

Neurodegenerative Markers are Increased in Post-Mortem BA21 Tissue
from African Americans with Alzheimer's disease

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ABSTRACT

Background: Alzheimer's disease (AD) presents with an earlier onset age and increased symptom severity in African Americans and Hispanics.

Objective: Although the prevalence of plaques and tangles may not exhibit ethnicity-related differences, levels of neurodegenerative proteins have not been described.

Methods: Here, levels of 5 proteins (i.e., S100B, sRAGE, GDNF, A β 40, and A β 42) and the A β 42/A β 40 ratio were measured in post-mortem samples of the middle temporal gyrus (BA21) from age-matched African Americans and Caucasians with AD (n=6/gender/ethnicity).

Results: S100B levels were increased 17% in African Americans ($p < 0.003$) while sRAGE was mildly decreased ($p < 0.09$). A β 42 levels were increased 121% in African Americans ($p < 0.02$), leading to a 493% increase in the A β 42/A β 40 ratio ($p < 0.002$). Analysis of GDNF levels did not indicate any significant effects. There were no significant effects of gender and no significant ethnicity with gender interactions on any analyte. Effect size calculations indicated "medium" to "very large" effects.

Conclusion: S100B is typically elevated in AD cases; however, the increased levels in African Americans here may be indicative of increased severity in specific populations. Increased A β 42/A β 40 ratios in the current study are compatible with increased disease severity and might indicate increased AD pathogenesis in African Americans. Overall, these results are compatible with a hypothesis of increased neuroinflammation in African Americans with AD.

Keywords: Racial differences, Ethnic groups, Temporal gyrus, Amyloid beta, African American, Caucasian, S100B, sRAGE

INTRODUCTION

In 2010, Harry Johns, then Alzheimer's Association President and CEO, noted that relative to Caucasians "the threat (*of AD*) is even more substantial in the African-American and Hispanic communities" (http://www.alz.org/news_and_events_18984.asp). This is partially related to the higher incidence of dementia and AD [1-7] as well as increased severity of symptoms in African Americans [8-10]. For example, 21% of African Americans aged 71 years and older have AD relative to only 11% of Caucasians [11] and first degree relatives of African Americans with AD have a higher risk than Caucasians [12]. That increased incidence or risk remains high despite adjustments for education, family dementia histories, and hypertension co-morbidity [13]. Socioeconomic status, other health conditions, health care access, and delays in physician consultation likely contribute to those disparities [14, 15]. However, regardless of those socio-environmental factors, it is compelling that higher percentages of African ancestry were detected in African Americans with AD than those without AD [16]. As Kukull and Martin bluntly stated "being African American (without the *APOE* ϵ 4 allele) may be one of the strongest risk factors for AD yet observed" [17].

Increased research of African Americans and other minority populations with AD is crucial to the goals of precision medicine and yet this is traditionally an understudied population [18]. This does, however, appear to be changing. Although the prevalence of the characteristic plaques and neurofibrillary tangles was reported not to differ between African Americans and Caucasians with AD [19-21], a more recent study described increased AD-associated neuropathology in African Americans [22]. Further, there are now 4 late onset Alzheimer's disease (LOAD) risk genes identified for African Americans from genome-wide association studies and one of those gene variants is found only in African Americans [23, 24]. Such studies indicate the advantage of using different approaches to understanding what is likely to be a complex picture of AD-related ethnicity differences. Several studies have reported elevated CNS levels of A β 42, S100B, and others in those with neurodegenerative diseases such as AD, Down syndrome, and schizophrenia [25-27]. However, it is not clear if such levels differ by ethnicity in AD cases.

In this study, five neurodegeneration-associated protein levels were measured in post-mortem African American and Caucasian brain tissue from both genders to explore ethnicity-related differences. The selected proteins include those thought to be critically involved in AD (e.g., A β 42). Those proteins were examined in samples of the middle temporal gyrus (Brodmann's area 21) that were matched for age at death. This region was specifically selected as it

is critically involved in language processing and generation [28-30] and has been shown to be significantly affected by AD [31-33]. A Luminex MAP multiplex assay platform allowed the simultaneous measurement of those five analytes in a small sample volume (for a detailed description of this methodology, see [34]). This methodology has been used to successfully measure cytokines and inflammatory proteins in post-mortem AD human brain tissue [35-37]. Further, this state-of-the-art technology has proven useful in delineating control and AD patients via cerebrospinal fluid levels of tau, phosphorylated tau and A β ₄₂ [38].

MATERIALS AND METHODS

Tissue samples

De-identified frozen AD-confirmed samples of BA21 (pulverized tissue or tissue slices) (n=6/ethnicity/gender) were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA), the Human Brain and Spinal Fluid Resource Center (VA Greater Los Angeles Healthcare System, Los Angeles, CA), the Mount Sinai NIH Brain and Tissue Repository (Bronx, NY), the University of Maryland Brain and Tissue Bank (Baltimore, MD), and the University of Miami Brain Endowment Bank (Miami, FL). Confirmation of AD for each subject was done via microscopic examination of the tissue per consensus guidelines using CERAD, Thal, or Braak stage criteria. Detailed information including ethnicity and gender is shown in Table 1. Although ethnicity was “self-reported”, a PCA plot of variant calls indicated a clear separation of the African American and Caucasian samples (unpublished data). All samples were stored at -80°C.

Ethics approval and consent

Each of the brain banks listed above operated under their institution’s IRB approval. All experimental procedures described here were approved by the appropriate committees at the National Center for Toxicological Research/FDA, including the NCTR Office of Research, the Regulatory Compliance and Risk Management Director and the FDA Research Involving Human Subjects Committee (RIHSC). As determined by the FDA RIHSC, this study did not reach the definition of “Human Subject Research” at 45 CFR 46.102(f) and thus, 45 CFR Part 46 does not apply.

Sample preparation

All study procedures were conducted using recommended BSL-2 standards and in accordance with the NCTR/FDA biosafety procedures. In a precooled mortar and pestle set on dry ice, approximately 50-100 mg tissue was pulverized into a fine powder in liquid nitrogen and immediately transferred to a precooled microcentrifuge

tube on dry ice. Powdered tissue was maintained at -80°C until further processing.

For all assays, a Bio-Plex Cell Lysis kit (#171304011, Bio-Rad, Hercules, CA) was used and prepared according to the manufacturer's protocol. Lysis buffer was added to the powdered tissue samples (350 μl of buffer for samples ≤ 50 mg or 500 μl for samples > 50 mg). Tissue and buffer were then homogenized using a MP FastPrep homogenizer (MP Biomedicals, Santa Ana, CA). Samples were spun in a refrigerated microcentrifuge for 20 min at 15,000 RPM and 4°C . The supernatant was aliquoted and stored at -80°C .

Protein assays were conducted using a Pierce BCA protein assay kit (#23225, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol (www.thermo.com/pierce). A 96 well plate reader (Spectramax M2^e with SoftMax Pro 5 software, Molecular Devices, LLC, Sunnyvale, CA) was then used to generate a standard curve and analyze the protein content of each sample with absorbance measured at 562 nm.

Multiplex assay

The multiplex assay was conducted using a Bio-Plex 200 immunoassay system (Bio-Rad, Hercules, CA). When wash steps were required, an automated Bio-Plex Pro wash station (Bio-Rad, Hercules, CA) was utilized. A magnetic plate was used to decant assays when specified by the manufacturer. Standard curves for all assays were fitted using logistic 5PL regression. Equal amounts of protein were used for each sample which was within the recommended protein range of the kit.

The Human Neurodegenerative Disease Panel 4 (HNDG4MAG-36K, EMD Millipore Corporation, Billerica, MA) simultaneously analyzed S100B, amyloid $\beta 40$ ($\text{A}\beta 40$), amyloid $\beta 42$ ($\text{A}\beta 42$), sRAGE (soluble receptor for advanced glycation end products), and GDNF (glial cell derived neurotrophic factor). Although this kit is validated by the manufacturer for use with cerebrospinal fluid only, it has been successfully used in studies of human brain tissue (e.g., [35]). Further, all analytes were measurable in all subjects. The plate was prepared using tissue lysates at a protein concentration of 500 $\mu\text{g}/\text{ml}$ and all procedures followed the manufacturer's protocol (see: http://www.emdmillipore.com/US/en/product/MM_NF-HNDG4MAG-36K#anchor_PR). From the $\text{A}\beta 40$ and $\text{A}\beta 42$ levels, the ratio ($\text{A}\beta 42/\text{A}\beta 40$) was calculated for each subject and included in statistical analyses.

Statistical Analysis

Levels of each analyte were analyzed via SigmaPlot (V. 13, Systat Software, Inc., San Jose, CA) using a two-way analysis of variance (ANOVA) with ethnicity, gender, and the interaction as factors. A base 10 log conversion was done on all data as several analytes were not normally distributed as assessed by the Kolmogorov-

Smirnov.

Reporting of effect sizes in addition to traditional null hypothesis significance testing has been strongly advocated [39]. Effect sizes describe the magnitude of the effect, are independent of sample size, scale-free, and aid in the interpretation of the substantive or practical significance of a result. Here, Cohen's d was determined for the effect of ethnicity for each analyte. Those endpoints which resulted in a large effect size (≥ 0.80 as defined by Cohen [40]) were considered to definitively distinguish the two ethnicities.

RESULTS

This study measured levels of neurodegenerative markers in the BA21 brain region in $n=6/\text{sex}$ of African Americans or Caucasian who were matched for age. The mean and standard error of each protein by ethnicity and gender are listed in Table 2.

A number of quality checks were performed for the multiplex assay. As recommended by the technical support specialists at Bio-Rad and a Bio-Rad field application specialist (see: http://users.stlcc.edu/Departments/fvbio/Array_Reader_BestPractices_AReid.pdf), several checks were completed to ensure the quality of the data. These checks were: 1) all bead counts were substantially above the recommended level of 30 and over 98% were above 50; 2) there were no sampling errors; 3) recovery values ranged 96-117% which is within the recommended range of 70-130%; 4) all controls fit well within the range of the standards; 5) percent differences between the observed concentrations values for the 7 standards for each analyte and those values listed in the manual were calculated and ranged 0.03 to 7.43%, indicating that the values for the standards were very close to what should have been measured.

S100B was augmented in African Americans with AD compared with age-matched Caucasians

Comparison of the protein expression of S100B in the BA21 brain region of African Americans versus Caucasians revealed that the expression of S100B was significantly increased in African Americans. Levels of S100B were increased approximately 17% in African Americans ($F(1, 20)=13.33, p<0.003$) (Cohen's $d=1.47$ or "very large") (see Fig. 1). There was no significant effect of gender or a significant interaction of gender with ethnicity.

Elevated $A\beta_{42}/A\beta_{40}$ ratios in African Americans compared with Caucasians

A prominent difference was detected in the $A\beta_{42}/A\beta_{40}$ ratio which was increased approximately 493% in African Americans ($F(1, 20)=12.39, p<0.002$) (Cohen's $d=0.88$ or "large") (see Fig. 2, bottom). The significant

elevation in the A β 42/A β 40 ratio was due in part to an approximately 121% increase in A β 42 levels in African Americans ($F(1, 20)=7.86, p<0.011$) (Cohen's $d=0.96$ or "large") (see Fig. 2, top). However, no significant effects of ethnicity, gender, or the interaction were detected in the analysis of A β 40. Furthermore, no significant effects of gender or the interaction of gender with ethnicity were observed in the analyses of A β 42 levels or the A β 42/A β 40 ratio.

sRAGE and GDNF do not show ethnicity-specific differences in AD

Levels of sRAGE were marginally decreased in African Americans ($F(1, 20)=3.37, p<0.09$) (Cohen's $d=0.69$ or "medium"). There were no significant effects of gender or the interaction of ethnicity and gender.

There were no significant effects detected in the analysis of GDNF levels (Cohen's $d=0.19$ or less than "small") and no significant effects of gender or the interaction of ethnicity and gender.

DISCUSSION

The underlying sources and mechanisms of the increased AD prevalence and severity in African Americans are only beginning to be studied and are likely to be complex and multifaceted. This study is the first describing significant elevations of S100B, A β 42, and the A β 42/A β 40 ratio in the middle temporal gyrus (BA21 region) of African Americans with AD relative to Caucasians. sRAGE levels were also marginally decreased in African Americans. Age at death, Braak Stage, and health status (where available) were similar across ethnicities. Although the sample size here was not large and the results must be replicated, the effect sizes are extremely encouraging with respect to the validity. Further, these results are consistent with increased AD severity in African Americans relative to Caucasians.

The calcium binding protein, S100B, which is primarily produced by astrocytes is known to regulate various cellular process, and is reported to be altered in AD [41]. Here, S100B levels were significantly elevated in African Americans and indicated a very large effect size. Although S100B levels increase with normal aging [42, 43], hippocampal and temporal lobe levels of S100B are significantly elevated in AD cases [26, 44, 45]. At micromolar extracellular concentrations, S100B can act as a cytokine, causing apoptosis and the production of reactive oxygen species (reviewed in [46, 47]). Like A β , S100B is a ligand for the receptor for advanced glycation end products (RAGE) which when activated can lead to increases in several AD-related signaling pathways. Furthermore, the extracellular release of S100B can be triggered by a number of stimuli, including TNF- α , IL-1 β , and A β [48-50]. While many studies have strongly implicated a role for S100B in AD (e.g., see [26, 51]), the results

here suggest that elevated S100B levels may be related to increased severity in specific populations.

Advanced glycation end products and its soluble receptor have been implicated in the pathogenesis of AD [52, 53]. Although not statistically significant, levels of the soluble form of RAGE (sRAGE) were decreased 48% in African Americans and indicated a medium effect size. sRAGE lacks the transmembrane domain and intracellular tail and is thought to act as an extracellular decoy by sequestering RAGE ligands [47, 54-56]. That sequestration can then prevent the activation of RAGE and its downstream pathological signaling effects. Reports of plasma/serum measures of sRAGE in AD cases have not been consistent [57-59]. Further, overall sRAGE levels in post-mortem AD brain tissue have not been described, although an alternative splicing isoform of sRAGE (i.e., sRAGE Δ) was described as decreased in AD cases relative to controls in several brain regions [60]. A slightly different form of sRAGE, endogenous secretory RAGE (esRAGE) formed from proteolysis, was reported to be lower in hippocampal neurons in areas CA1 and CA3 of AD cases [61]. Most sRAGE assays, including that reported here, measure both sRAGE and esRAGE. The hypothesis that sRAGE levels might attenuate disease severity/pathology has led to a focus on potential therapeutic sRAGE interventions (see review by [62]).

A β 42 is the predominant form of amyloid β found in the brain of those with Alzheimer's disease. An increase in A β 42 is correspondingly accompanied by a decrease in A β 40, and the ratio of A β 42/A β 40 is a well-accepted marker of Alzheimer's disease. Levels of A β 42 and the A β 42/A β 40 ratio were significantly increased in African Americans with AD relative to Caucasians, and both endpoints indicated a large effect size. Although not statistically significant, the calculated effect size for A β 40 levels was large as well, indicating lower levels in African Americans. A β 42 is typically considered the more neurotoxic protein, as increased A β 42/A β 40 ratios cause an earlier onset and more severe form of AD [63]. The A β 42/A β 40 ratio is increasingly recognized as a better diagnostic marker of AD [64]. Further, regional brain measurements of A β 42 can provide increased dementia predictability [65].

The choice of the BA21 or middle temporal gyrus for analysis was based on its reported involvement in AD. This region, particularly the left portion, is critically involved in language processing and semantic memory [28-30, 66]. In fact, higher A β levels in the temporal cortex are associated with a faster decline in language, average cognition and executive/speed scores in non-AD non-demented African Americans, but not Caucasians [67]. We are unaware of any evidence that this brain region would be selectively impaired in African Americans with AD, but the current results could indicate ethnicity-related differences in language processing in those with AD. However,

there is little information in this area, and the multiple potential confounders, such as education quality, do not allow for easy interpretation of some results. For example, some studies have indicated ethnicity-related lower language test scores in the cognitively normal elderly and in those with AD [68, 69]. Yet when adjusted for reading level, no significant ethnicity-related differences in language assessments of African Americans and Caucasians with AD were apparent [70].

The results described here are promising and strongly suggest avenues for future research; however, they are not without limitations. Information on any cognitive evaluations was not available. The small sample size here might be cause for concern; however, the direction of the significant effects indicates increased neurodegeneration on African Americans which is consistent with the observed symptomology. In general, African Americans are much less likely to agree to brain donation [71] which contributed to the difficulty in obtaining these samples. However, efforts are underway to increase such donations [72, 73]. Only one of the brain banks that contributed tissue to this study collected information on current medications; thus, it is not clear if ethnicity-related differences in medication use may have impacted the results. However, fully 50% of the analyses were statistically significant (i.e., analysis of the 5 analytes and the A β 42/A β 40 ratio), an effect not likely due to chance. Finally, levels of tau were not measured here which may have provided a clearer overall analysis of the increased AD severity in African Americans.

CONCLUSION

The ethnicity differences reported here from the middle temporal gyrus are consistent with increased neuropathology in African Americans with AD. Specifically, African Americans exhibited increased levels of s100B, A β 42, and the A β 42/A β 40 ratio but somewhat lower levels of sRAGE. The current results add to the growing number of studies describing AD ethnicity-related differences in a variety of endpoints, including micro RNAs, SNPs, alleles, genetic variants determined from genome-wide association studies (GWAS) and exonic sequencing, and long runs of homozygosity (ROHs) as well as polymorphisms in *ESR* genes. Given the multifactorial nature of AD, the current results may be partially related to ethnicity-related differences in AD therapeutic drug use [74, 75], socioeconomic or other potential risk factors; however, they indicate increased neuropathology in African Americans with AD. Overall, they are compatible with a hypothesis of increased neuroinflammation in African Americans with AD and could have significant future ramifications.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

DISCLAIMER

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TABLE 1: Subject characteristics

Ethnicity	Gender	Age at Death	Post-Mortem Interval (hr)	Brain Bank	Comments
AA	Male	76	25	UMD	Braak stage V-VI; Coronary heart disease, COPD, hyperlipidemia, hypertension
AA	Male	78	23	MS	4.2 plaque/mm ² *
AA	Male	79	8	MS	10.46 plaque/mm ²
AA	Male	80	22.3	MS	20.72 plaque/mm ²
AA	Male	83	27	UMD	
AA	Male	87	8	UMD	Braak stage V-VI; Pneumonia, hypertension, diabetes, history of deep venous thrombosis
AA	Female	77	46	UMD	
AA	Female	78	3.7	MS	20.24 plaque/mm ²
AA	Female	82	14.6	MS	7.53 plaque/mm ²
AA	Female	84	16.1	MS	3.85 plaque/mm ²
AA	Female	84	9	MS	6.21 plaque/mm ²
AA	Female	85	26.8	MS	7.9 plaque/mm ²
C	Male	76	N/A	HBSFRC	Atherosclerosis
C	Male	83	N/A	HBSFRC	
C	Male	87	N/A	HBSFRC	TIA, coronary heart disease, congestive heart failure, hypertension, hyperlipidemia, hypothyroidism, atherosclerosis
C	Male	78	N/A	HBTRC	Braak stage VI
C	Male	80	N/A	HBTRC	Braak stage V
C	Male	80	27.8	HBTRC	Braak stage IV
C	Female	77	N/A	HBSFRC	Atherosclerosis
C	Female	78	N/A	HBTRC	Braak stage V
C	Female	82	N/A	HBTRC	Braak stage V
C	Female	85	N/A	HBTRC	Braak stage V
C	Female	88	10.88	HBTRC	Braak stage V
C	Female	85	N/A	UMBEB	Braak stage V-VI; hypertension, hypothyroidism, carotid artery stenosis

Abbreviations: AA: African American; C: Caucasian; UMD: University of Maryland; MS: Mount Sinai; UMBEB: University of Miami Brain Endowment Bank; HBTRC: Harvard Brain Tissue Resource Center; HBSFRC: Human Brain and Spinal Fluid Resource Center

*Plaque/mm² was measured in the middle frontal gyrus, orbital frontal cortex, superior temporal gyrus, inferior parietal lobule, and occipital cortex by the brain bank (Mount Sinai).

TABLE 2: Results of neurodegenerative disease panel (mean±standard error; n=6/gender/ethnicity)

Neurodegenerative marker	AA Males	AA Females	C Males	C Females
S100B (pg/ml)	4,551±273	4,766±176	3,897±90	4,059±187
Aβ40 (pg/ml)	34.32±13.86	39.63±17.44	132.93±82.66	190.73±84.93
Aβ42 (pg/ml)	744±230	452±73	252±41	289±132
Aβ42/Aβ40	56.40±29.73	25.63±10.27	7.56±3.47	6.27±2.64
sRAGE (pg/ml)	13.52±2.59	10.42±0.54	16.85±1.82	18.60±6.32
GDNF (pg/ml)	1.3050±0.1630	1.1550±0.0428	1.1517±0.0430	1.2250±0.0490

FIGURE LEGENDS

Figure 1: Levels of S100B were significantly increased in African Americans with AD ($p < 0.003$). The dashed and solid lines inside each box represent the mean and median, respectively. The “whiskers” at the top and bottom of each box are the 90th and 10th percentiles, respectively. Solid points are those levels lying outside those percentiles.

Figure 2: Top: Levels of A β 42 were significantly increased in African Americans with AD ($p < 0.011$). Bottom: The A β 42/A β 40 ratio was significantly increased in African Americans with AD ($p < 0.002$). Plot symbols are defined as in Figure 1.

Fig. 1: S100B levels are increased 17% in African Americans

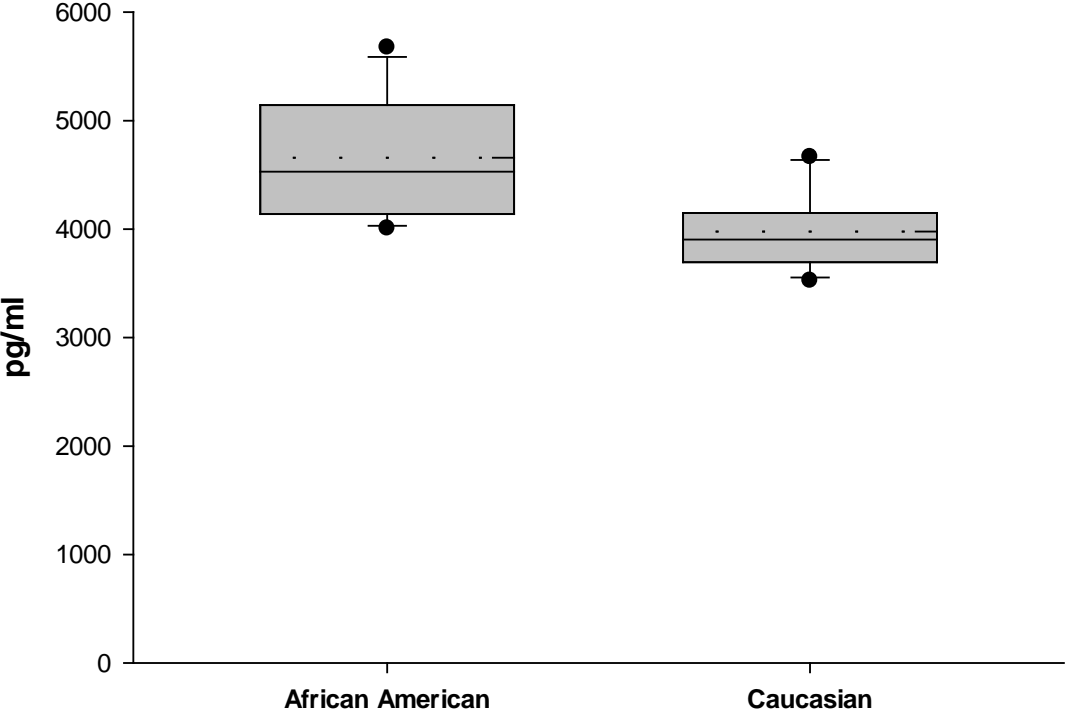


Fig. 2 (top): A β 42 levels are increased 121% in African Americans

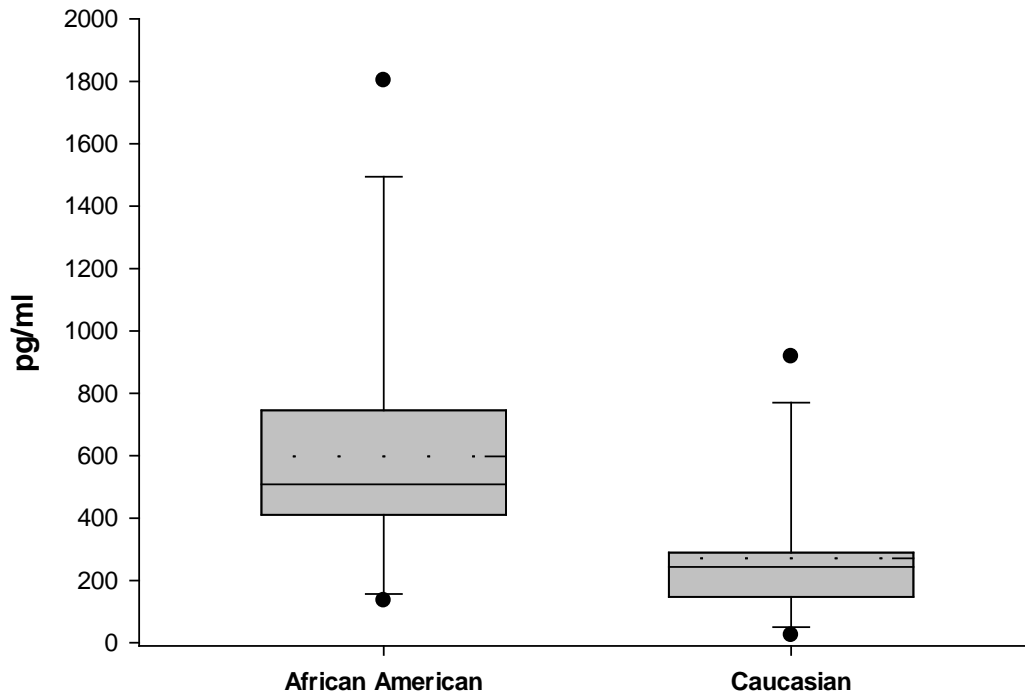


Fig. 2 (bottom): The A β 42/A β 40 ratio is increased 493% in African Americans

