Supplemental materials for article Kushnir MM, Michno W, Rockwood AL, Blennow K, Strathmann FG, Hanrieder J. "Association of PTHrP Levels in CSF with Alzheimer's Disease Biomarkers"

MATERIALS AND METHODS

Reagents, standards.

Calibration standards of PTHrP were prepared from recombinant PTHrP (AAS 37-122; AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTAEIRATSEVSPNSKPSPNTKNHPVRFGS DDEGRYLTQETNKVETYKEQPLKTP) purchased from PeproTech (Rocky Hill, NJ)) in fetal bovine serum, lyophilized and stored frozen at -70°C. The working calibrators were prepared in 0.1% BSA; at concentrations 1,2,5,10 and 30 pmol/L. Rabbit polyclonal anti-PTHrP antibody was purchased from PeproTech. Plasma quality control samples were pooled human plasma and contained 4 and 11 pmol/L of PTHrP. The recombinant ^{15}N labeled internal standard (IS) was diluted in BSA to a concentration of 1 nmol/L and frozen at −20°C until used. Trypsin (purity 99%, activity 15,000 BAEE units), Tris(hydroxymethyl)aminomethane (TRIS), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), formic acid, acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest purity commercially available. Solvents were of HPLC grade, purchased from JT Baker (Phillipsburg, NJ).The polyclonal rabbit anti-PTHrP antibody (PeproTech) was conjugated to Tosyl activated magnetic beads (Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations. Briefly, the beads were washed with buffer and re-suspended in one molar ammonium sulfate solution containing 20 μg of the antibody per milligram of beads. Beads were incubated at 37°C for 20 hours, washed and incubated for 1 hour with blocking buffer containing 0.5% BSA, and reconstituted to a concentration of 20 mg/mL.

Sample preparation.

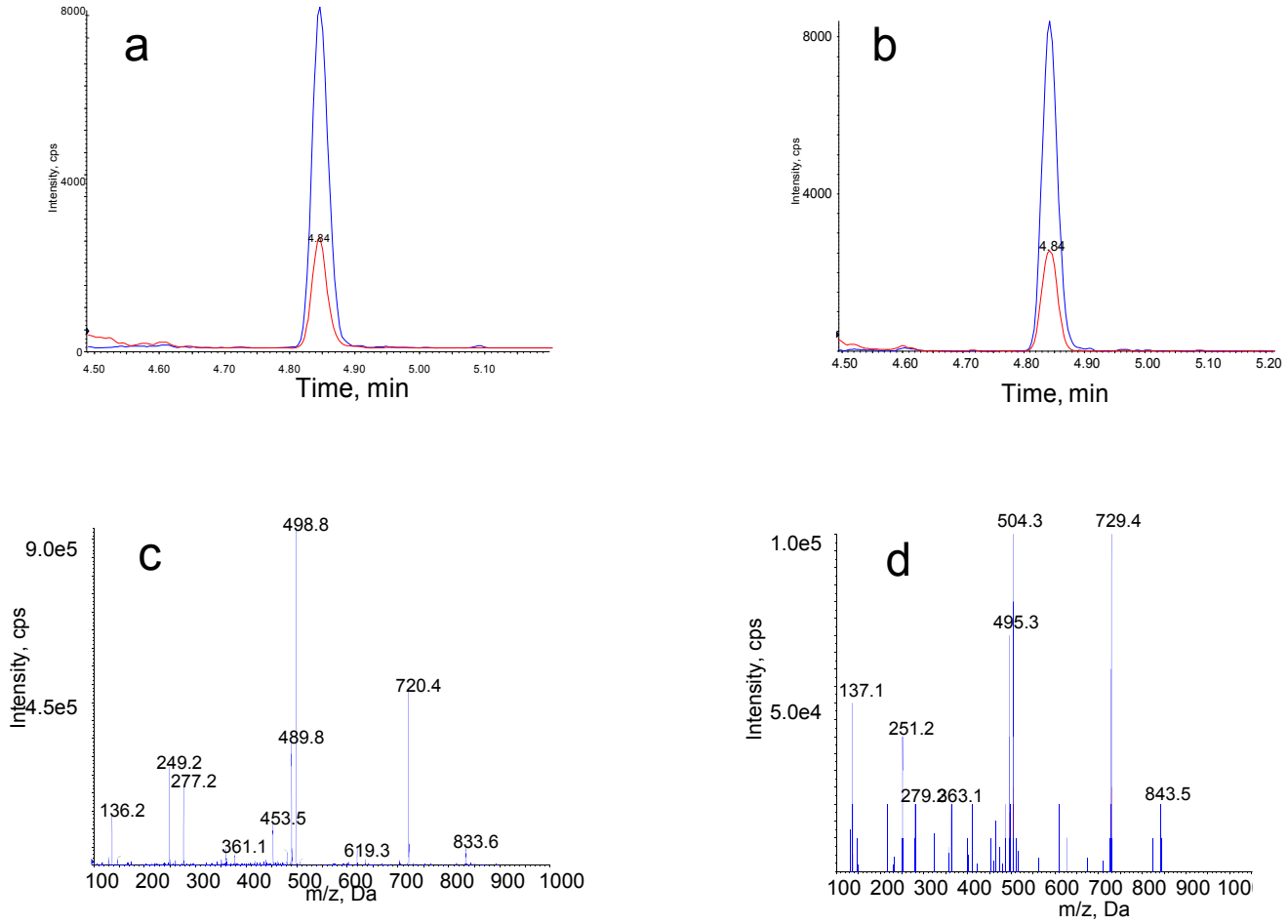
Sample preparation was performed on a liquid handler Janus (Perkin Elmer, CA). To a 200μL aliquot of plasma sample 600 μL of HEPES buffer (pH 7.4) and 20 μL of IS were added and the samples were incubated for 15 minutes. After the incubation, 5 μL of the magnetic beads suspension was added, and the samples were incubated with agitation at 10°C for 3 h. The beads were washed three times with TRIS buffer (pH 7.4), then 200 μL of 25mM bicarbonate buffer, and

10 µL of trypsin (4 µg/µL) were added, and the samples were incubated for 3 hours at 37°C. After the digestion 10 µL of 10% formic acid was added to the samples, the tubes were vortexed, the samples were transferred in wells of a 96-well plate, and 70 µL aliquots were injected in LC-MS/MS. Magnetic beads were processed using magnetic stand for 96-well plates. Effect of the conditions of the denaturing, reduction, alkylation, digestion, affinity enrichment, magnetic beads washes and peptide elution was evaluated in order to optimize the method's performance. Experiments showed no effect of the denaturing, cysteines reduction and alkylation on the recovery of the targeted peptide; therefore reduction and alkylation were not used in this method.

Instrumental analysis.

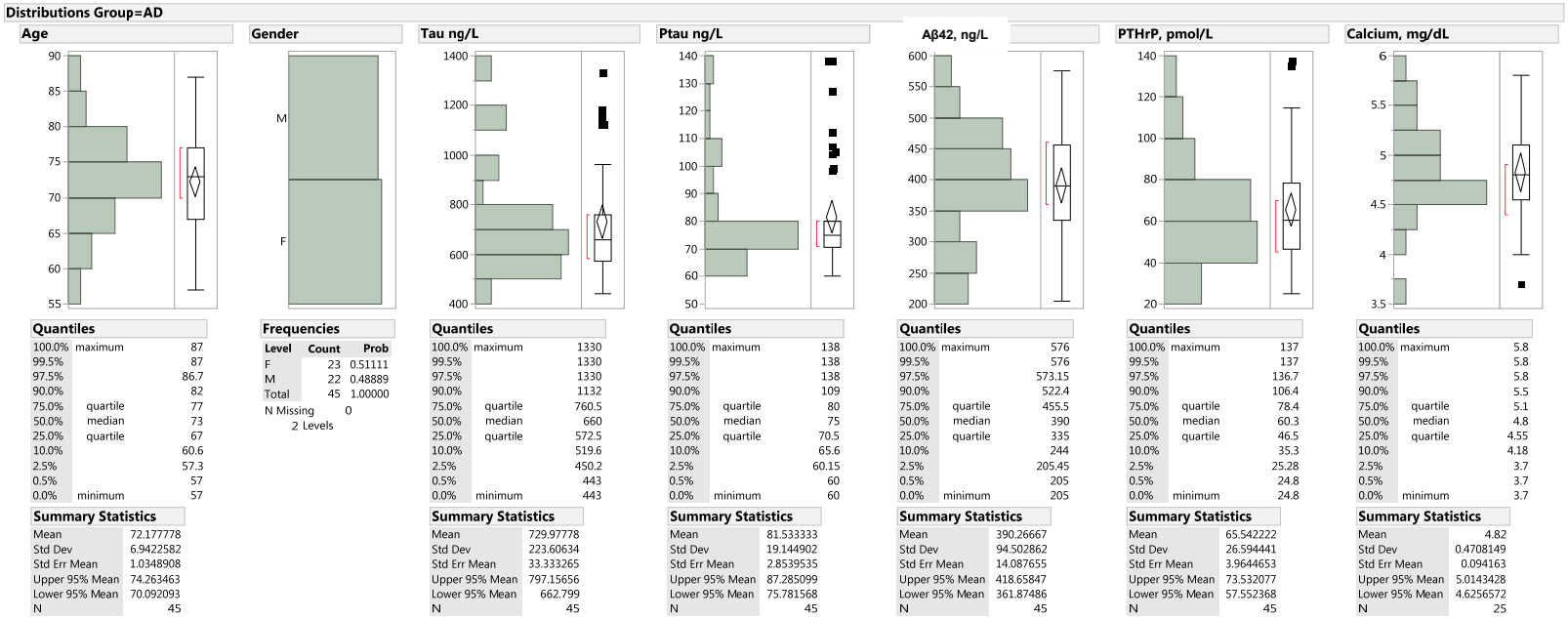
The two-dimensional (2D) HPLC separation was performed on an HPLC system consisting of series 1260 and 1290 pumps (Agilent Technologies, Santa Clara, CA). A Synergy Polar RP, 50x3mm, 4 µm HPLC column (Phenomemex, Torrance, CA) was used for the 1stD separation with gradient: 99% to 87% A in 2.7 min (A, 10 mM formic acid in water; B, 10 mM formic acid in acetonitrile); the 2ndD separation was on a Synergy Max-RP, 100x3mm, 2.5µm, column (Phenomemex) using gradient 97% to 85% A in 2 min (A, 5 mM acetic acid in water; B, 5 mM acetic acid in acetonitrile); the LC separation was performed at 40°C.

Quantitative analysis was performed on an AB6500 mass spectrometer (AB Sciex, Framingham, MA) with a V-spray ionization source in a positive ion, multiple reaction monitoring (MRM) mode. Mass transitions monitored were *m/z* 498.75→720.35, 499.25→721.35, for the YLTQETNK peptide, and *m/z* 504.25→729.35, 504.75→730.25, for the IS. The instrument settings were adjusted to maximize the sensitivity and the specificity of detection. The heating gas temperature was 650°C; the nebulizing, curtain and collision gases (nitrogen) were 90 psi, 70 psi and 9 arbitrary units. The declustering potential, collision energy, collision cell exit potential, and entrance potential were 60V, 23V, 26V, and 10V. The dwell time for the mass transitions was 40 ms. The Q1 and Q3 quadrupoles were set to unit resolution. The total analysis time per sample was 6.5 min. The instrument control and the data were performed using Analyst 1.6.2 (AB Sciex).

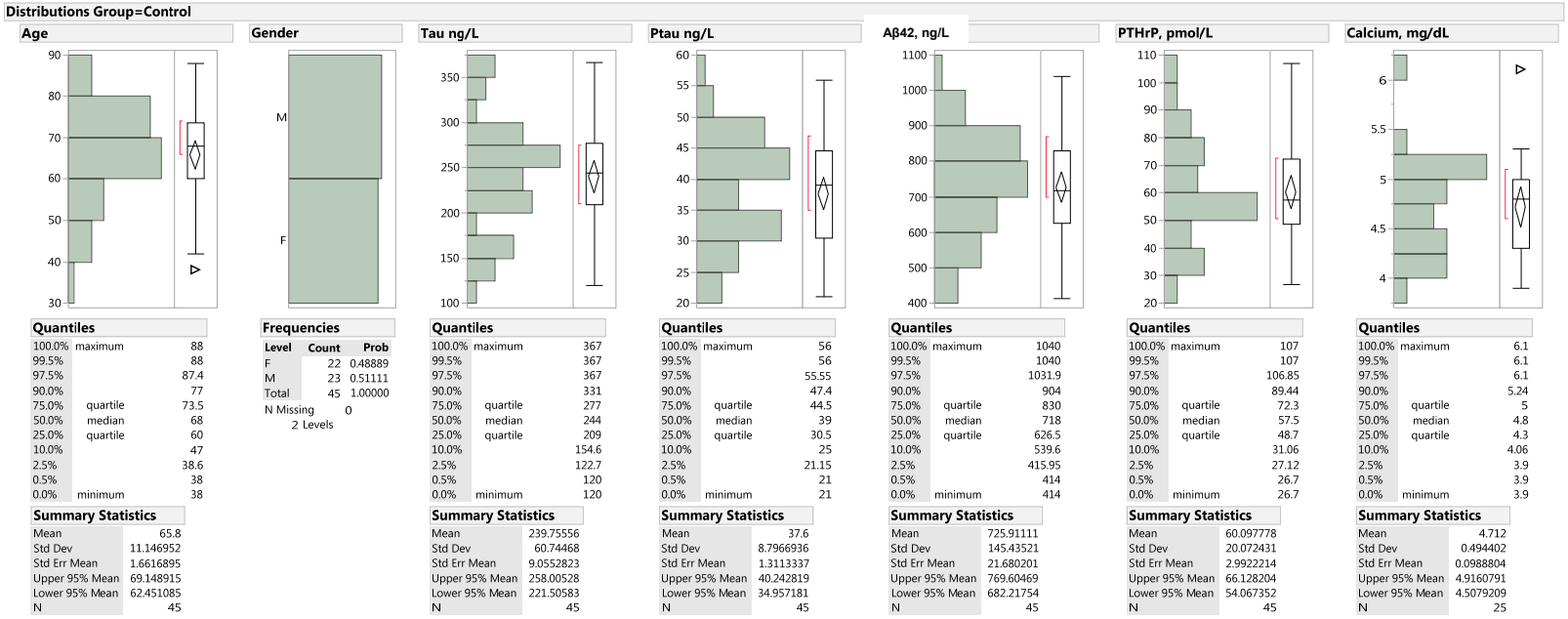


Supplemental Figure 1. Representative chromatograms of patient CSF sample containing 55 pmol/L of PTHrP (a) two mass transitions of the PTHrP specific peptide, (b) two mass transitions of the ^{15}N labeled internal standard. Product ion mass spectrum of the PTHrP-specific peptide YLTQETNK (c, parent ion *m/z* 498.8), and its analog used as internal standard (all nitrogens in amino acids replaced with ^{15}N (d, parent ion *m/z* 504.3)).

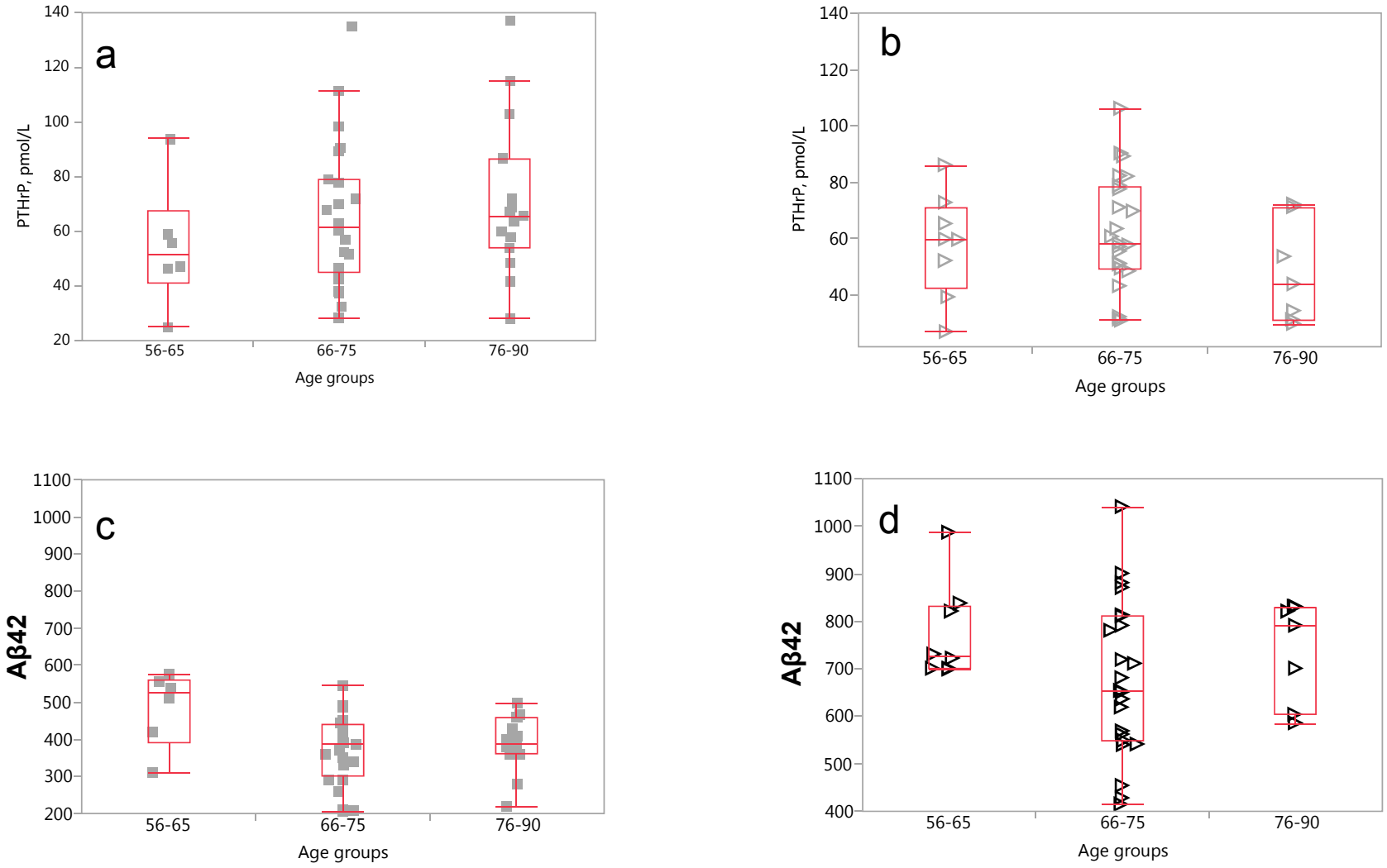
a



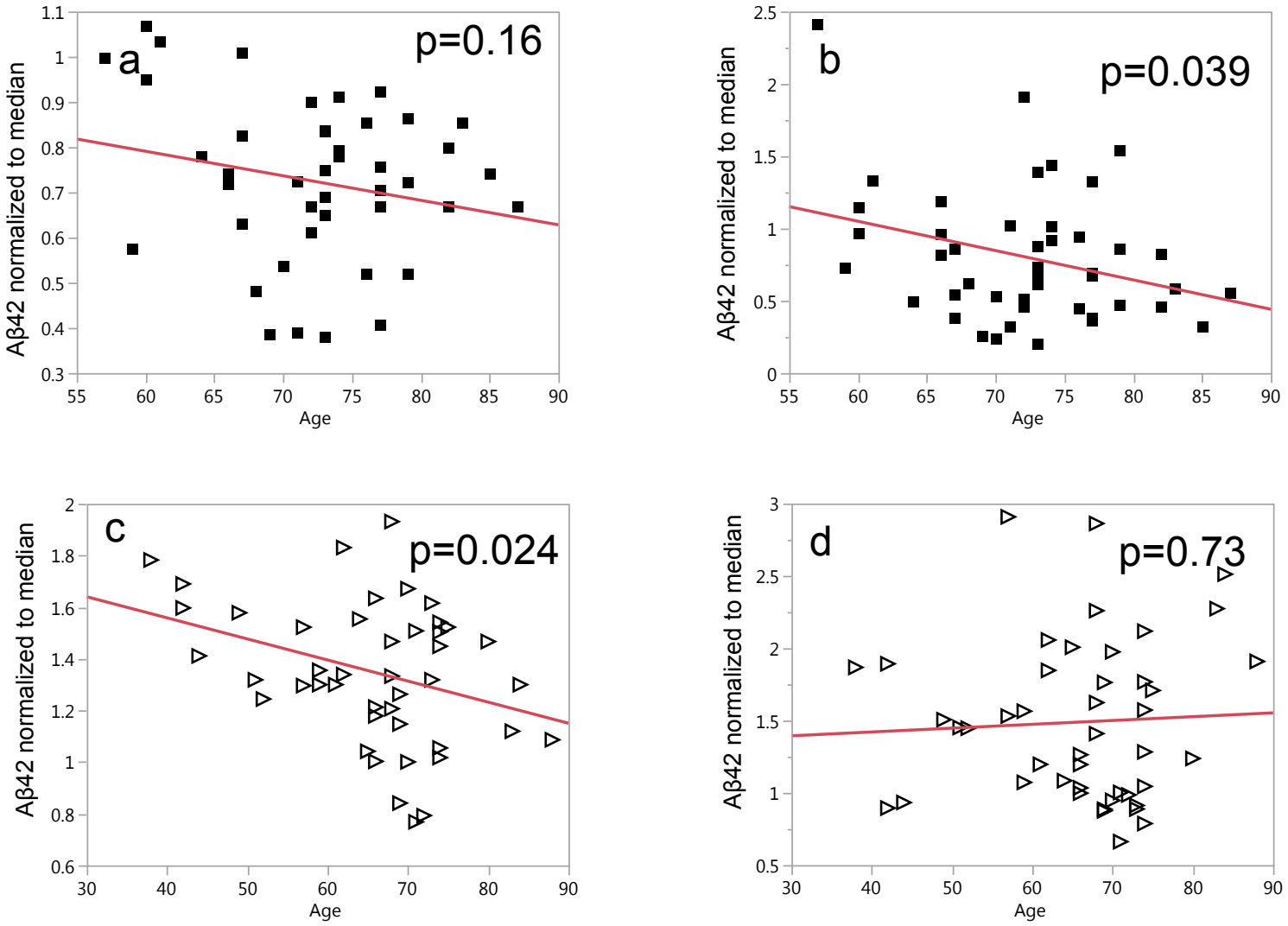
b



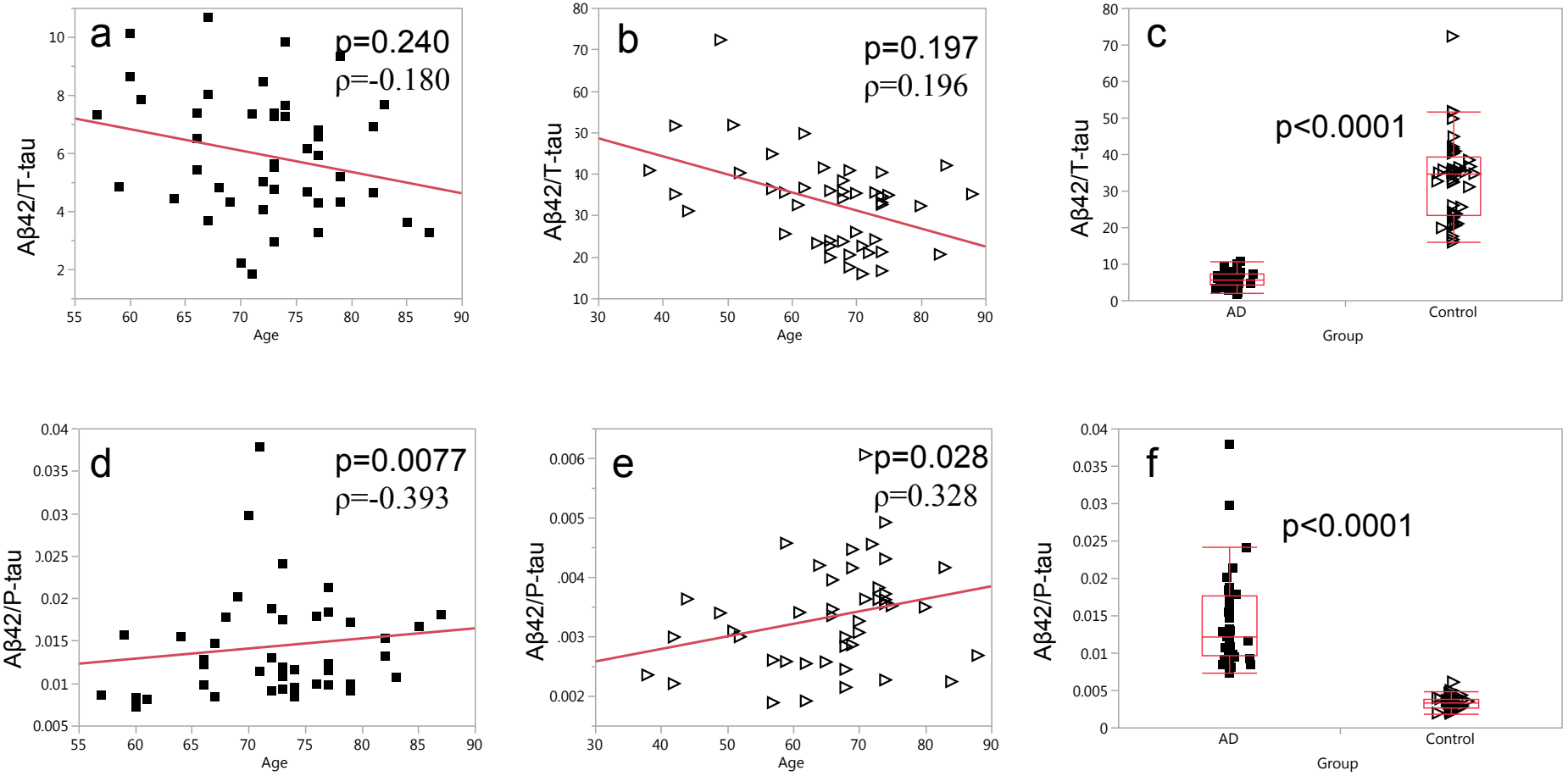
Supplemental Figure 2. Histograms with distribution of age, gender, and concentrations of T-tau, P-tau, Aβ42, PTHrP and calcium in (a) AD and (b) control groups.



Supplemental Figure 3. Box plots with distribution of PTHrP (a,b) and Aβ42 (c,d) concentrations in AD (a,c) and controls (b,d) by age groups.



Supplemental Figure 4. Association of the normalized to median values of Aβ42 concentrations (a,c) and Aβ42/PTHrP ratios (b,d) with age in AD patients (a,b) and controls (c,d).



Supplemental Figure 5. Association of Aβ42/T-tau (a, b, c) and Aβ42/P-tau (d, e, f) ratios with age in a, d– AD group (n=45) and b, e– control group (n=45); c, f – box plots with distribution of the Aβ42/T-tau (c) and P-tau ratios in the AD and the control groups.