421 | MRPL51 | 0.35 | 0.27 | 0.20 | 0.29 |
| ILMN_1784286 | NDUFA1 | 0.40 | 0.26 | 0.13 | 0.21 |
| ILMN_1776104 | NDUFS5 | 0.37 | 0.27 | 0.17 | 0.26 |
| ILMN_1726603 | ATP5I | 0.34 | 0.28 | 0.22 | 0.30 |
| ILMN_2187718 | COX17 | 0.29 | 0.28 | 0.31 | 0.36 |
| ILMN_2128128 | SHFM1 | 0.22 | 0.27 | 0.41 | 0.43 |
| ILMN_1799030 | CMTM2 | 0.34 | 0.29 | 0.24 | 0.30 |
| ILMN_1703538 | AIF1 | 0.23 | 0.28 | 0.41 | 0.43 |
| ILMN_2166865 | ENY2 | 0.46 | 0.27 | 0.095 | 0.2 |
| ILMN_1732328 | LOC646200 | 0.40 | 0.25 | 0.12 | 0.21 |
| ILMN_1680314 | TXN | 0.53 | 0.28 | 0.058 | 0.15 |
| ILMN_1726239 | TBCA | 0.51 | 0.26 | 0.054 | 0.15 |
| ILMN_2232936 | UQCRH | 0.53 | 0.27 | 0.048 | 0.15 |
| ILMN_1746516 | RPS25 | 0.42 | 0.26 | 0.11 | 0.21 |
| ILMN_2189936 | RPL36AL | 0.071 | 0.26 | 0.79 | 0.79 |
| ILMN_1791332 | ATP5O | 0.62 | 0.26 | 0.022 | 0.15 |
| ILMN_1792528 | LOC401206 | 0.50 | 0.26 | 0.055 | 0.15 |
| ILMN_2189933 | RPL36AL | 0.31 | 0.26 | 0.24 | 0.30 |
| ILMN_2225887 | ATP5EP2 | 0.58 | 0.27 | 0.036 | 0.15 |
| ILMN_1661945 | C14orf156 | 0.62 | 0.27 | 0.027 | 0.15 |
| ILMN_1745343 | ZMAT2 | 0.54 | 0.27 | 0.045 | 0.15 |
| ILMN_1680967 | CIP29 | 0.51 | 0.28 | 0.07 | 0.16 |
| ILMN_1652073 | LOC653658 | 0.57 | 0.25 | 0.025 | 0.15 |

**Table 3:** Demographic information for subjects included in the proteomic analysis.

|  | AchEI | Non-AchEI | p-value |
|---|---|---|---|
| **Total Number** | 129 | 39 | - |
| **Males/Females** | 48/81 | 9/30 | 0.12 |
| **Median Age (IQR)** | 77 (10) | 78 (9.5) | 0.45 |
| **Median MMSE (IQR)** | 21 (8) | 21 (8) | 0.63 |
| *APOE* ε4 **Status (0/1/2)** | 55/48/26 | 19/18/2 | 0.074 |

Abbreviations: AchEI, Group taking acetylcholinesterase inhibitors; Non-AchEI, Group not on any AD cognitive enhancing medication; MMSE, Mini-Mental State Examination.

**Table 4:** Results for the proteomic analysis showing the four proteins analyzed with details of the UniProt ID, coefficient value, standard error, p-value and q-value. * Q-values are approximately equal, exact equality of these Q-values is an artifact of the approximation method used by the R function 'p.adjust'.

| UniProt ID | Protein Name | Coefficient | Standard Error | p-value | q-value |
|---|---|---|---|---|---|
| P07288, P01011 | PSA-ACT | -0.049 | 0.19 | 0.80 | 0.80* |
| Q9UGM5 | Fetuin B | 0.083 | 0.19 | 0.66 | 0.80* |
| P10909 | Clusterin | 0.12 | 0.19 | 0.54 | 0.80* |
| P01298 | Pancreatic Prohormone | 0.17 | 0.19 | 0.37 | 0.80* |

Abbreviations: PSA-ACT, prostate specific antigen complexed to $\alpha$1-antichymotrypsin
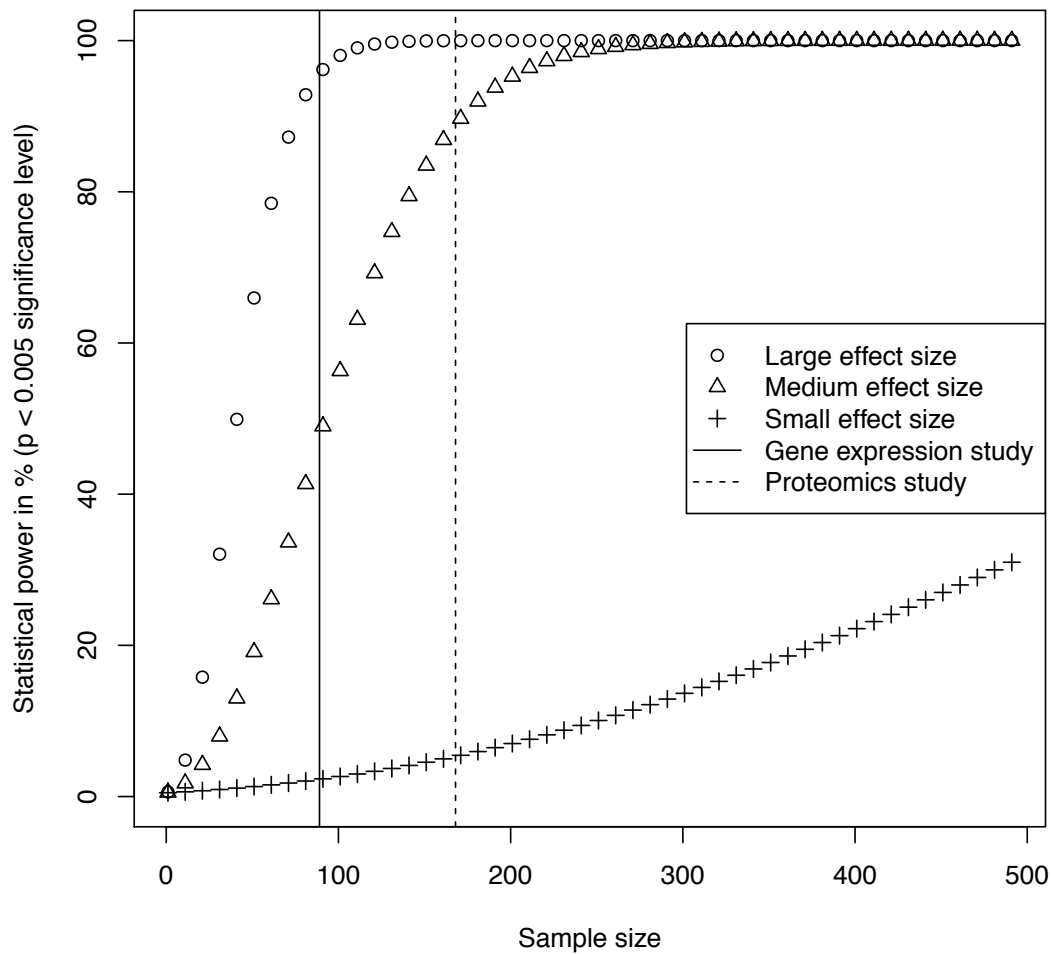
**Figure 1:** Statistical power curves for the detection of the effect of AchEIs on 10 blood markers (midway between number of genes and proteins investigated). Points indicate statistical power for different effect sizes (small, medium and large) in a model with 6 degrees of freedom for the numerator. Black lines indicate sample size of the gene expression study (n = 89) and the proteomics study (n = 168). A significance threshold of $p < 0.005$ was used to correspond to a $p < 0.05$ significance level after Bonferroni multiple testing correction for 10 markers/tests.
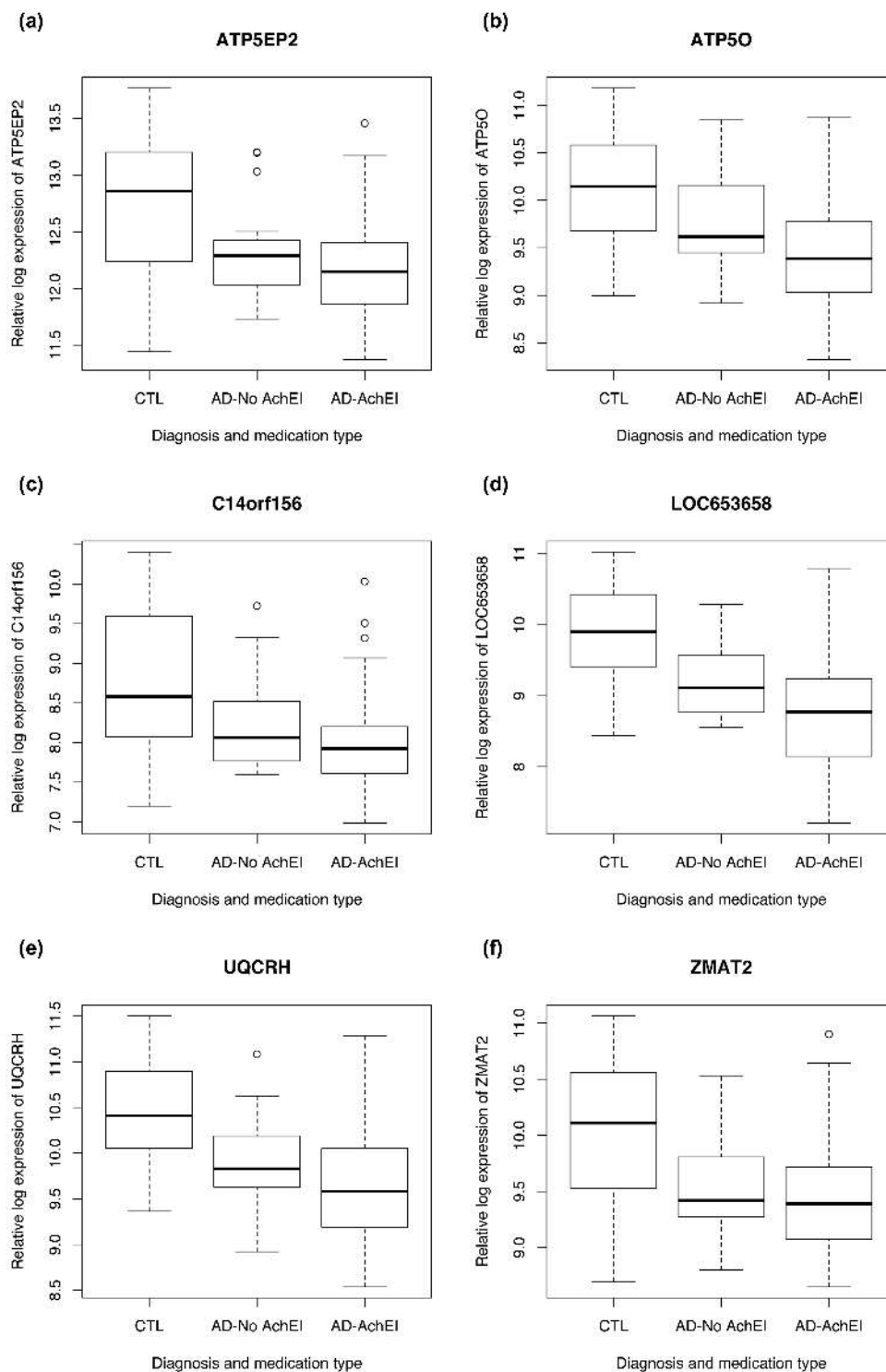
**Figure 2:** Box plots of the six nominally significant genes found in the gene expression analysis. AD subjects on AchEIs (AchEI) are compared with AD subjects not on AchEIs (Non-AchEI) and control subjects without AD (CTL).

There do not appear to be a significant difference in gene expression between AD subjects with and without AchEIs.