

Original Article

Peripheral biomarkers in Autism: secreted amyloid precursor protein- α as a probable key player in early diagnosis

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Abstract: Autism is a pervasive developmental disorder characterized by impairments in socialization and communication. There is currently no single molecular marker or laboratory tool capable of diagnosing autism at an early age. The purpose of this study is to explore the plausible use of peripheral biomarkers in the early diagnosis of autism via a sensitive ELISA. Here, we measured plasma secreted amyloid precursor protein alpha (sAPP- α) levels in autistic and aged-matched control blood samples and found a significantly increased level of sAPP- α in 60% of the known autistic children. We then tested 150 human umbilical cord blood (HUCB) samples and found significantly elevated levels of plasma sAPP- α in 10 of 150 samples. As an additional confirmatory measure, we performed Western blot analysis on these samples which consistently showed increased sAPP- α levels in autistic children and 10 of 150 HUCB samples; suggesting a group of autistic patients which could be identified in early childhood by levels of sAPP- α . While there is need for further studies of this concept, the measurement of sAPP- α levels in serum and human umbilical cord blood by ELISA is a potential tool for early diagnosis of autism.

Key Words: Autism; autism spectrum disorders (ASD); secreted amyloid precursor protein- α (sAPP- α); brain derived neurotrophic factor (BDNF)

Introduction

First described by Dr. Leo Kanner, autism is a pervasive developmental disorder (PDD) characterized by the presence of limited interests and activities, as well as by impairments in socialization and communication [1, 2]. Despite the fact that there are many suggestions for the causes of this disorder, including, but not limited to, genetics, the environment, and vaccinations, there is no one cause of autism [3]. With the advent of electroencephalography (EEG), observations of aberrant patterns in autistic

patients contributed the contemporary understanding of the syndrome as a brain-based disorder [4]. The heterogeneity of the clinical syndrome would seem to indicate that the disorder termed autism may arise from a constellation of different etiologies [5, 6]. For example, about one quarter of autistic patients have comorbid epilepsy [7]. Studies suggest another subgroup, some 40 - 55% of autistic patients, suffers mental retardation [8-10]. Furthermore, even though the heritability of autism is relatively high, only some 10% of cases can be attributed to a known genetic aberration [5].

Although there are diagnostic criteria listed in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), the International Statistical Classification of Diseases and Related Health Problems (ICD-10), the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R), the disorder is currently diagnosed solely using core behavioral criteria selected to define autism, typically during the toddler or preschool years at the earliest [11]. There is presently no clinical laboratory test for diagnosing autism [12]. To begin intervention at the earliest possible time, the development of biological quantitative methods to predict the presence or risk of autism is necessary.

A common biological correlate of autism, which may be used in the future as a quantitative diagnostic tool, is abnormal brain overgrowth [13-15]. Macrocephaly is said to occur in 15-35% of autistic children [10]. Specifically, observations of brain volume through magnetic resonance imaging (MRI) as well as head circumference studies suggest that regulation of brain growth is abnormal in autism, as indicated by early brain overgrowth followed by abnormally slow brain growth [14, 16]. Moreover a positive association between increasing radiate white matter volume and motor skill impairment in children with autism has also been shown [17]. However, brain overgrowth observations by way of head circumference or brain volume measurements via MRI would be an inaccurate method of diagnosis for autism.

Brain overgrowth may be detected by looking for signs of a central nervous system (CNS) anabolic state during childhood. Indeed this has been suggested by studies of brain-derived neurotrophic growth factor (BDNF, a modulator of neuronal development and maintenance) levels in both brain and blood of autistic children [18]. Observed BDNF levels were three times as high in basal forebrain of autistic patients compared to adults of comparable age [19, 20]. Likewise, Nelson and colleagues found elevated neonatal concentrations of BDNF in autistic spectrum children compared to controls [21]. It has been suggested that serum levels of BDNF, in addition to another neurotrophin, NT-4, be measured in order to aid in the diagnosis of autism and mental retardation [22]. Conversely, a later study reports a delayed increase in serum BDNF levels of autism

patients with development [23]. This inconsistency implies that BDNF is not the best candidate for a peripheral biomarker that can be used in autism diagnosis.

Proteolytic cleavage of amyloid precursor protein (APP) by the sequential actions of β - and γ -secretases form the neurotoxic amyloid beta ($A\beta$) peptide, which typically consists of 40 or 42 amino acid residues (the amyloidogenic pathway). On the other hand the non-amyloidogenic pathway consists of APP cleavage by α -secretase [24] which yields the neurotrophic product, secreted APP- α (sAPP- α). As α -secretase cleaves APP within the $A\beta$ sequence, $A\beta$ formation is subsequently prevented. In a recent report Sokol and colleagues demonstrated, in children with severe autism and aggressive behavior, that serum sAPP- α levels were more than twice that of children without autism and up to four times higher than observed in children with mild autism [18].

Based on the Sokol study, we speculated that sAPP- α is a peripheral biomarker that can be used for the diagnosis of autism. In addition, we have recently developed a sensitive enzyme-linked immunosorbent assay (ELISA) to specifically measure sAPP- α secretion in human plasma and umbilical cord blood and we hypothesize that this ELISA will show a significant difference in sAPP- α levels of autistic patients when compared to healthy individuals. Our goal is to design a laboratory tool for early diagnosis of autism.

Methods

Blood Samples

Blood samples were acquired from the Autism Genetic Resource Exchange (AGRE, California, USA) which provides plasma from children ($n = 25$, 2 - 4 years of age) diagnosed with autism via the Autism Diagnostic Interview-Revised (ADI-R). Additionally, age-matched healthy controls ($n = 25$) were acquired from the same source. Controls were not genetically or otherwise susceptible to autism. Human umbilical cord blood (HUCB) samples ($n = 150$, 85 male and 65 female) were provided by Saneron CCEL Therapeutics, Inc. (Tampa, FL). Cord blood samples were donated by mothers of newborn babies for whom an autism diagnosis was not yet obtained.

sAPP- α ELISA

The sAPP- α ELISA method used was an adapted sandwich ELISA protocol previously described by Olsson [25]. High binding 96-well plates (Nunc, Denmark) were coated with monoclonal antibody 22C11 diluted in 100 μ L (1 μ g/mL) of carbonate buffer (pH 9.6) and

incubated overnight at 4°C. The plate was washed five times with PBS-Tween buffer (0.05% Tween 20) and blocked with 300 μ L of blocking buffer (1% BSA, 5% Horse Serum in PBS) for 2 hrs at 37°C. Synthetic sAPP- α protein (Abgent, San Diego, CA) was used as the positive control for this ELISA. All samples were analyzed in duplicate. Samples of cell

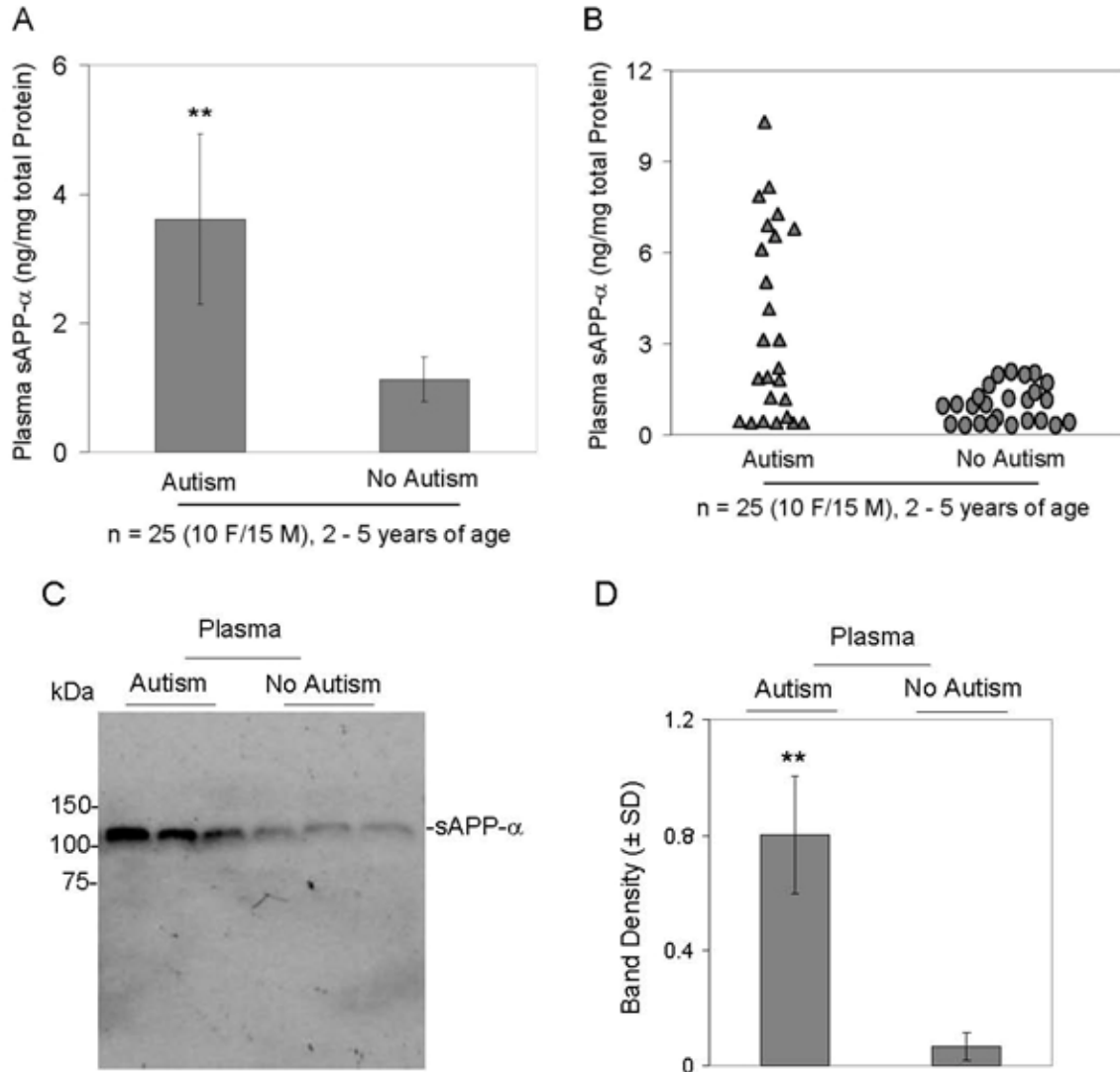


Figure 1. Levels of sAPP- α are elevated in autistic children. (A and B) Plasma sAPP- α levels were measured by sAPP- α ELISA. Data are presented as mean \pm SEM (n = 25 for autistic children, 15 ♂/10 ♀; n = 25 for healthy age matched children, 15 ♂/10 ♀) of sAPP- α (ng/mg total plasma protein). (C) Western blotting analysis consistently shows increased sAPP- α levels in autistic children versus age-matched healthy controls as indicated. Blood plasma samples for both groups of children were randomly selected. The selected samples were then pooled and loaded in triplicate for electrophoresis. The lack of similarity between all three lanes for the autism samples is not due to differences in the individual samples. (D) As quantified in comparison to total protein (normalization), densitometry analysis shows significantly increased density in Western blotting band density as indicated. Data are presented as mean \pm SEM [n = 15 (autistic children), 11 ♂/4 ♀; n = 15 (healthy controls), 8 ♂/7 ♀] of Western blotting band density.

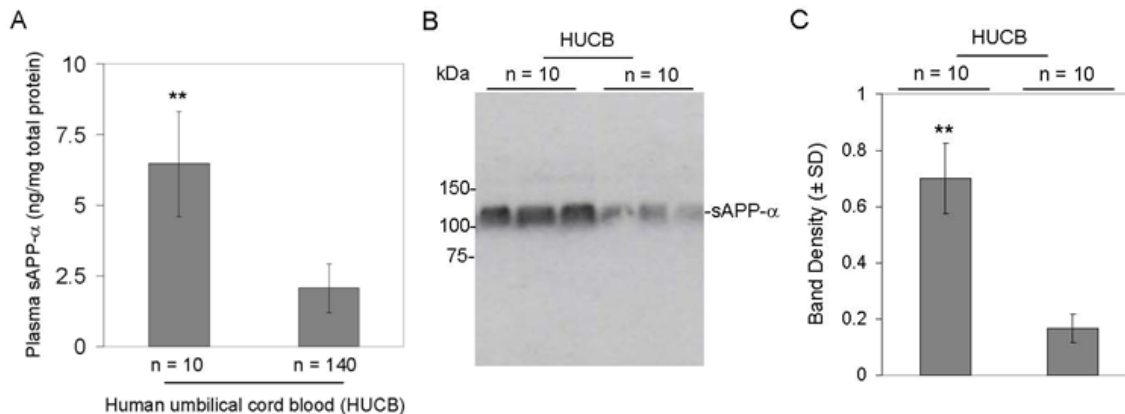


Figure 2. A significantly increased level of sAPP- α is observed in approximately 7% of 150 human umbilical cord blood (HUCB) samples. (A) The plasma was isolated, prepared, and subjected to sAPP- α ELISA. Data indicated elevated levels (> 3 ng/mg total plasma protein) of plasma sAPP- α in 10 of 150 samples. Data are presented as mean \pm SEM of sAPP- α (ng/mg total protein). (B) Western blotting analysis demonstrated increased sAPP- α levels in the same samples which demonstrated elevated sAPP- α by ELISA. (C) As quantified with total protein (normalization), densitometry analysis shows significantly increased density of Western blotting band as indicated. Likewise, samples demonstrating a < 3 ng/mg difference in sAPP- α by ELISA demonstrated the same changes upon Western blotting analysis. HUCB samples from both groups of infants were randomly selected. The selected samples were then pooled and loaded in triplicate for electrophoresis. Data are presented as mean \pm SEM ($n = 10, 8 M/7 F$) of band density.

cultured media or plasma were diluted 1:1 and 1:2 respectively in reagent diluent (1% BSA in PBS) and added to each well of the plate. The plate was incubated for 2 hrs at 37°C. After washing 5 times, 100 μ L of goat anti-human antibody 6E10 (Biosource; diluted 1:3,000 in reagent diluent) was added to each well of the plate. Following 2 hour-incubation at 37°C and 5-time washing, 100 μ L of anti-goat IgG conjugated with HRP (1:1500) was added to each well of the plates. The plate was incubated for 1 hr at 37°C. Following 5-time washing, 100 μ L of substrate solution (TMB) was added to each well and plate was incubated at room temperature. Twenty minutes later, 50 μ L of stop solution (2 N H₂SO₄) was added to each well of the plate. The optical density was determined immediately by a microplate reader at 450 nm. Data were reported as ng of sAPP- α /mg of total intracellular protein or total plasma protein.

Western Blot Analysis

Blood plasma was prepared from peripheral and human umbilical cord blood. Synthetic sAPP- α (Abgent, San Diego, CA) was used as the positive control. Plasma aliquots from

these blood samples corresponding to 100 μ g of total protein were electrophoretically separated using 10% Tris-glycine gels. Electrophoresed proteins were then transferred to PVDF membranes (Bio-Rad), washed in ddH₂O, and blocked for 1 hr at ambient temperature in Tris-buffered saline (TBS; Bio-Rad) containing 5% (w/v) non-fat dry milk. After blocking, membranes were hybridized for 1 hr at ambient temperature with a primary antibody (anti-N-terminal A β ₁₋₁₇ antibody). Membranes were then washed 3 times for 5 min each in ddH₂O and incubated for 1 hr at ambient temperature with the appropriate HRP-conjugated secondary antibody (1:1,000, Pierce Biotechnology, Inc. Rockford, Illinois). All antibodies were diluted in TBS containing 5% (w/v) non-fat dry milk. Blots were developed using the luminol reagent (Pierce Biotechnology). Densitometric analysis was done using the Fluor-S Multimager™ with Quantity One™ software (Bio-Rad).

Statistics

Data are presented as mean \pm SD. All statistics were calculated using one-way analysis of variance (ANOVA) for multiple comparisons. A *P* value of < 0.05 was

considered significant. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, Illinois) was used for all data analysis.

Results

We have recently developed a sensitive ELISA to specifically measure sAPP- α secretion in plasma. In order to validate this assay, we measured plasma sAPP- α levels in autistic and age-matched control blood samples using our novel sAPP- α ELISA and found a significantly increased level of sAPP- α in 60% of the known autistic children, compared to healthy age-matched children (**Figure 1A and B**, $P < 0.05$). Post hoc analysis revealed no association between the severity of aggression, social, or communication sub-scores (Revised Autism Diagnostic Instrument; ADI-R) and elevations in sAPP- α ($P > 0.05$). Such findings point to a group of autistic patients which could be identified in early childhood by levels of sAPP- α . As an additional confirmatory measure, we performed Western blot analysis on these samples. As shown in **figures 1C, D**, Western blot analysis consistently showed increased sAPP- α levels in autistic children versus age-matched healthy controls.

In order to further evaluate our optimized sAPP- α ELISA, we acquired 150 human umbilical cord blood samples from Saneron CCEL Therapeutics, Inc. (Tampa, FL). These HUCB samples were screened and found to be free of infectious diseases. The plasma was isolated, prepared, and subjected to sAPP- α ELISA. Data indicated significantly elevated levels (>3 ng/mg total plasma protein) of plasma sAPP- α in 10 of 150 samples (**Figure 2A**). To confirm the ELISA results, we performed Western blot analysis on 20 samples from the original pool of 150. Ten of these plasma samples had concentrations sAPP- α greater than or equal to 3 ng per mg of total protein by ELISA while the other 10 samples had less than 3 ng of sAPP- α per mg of total protein. Consistent with our ELISA data (**Figure 2B, C**), Western blot analysis demonstrated increased sAPP- α levels in the same 10 samples originally identified as "elevated" by our ELISA. Likewise, samples demonstrating less than 3 ng of sAPP- α per mg of total plasma protein by ELISA demonstrated the same changes upon Western blot analysis (**Figure 2C**). These

results indicate that significant differences in levels of sAPP- α production can be measured at birth using our ELISA.

Discussion

The goal of this study was to explore the plausible use of peripheral biomarkers in the early diagnosis of autism via a sensitive ELISA. Taking into account previous studies showing that brain-derived neurotrophic growth factor (BDNF) is associated with macrocephaly in autistic patients [19-21], we considered BDNF a likely biomarker candidate for our study. After further literature search, we discovered that not only is BDNF unreliable [23], but it is also not mutually exclusive to autism patients [22] and so it would not make an effective biomarker for diagnostic purposes. The discovery of elevated levels of secreted amyloid precursor protein alpha (sAPP- α) in children with autism by Sokol and colleagues [18] led to our speculation that sAPP- α could be used as a diagnostic biomarker.

Ours is only the second investigation which has uncovered elevated sAPP- α in autistic patients. Our preliminary results suggest a possible biomarker for affected individuals. The potential implications of these findings in the context of the known neurotrophic properties of sAPP- α , the observed brain overgrowth in certain brain regions of children with autism, and the lack of cerebral plaques found on histological examination of brains of autistic individuals [26-29] are quite significant. Although sAPP- α has not yet been shown to be directly pathogenic, as a plasma biomarker, it may help delineate a subset of children in which early regional brain overgrowth is necessary and sufficient for the development of autism and may even represent a mechanism by which overgrowth may occur. Indeed it has previously been shown that sAPP- α , is able to potentiate nerve growth factor (NGF)/retinoic acid (RA)-induced transdifferentiation of bone marrow-derived adult progenitor cells (MAPCs) into neural progenitor cells and, more specifically, augments their differentiation into a cholinergic-like neuronal phenotype [30]. Interestingly, cholinergic hypertrophy is a common feature of autism [18]. Whether the neurotrophic ability of this peptide is present during neurogenesis remains an unanswered key question.

In addition to using our ELISA to measure sAPP- α levels in serum, we sought to test its ability to measure sAPP- α levels in human umbilical cord blood, thereby examining the possible use of the ELISA for such measurements at birth. A significant elevation in sAPP- α levels in 7% of our samples confirm that sAPP- α can indeed be identified in HUCB via our ELISA. It has previously been shown by in vitro methods [31] that BDNF confers a 1.7-fold increase in sAPP- α secreted from neuron-like cells [31], and in light of our findings of a high incidence (7%) of significantly elevated sAPP- α in HUCB samples, relative to the incidence of autism in the general population, we can not rule-out that elevations in sAPP- α do not represent a normal or non-autistic variant of perinatal development. Furthermore, a subset of these 7% of HUCB samples may actually represent a group of children in which sAPP- α remains elevated into childhood and potentially leads to abnormal brain development. This hypothesis, and whether children with other conditions such as mental retardation and Down syndrome may also express comparably high plasma levels of sAPP- α , remains to be elucidated.

A major drawback of both our study, and that of Sokol and colleagues, is small sample size. Further studies of sAPP- α as a plasma protein marker in larger samples of autistic children are needed to more adequately characterize sAPP- α as a sensitive and useful biomarker and possibly a key player in the pathogenesis of autism. Further shortcomings of our study include a limited knowledge of the background and future condition of the patients from whom we received our HUCB samples. A follow-up study on HUCB donors would be the best means of determining the meaning of elevated sAPP- α levels in HUCB.

The substantial gaps in our knowledge of the neurodevelopmental mechanisms underlying autism arise largely from the difficulty of characterizing the circuitry subserving higher mental functions, the complexity of the genetic underpinnings of "normal" versus "abnormal" behavioral variation in childhood, the unsatisfactory nature of current autism animal models [5], and lack of quantifiable diagnostic and prognostic markers. Importantly however, earlier intervention in autism has been linked to improved long-term outcomes [32]. As in other diseases such as congenital

hypothyroidism, and Down syndrome, biomarkers can aid in this endeavor by helping to identify affected children in the neonatal or perinatal time period. Additionally, biomarkers for autism, in combination with clinical stigmata, may also define different disease subtypes and reveal new subtype-specific therapeutic strategies and targets. The availability of only a small number of biomarkers and the early stage of our understanding of the molecular pathophysiology of autism have, reasonably enough, impeded the development of more effective therapeutic interventions. The identification and characterization of biomarkers such as sAPP- α may facilitate the future creation of a diagnostic system for autism that incorporate molecular biomarkers and clinical symptoms, thus providing for differentiation of autism subtypes and customized treatment.

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