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Extra-axial cerebrospinal fluid in high-risk and normal-risk children with autism aged 2–4 years: a case-control study

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Dedication:

This paper is dedicated to the late Dr. Sandra L. Wootton-Gorges (19582017), a pediatric radiologist at UC Davis who was instrumental in the initial detection of increased extra-axial CSF in autism, and who made critical contributions as a co-author to the 2013 paper on these initial findings.[1] Her expertise has continued to guide our current research, her care for her patients has inspired our own efforts to improve the lives of individuals with autism, and her mentorship has influenced many residents, students and trainees, including her own daughter who contributed to this paper.

Declaration of interests

DGA is on the Scientific Advisory Boards of Stemina Biomarkers Discovery Inc. and Axial Biotherapeutics Inc. All other co-authors declare no competing interests.

Contributors

MDS: study concept and design; acquisition of data; image analysis; development of methods; statistical analysis; interpretation of results; wrote the manuscript; critical revision of the manuscript for important intellectual content. As corresponding author, Dr. Shen had full access to all the data in the study and had final responsibility for the decision to submit for publication.
CWN: study concept and design; acquisition of data; interpretation of results; co-wrote the manuscript; critical revision of the manuscript for important intellectual content; study supervision
DDL: acquisition of data; image analysis; development of methods
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KA: acquisition of data; interpretation of results; critical revision of the manuscript for important intellectual content
RWE: development of methods, statistical analysis; critical revision of the manuscript for important intellectual content
SJR: study concept and design; acquisition of data; interpretation of results; critical revision of the manuscript for important intellectual content; study supervision
SO: study concept and design; acquisition of data; interpretation of results; critical revision of the manuscript for important intellectual content; study supervision
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Summary

Background: We previously showed, in two separate cohorts, that high-risk infants who were later diagnosed with autism spectrum disorder had abnormally high extra-axial cerebrospinal fluid (CSF) volume from age 6–24 months. The presence of increased extra-axial CSF volume preceded the onset of behavioural symptoms of autism and was predictive of a later diagnosis of autism spectrum disorder. In this study, we aimed to establish whether increased extra-axial CSF volume is found in a large, independent sample of children diagnosed with autism spectrum disorder, whether extra-axial CSF remains abnormally increased beyond infancy, and whether it is present in both normal-risk and high-risk children with autism.

Methods: In this case-control MRI study, children with autism spectrum disorder or with typical development aged 2–4 years were recruited from the community to the UC Davis MIND Institute Autism Phenome Project, based in Sacramento, CA, USA. The autism spectrum disorder group comprised children with autism spectrum disorder who were either normal risk (ie, from simplex families) or high risk (ie, from multiplex families). Measurements of extra-axial CSF volume, brain volume, head circumference, sleep problems, and familial risk status were derived from MRI and behavioural assessments. We applied a previously validated machine learning algorithm based on extra-axial CSF volume, brain volume, age, and sex to the current dataset.

Findings: Between July 20, 2007, and Dec 13, 2012, 159 children with autism spectrum disorder (132 male, 27 female) and 77 with typical development (49 male, 28 female) underwent MRI scans. The autism spectrum disorder group had an average of 15·1% more extra-axial CSF than controls after accounting for differences in brain volume, weight, age, and sex (least-squares mean 116·74 cm^3 [SE 3·33] in autism group vs 101·40 cm^3 [3·93] in typical development group; p=0·007; Cohen's d=0·39). Subgroups of normal-risk (n=132) and high-risk (n=27) children with autism spectrum disorder had nearly identical extra-axial CSF volumes (p=0·78), and both subgroups had significantly greater volumes than controls. Both extra-axial CSF volume (p=0·004) and brain volume (p<0·0001) uniquely contributed to enlarged head circumference in the autism spectrum disorder group (p=0·04). Increased extra-axial CSF volume was associated with greater sleep disturbances (p=0·03) and lower non-verbal ability (p=0·04). The machine learning algorithm correctly predicted autism spectrum disorder diagnosis with a positive predictive value of 83% (95% CI 76·2–88·3).

Interpretation: Increased extra-axial CSF volume is a reliable brain anomaly that has now been found in three independent cohorts, comprising both high-risk and normal-risk children with autism spectrum disorder. Increased extra-axial CSF volume is detectable using conventional structural MRI scans from infancy through to age 3 years. These results suggest that increased extra-axial CSF volume could be an early stratification biomarker of a biologically based subtype of autism that might share a common underlying pathophysiology.

Keywords

autism; neuroimaging; MRI; extra-axial fluid; cerebrospinal fluid; CSF; hydrocephalus; brain development; sleep problems; developmental disorders; familial risk

INTRODUCTION

Increased extra-axial cerebrospinal fluid (EA-CSF) is a brain anomaly characterized by excessive CSF in the subarachnoid space surrounding the cortical surface.[3–5] We previously reported that high-familial-risk infants (based on having an older sibling with autism) who were later diagnosed with autism spectrum disorder (ASD) had an increased amount of EA-CSF from 6 to 24 months of age, which preceded the age of diagnosis at ~33 months.[1] In a second study, we reproduced this finding in a much larger independent sample of high-risk infants who developed ASD. The amount of EA-CSF at 6 months of age preceded the onset of autism symptoms, and was predictive not only of their later ASD diagnosis but also of the severity of their ASD symptoms two years later.[2]

The presence of increased EA-CSF in infancy had been considered “benign” because it had not previously been associated with a clinical syndrome. While these previous two reports were the first to link the condition to autism, past reports have associated increased EA-CSF with other neurological problems.[6–9] Recent research[10] has highlighted the importance of normal CSF circulation to transport trophic factors that regulate normal brain development,[11] and in turn, to clear metabolites that affect brain development and function.[12–14] For example, altered CSF circulation has been shown to impair the clearance of neurotoxic proteins implicated in neurodegenerative diseases (e.g., amyloid-beta protein[14]). This neuroprotective process is particularly affected by disturbances in sleep.[13]

Several outstanding questions remained from the previous studies, both of which utilized the infant sibling study design in which the participants were at high familial risk for autism because they had an older sibling with ASD.[12] The goal of the present study was to determine whether increased extra-axial CSF is found in a large, independent sample of children diagnosed with ASD and ascertained from the community at 2–3·5 years of age. Specifically, we aimed to determine whether extra-axial CSF remains abnormally elevated beyond infancy in ASD children at either normal- or high-risk for ASD. Based on our previous reports and the extant literature, we conducted this study guided by several hypotheses. First, we hypothesized that the ASD group would show excessive EA-CSF at 2–3·5 years of age, regardless of familial risk status, compared to children with typical development (TD). Second, we expected that head circumference would be increased in the ASD group and that both EA-CSF volume and brain volume would independently contribute to head size. Third, given the biological evidence that normal sleep is associated with proper CSF circulation[13], we predicted that increased EA-CSF volume would be associated with sleep problems. Finally, based on our previous report that EA- CSF could predict ASD diagnosis among high-risk children,[2] we hypothesized that applying the same prediction algorithm to normal-risk children could also accurately predict ASD diagnosis on an individual level.

METHODS

Participants

Children were recruited through the UC Davis MIND Institute Autism Phenome Project, a multidisciplinary, longitudinal study investigating subtypes of ASD. (See Supplementary

Methods.) Participants entered the study at 2–3·5 years of age and underwent an MRI scan, behavioral assessment, and medical record review. MRI and behavioral data were collected on N=236 children, 159 with ASD (132 male; 27 female; age M[SD]=3·1 [0·48] years) and 77 with TD (49 male; 28 female; age=3·0 [0·42] years). Medical history interview and chart review were used to determine whether the 159 children with ASD were either "normal-risk" (i.e., came from a simplex family in which they were the only child with ASD; N=132) or "high-risk" (i.e., came from a multiplex family with more than one child with ASD in the family; N=27).

Behavioral Assessments and Physical Measures

Diagnosis was confirmed using the Autism Diagnostic Observation Schedule[15] (ADOS) and Autism Diagnostic Interview-Revised.[16] The Mullen Scales of Early Learning was used to measure cognitive ability. Weight and occipitofrontal head circumference were measured at the visit. Head circumference was not available for 4 of the 159 participants in the ASD group. Analyses indicated that there were no differences between these four participants and the rest of the ASD group with available head circumference data (n=155) in EA-CSF volume (t_{157}=0·32, p=0·75), sex (X^2(1)=018, p=0·67), age (t_{157}=1·20, p=0·23), cognitive ability (t_{157}=1·07, p=0·29), or ADOS severity score (t_{157}=1·48, p=0·14).

Sleep Assessments

Sleep problems were assessed using the Children's Sleep Habits Questionnaire[17] (CSHQ), a parent-report questionnaire designed as a screening instrument for sleep disorders. It has been validated for preschool children with ASD and has a high degree of correspondence with actigraphy measures.[18] The CSHQ measures 8 subscales (sleep onset, duration, night wakings, parasomnias, sleep-disordered breathing, bedtime resistance, sleep anxiety, daytime sleepiness) and yields a total score used for data analysis. Sleep problems were also assessed using the Sleep Problems subscale of the Child Behavior Checklist[19] (CBCL). Higher scores on the CSHQ and CBCL indicate more sleep problems.

Data from Birth Records

Medical charts from labor and delivery were obtained in a subset of our MRI sample (N=136; ASD N=92; TD N=44). There were no differences between the proportion of ASD children (57·9%; 92 of 159) vs. TD children (57·1%; 44 of 77) who had available birth data from medical records (X^2 (1)=0·01, p=0·92). There were no differences in cognitive ability measured by the Mullen Scales of Early Learning[20] ($F_{1,234}$=0·08; p=0·78) or autism severity measured by the ADOS[21] ($F_{1,157}$ 0·06; p=0·80) between children with and without available medical records. Birth weight, length, head circumference, and gestational age at birth were extracted from the records. Normalized birth head circumference was defined as birth head circumference (in cm) divided by gestational age (in weeks).

MRI Acquisition and Analysis

Participants were scanned during natural nocturnal sleep[22] on a 3T Siemens TIM Trio scanner. Tl-weighted structural scans (1 mm^3) were acquired from each participant[23]. Quantitative measurements of total cerebral volume (TCV) and EA-CSF were obtained

using previously published protocols.[1] (See Supplementary Methods and Figure 1 for more details.)

Statistical Analysis

Analysis of covariance (ANCOVA) was utilized to test for interactions and main effects of group, while controlling for differences in age, sex, and body weight. The primary dependent variables (DV) were EA-CSF volume and head circumference. Group differences for each DV were determined using a model that included group as the primary independent variable of interest, covariates (age, sex, weight), and group interactions with each. Body weight was included as a covariate since body size is related to brain and head size in ASD[24]. In analyses examining EA-CSF, TCV was included as an additional covariate to determine whether EA-CSF volume was elevated regardless of brain size. Regression analyses tested continuous associations between EA-CSF and head circumference and sleep problems. All tests were two-tailed with $\alpha = 0{\cdot}05$. All analyses were performed using SAS JMP software (SAS Institute, Cary, NC).

In our recent infant sibling study,[2] a fully cross-validated machine learning prediction algorithm was used to test whether EA-CSF volume and TCV could accurately classify which high-risk children would be diagnosed with ASD. In the current study, we applied the identical prediction model—which required using the same predictors (EA-CSF volume, TCV, age, sex), model parameters, and threshold of the area under the receiver operating characteristic curve (AUC=.69)—to the current dataset to classify children with ASD. The model appropriately accounted for sex imbalance between groups, by virtue of implementing a balance-boosted trees ensemble algorithm, *RUSBoost Trees*,[45] which continuously identifies balanced samples, thereby testing the classifier on evenly distributed samples. The goal of conducting the machine learning prediction analysis in the current paper was to externally validate a previously defined model, in order to test whether the model was both robust and applicable to an independent dataset. As Gabrieli and colleagues have noted,[25] building a cross-validated prediction model on one dataset, and then applying the identical model to a new, independent, out-of-sample dataset is the most rigorous prediction approach. Applying the original prediction model to the current dataset yielded the positive predictive value (PPV), sensitivity, specificity, and 95% confidence intervals for the current dataset. (See Supplementary Methods for more details.)

RESULTS

Participant characteristics and cognitive measures are presented in Table 1. There were no group differences in age. As expected, the ASD group had significantly lower overall cognitive, verbal, and nonverbal abilities compared to TD controls.

Extra-axial CSF Volume

The ASD group had significantly greater EA-CSF volume compared to the TD group. There was a significant main effect of group ($\beta = 7{\cdot}67$, $F_{1,213} = 7{\cdot}33$, $p < 0{\cdot}01$), after controlling for TCV, weight, age, and sex. There were significant positive effects of TCV ($\beta = 0{\cdot}099$, $F_{1,213} = 14{\cdot}63$, $p < 0{\cdot}0005$) and weight ($\beta = 113$, $F_{1,213} = 3{\cdot}95$, $p < 0{\cdot}05$) on EA-CSF volume, and

no interactions with group (group x TCV: $F_{1,213}$=2·16, p=0·14; group x weight: $F_{1,213}$=1·40, p=0·24), indicating that EA-CSF was elevated in the ASD group regardless of differences in brain or body size. There were no significant main effects or group interactions with sex (β=0·44, $F_{1,213}$=0·03, p=0·85; group x sex: $F_{1,213}$=2·17, p=0·14) or with age (β=−2·77, $F_{1,213}$=0·27, p=0·61; group x age: $F_{1,213}$=2·46, p=0·12), indicating that the elevated extra-axial CSF found in the ASD group did not differ significantly between male and female children. Even when the analysis excluded the four ASD participants with the highest EA-CSF volumes, there was still a significant main effect of group (β=7·23, $F_{1, 209}$ 7·33, p<0·01), controlling for the same covariates. An example of a TD child with a normal level of EA-CSF, compared to a child with ASD who had elevated extra-axial CSF at 3 years of age is illustrated in Figure 1A-B. Figure 2 illustrates a plot of the raw values of EA-CSF volume for each individual and model-adjusted least squares means (LSM) for each group. On average, the ASD group had 15·1% more EA-CSF than the TD group (LSM [SE]: ASD=116·74 cm^3 [3·33]; TD=101·40 cm^3 [3·93]). Figures 3 illustrates a plot of the *model-predicted* values of EA-CSF for each individual, which were computed from the beta weights of the variables in the full statistical model reported above.

Normal-risk vs. High-risk family status

Our previous studies were conducted with an infant sibling design, and as such, infants who developed ASD were by definition from multiplex families and considered “high-risk”.[1,2] In the current study, the ASD group (N=159) was ascertained from the community, and thus comprised of children from either simplex families (i.e., “normal-risk”; N=132) or multiplex families (i.e., “high-risk”; N=27). EA-CSF volumes were nearly identical (t_{157}=0·28, p=0·78; controlling for age, sex, and TCV) between ASD children from simplex families (LSM [SE]=115·0 cm^3 [4·14]) and ASD children from multiplex families (LSM [SE]=117·09 cm^3 [5·76]). Both ASD subgroups had significantly greater EA-CSF volumes compared to the TD group (t_{207}=2·21, p<0·05; t_{102}=2·06, p<0·05; controlling for age, sex, and TCV). See Table 2 for participant characteristics of the ASD normal-risk and high-risk subgroups.

Relationship to Head Circumference

There was a significant main effect of group on head circumference ($F_{1,215}$=4·24, p<0·05), with the ASD group having significantly larger head circumference after controlling for age, weight, and sex. (See Supplementary Figure 1B.) There were significant positive effects of the control variables, age ($F_{1,215}$=4·61, p<0·05), weight ($F_{1,215}$=19·85, p<0·0001), and sex ($F_{1,215}$=9·22, p<0·005), but no interactions with group. Furthermore, we added TCV and EA-CSF in the model to examine the effects that brain size and EA-CSF volume had on head circumference. Both EA- CSF volume (β=0·011; se=0·003; $F_{1,215}$=8·65, p<0·005) and TCV (β=0·009; se=0·003; $F_{1,211}$=47·31, p<0·0001) were found to be highly significant predictors of head circumference, above and beyond age, weight, and sex. There were no interactions with group (group x EA- CSF: $F_{1,211}$=0·16, p=0·69; group x TCV: $F_{1,211}$=0·40, p=0·53). Taken together, these findings indicate that both EA-CSF and TCV have independent effects on head circumference and both contribute to individual variability in head circumference. Both groups showed significant correlations of head circumference with TCV (Fig. 4A) and with EA-CSF (Fig. 4B; Supplementary Figure 2).

Relationship to Head Size at Birth

There were no significant differences between groups in birth weight ($F_{1,134}$=0·24; p=0·62), birth length ($F_{1,133}$=0·01; p=0·98), gestational age ($F_{1,134}$=2·11; p=0·15), or birth head circumference ($F_{1,134}$=0·02; p=0·88; see Supplementary Figure 1C). We tested whether there was an association between normalized head circumference at birth and TCV and EA-CSF volume at 3 years of age. Both groups showed a significant correlation between birth head circumference and TCV three years later (Fig. 4C; ASD: r=0·39, p<0·0001; TD: r=0·41, p<0·01). These associations remained significant after covarying for birth weight and sex (p<0·0005 and p<0·05, respectively). The ASD group showed a significant correlation between birth head circumference and EA-CSF volume (Fig 4D; r=0·37, p<0·0005), but not the TD group (r=0·16, p=0·29). These associations remained consistent after covarying for birth weight and sex (p<0·0005 and p=0·44, respectively).

Association between Extra-axial CSF and Sleep Problems

We used a data-driven and previously published method[1] to establish a quantitative cutoff to subgroup children on the basis of both EA-CSF and brain volumes. A ratio of CSF-to-Brain volume was derived by dividing EA-CSF volume by TCV. Using this ratio, the ASD group had greater EA-CSF even when accounting for brain size in this manner ($F_{1,218}$=4·37, p<0·05; covariates: age, sex, weight). A cutoff of+1·5 SD above the mean of the TD group resulted in a cutoff ratio of CSF-to-Brain volume = 0·14, which was precisely the same cutoff found in our earlier infant study to yield a high sensitivity (78%) and specificity (79%) for predicting autism diagnosis[1]. This cutoff was used to split the ASD group into two subgroups, one with a higher volume of EA-CSF (ASD-high > +1·5 SD; n=21) and the other with lower levels of EA-CSF (ASD-normal < +1·5 SD; n=138) (Fig. 5A). The ASD-high subgroup had significantly more sleep problems on the CSHQ compared to both the ASD-normal subgroup ($F_{1,182}$=5·19, p<0·05) and the TD group ($F_{1,182}$=7·41, p<0·01) (Fig. 5B). The CBCL was examined as a second convergent measure of sleep problems, and showed the same pattern of results (Fig. 5C) (ASD- high vs. ASD-normal: $F_{1,218}$=3·59, p=0·06; ASD-high vs. TD group: $F_{1,218}$=9·98, p<0·005). We confirmed this association between EA-CSF and sleep problems by treating the EA-CSF ratio and sleep problems as continuous measures in a regression analysis and found a significant positive association between EA-CSF ratio and CSHQ sleep problems ($F_{1,182}$=4·79, p<0·05), even after controlling for differences between groups.

Association between Extra-axial CSF and Other Behavioral Measures

We tested the association between EA-CSF and nonverbal ability, given our earlier observation that EA-CSF in infancy was related to poorer motor ability.[2] EA-CSF had a significant negative effect on nonverbal ability (β=−0·04, $F_{1,228}$=4·20, p<0·05), controlling for general cognitive ability, overall group differences in nonverbal ability, and the same covariates included in the primary EA-CSF model (sex, age, weight, TCV). This association between EA-CSF and nonverbal ability, which remained significant even after controlling for general cognitive ability, suggests that EA-CSF may have a specific association with nonverbal ability. There were no other associations between EA-CSF and other Mullen or ADOS scores.

Extra-axial CSF as a predictor of diagnosis

In our recent infant-sibling paper,[2] a fully cross-validated machine learning prediction algorithm was applied to the EA-CSF and TCV data, and accurately classified which high-risk children were diagnosed with ASD.[2] In the current study, we applied the identical algorithm and predictors to the current dataset of preschool-age children, with the goal of externally validating the previously defined model to demonstrate that the model is both robust and applicable to an independent dataset. The prediction algorithm yielded a PPV of 83% (95% CI: 76·2–88·3), with an accuracy of 78% (183 of 236), correctly predicting 133 of 159 children with ASD (sensitivity=84%; 95% CI: 76·7–88·8) and 50 of 77 children without ASD (specificity=65%; 95% CI: 53·1–75·2).

DISCUSSION

In two previous studies, we found that infants at high risk for autism (because they have an older sibling with ASD) demonstrate increased EA-CSF as early as 6 months of age, which remained elevated until at least 24 months of age.[1,2] The two primary goals of the current study were to determine: (1) whether children with ASD from normal-risk families also have increased EA- CSF; and (2) whether increased EA-CSF is still present at three years of age. The current study confirms both of these findings in a large cohort of 2–3·5 year-old children. Group differences in EA-CSF remained significant after controlling for brain volume, indicating that EA-CSF was elevated above and beyond brain enlargement. There was substantial variability within the ASD group, but the sensitivity and specificity of the prediction results conducted at the individual-level were highly convergent between the current and previous samples,[1,2] which suggests that there is a subset of children with ASD who reliably demonstrate increased EA-CSF volume from as early as 6 months and until at least 3 years of age.

We confirmed that head circumference, on average, was larger in the ASD group. We have reported larger head circumference and greater total brain volume on a portion of these children in previous reports[23,26,27] but not in relation to extra-axial CSF findings. Several head circumference studies in ASD have highlighted the importance of studying large sample sizes to account for heterogeneity in autism and comparing to study-specific controls (instead of comparing to outdated population norms).[28] The current study, with its large sample of children with ASD (N=159) and study-specific controls, sufficiently accounted for these issues. Head size tends to be larger on average in males than females[29], correlates strongly with body size[24], and is often considered a proxy for brain size.[30] Our findings indicate that, above and beyond sex and body size, both EA-CSF and TCV have independent effects on head circumference and both contribute to individual variability in head circumference. Since monitoring head growth is a common clinical practice, these findings suggest the importance of emphasizing that head circumference is a reflection of both brain size and EA-CSF volume. Volume of EA-CSF at 3 years of age was also related to head size at birth in the ASD group, and coupled with our reports that increased EA-CSF is present at 6 months of age, this raises the possibility that increased EA- CSF may be present at birth.

Children with ASD with a pronounced elevation of EA-CSF had more sleep disturbances compared to other ASD and TD children with normal amounts of EA-CSF. This was

demonstrated in two validated screening assessments for sleep disorders in children.[18] We used a data-driven and previously published method[1] to establish the quantitative cutoff that determined the subgroup of children with ASD (13%) with a high level of EA-CSF compared to the subgroup with lower levels (87%). The ASD subgroup with high levels of EA-CSF had significantly greater sleep problems compared to both the ASD subgroup with normal levels of EA-CSF and the TD group. This association was confirmed by a positive linear relationship between higher EA-CSF and greater sleep problems.

Further research is needed to explore the potential relationship between sleep disturbances, altered CSF circulation, and the brain's ability to clear neurotoxic proteins. Circulation of CSF is critical for clearing metabolic byproducts and inflammatory proteins during natural, undisturbed sleep. Recent studies in animals models have demonstrated that during natural sleep there is a 60% increase in the influx of CSF compared to the awake state, as the exchange of CSF between the interstitial space and the subarachnoid space is accelerated during sleep.[13] This increased flow of CSF during sleep drives the increased clearance of neurotoxic proteins such as amyloid-beta (Aβ) and tau, which are continually secreted by neurons and need to be continually removed by the flow of CSF from the interstitial space to the subarachnoid space.[13] From the subarachnoid space, CSF carrying these proteins is then drained into the cranial sinuses and the newly discovered meningeal lymphatic system of the brain.[10,13,14] Thus, sleep has a restorative function of clearing neurotoxic metabolic byproducts that accumulate in the awake brain. Of course, the results of the current study do not elucidate the directionality of the association between sleep problems and excessive CSF in the subarachnoid space. One possible mechanism is that disrupted sleep hinders the flow of CSF (which has been confirmed in animal models[13]), and in turn results in an increased accumulation and altered composition of CSF proteins that would have a pathological effect on the brain.[31] Indeed, sleep problems are commonly found in ASD,[32,33] and there is mounting evidence of increased levels of Aβ in individuals with ASD, found in neurons from postmortem brain tissue, blood, and peripheral CSF.[34–37]

Limitations of the Current Study and Future Questions:

There are several questions that this study could not address. *What is the mechanism underlying increased extra-axial CSF, its effect on the brain, and its association with sleep?* The potential mechanism described above is only one of many plausible mechanisms. But, it is one that has emerged as a tractable hypothesis to test experimentally, by virtue of the experimental methods that have been established through the recent discovery of the glymphatic system of the brain.[13,14,38] At this point it is unclear whether the observation of increased extra-axial CSF is related to one etiology of ASD, or is an indication of a different underlying process that produces ASD and increased extra-axial CSF. Regarding the relationship to sleep, the directionality of the relationship between sleep and EA-CSF cannot be determined with these data, in part because the MRI data and sleep data were acquired contemporaneously, and therefore temporal precedence cannot be established. Thus, we chose to be conservative in interpreting this observed association. However, the data reported herein indicates that increased EA-CSF is associated with poorer sleep in two sleep measures, which is consistent with our hypothesis given the biological evidence linking CSF and sleep.[13,14,38] Our objective was to report this observation in a well-phenotyped clinical

population, so that it may open future mechanistic investigations into whether sleep problems in autism precede and contribute to EA-CSF, or vice versa.

What is the specificity and sensitivity for autism? It is currently unknown whether increased extra-axial CSF from infancy through preschool-age is specific to autism. It is possible (given the reported specificity metric) that increased extra-axial CSF *is not specific* to autism, and that there are other neurodevelopmental conditions that show increased EA-CSF from infancy through preschool-age. Several efforts are currently underway to evaluate EA-CSF in other conditions, and we welcome attempts at both replication in autism and extension to other conditions.

Given the heterogeneity of autism, it is unlikely that increased EA-CSF is present in *all* children with ASD (as evidenced by the reported sensitivity metric). On the other hand, the data across three cohorts indicate that the presence of increased EA-CSF in infancy through preschool-age may be a potential stratification biomarker that delineates one biological subtype of autism spectrum disorder that potentially shares a common underlying biology. The clinical utility of this potential stratification biomarker would be to parse the etiologic heterogeneity of autism into clinically significant subtypes that may map on to mechanistically-targeted treatments. In order to validate EA-CSF as a potential stratification biomarker, several future experiments must be conducted to determine its specificity (e.g., by comparing to other neurodevelopmental disorders), elucidate the underlying biology (using animal models and identifying genetic associations), and test potential mechanisms using experimental approaches such as those established by studies described above of the glymphatic system.

Is increased EA-CSF simply the result of brain tissue loss? In other conditions like dementia, increased extra-axial CSF arises because CSF fills in the subarachnoid space that was previously occupied by atrophic brain tissue.[39,40] However, in this study there was a *positive* association between total cerebral volume and extra-axial CSF volume; this was also found in our other two studies of EA-CSF in young children with autism.[1,2] If increased EA-CSF was due to brain tissue loss, then we would observe a *negative* association between CSF and brain volume. Thus, the observation of increased EA-CSF in young children with autism is unlikely to be related to degenerative disorders of the brain.

Conclusions:

In three independent cohorts, we have demonstrated that EA-CSF is elevated in children with ASD from 6 months through preschool age, regardless of familial risk. It is not currently clear whether increased CSF in the subarachnoid space may directly impact brain development and contribute to the pathology of psychiatric disorders such as ASD, or is an epiphenomenon that reflects another underlying etiology. Regardless, increased EA-CSF appears to be an early sign of altered neurodevelopment in at least one subtype of autism: it is an observable, structural brain anomaly that is reliably detected with conventional MRI across different cohorts, family risk backgrounds, neuroimaging parameters, and MRI scanners. Beyond group-level differences, which are susceptible to the heterogeneity of ASD, the consistency of individual-level prediction results across different samples and the

corresponding PPV suggest that increased EA-CSF could be a reliable stratification biomarker for a biologically-homogenous subtype in autism. There has been recent emphasis placed on the importance of finding biologically-based stratification markers in psychiatric disorders to aid in evaluating diagnosis, prognosis, treatment selection and response.[41–43] A major obstacle in this pursuit is the lack of consistency of putative biomarkers, small sample sizes, and clinical heterogeneity (for review[44]). The current findings suggest that increased extra-axial CSF is a potential stratification marker for a biologically-meaningful subtype in ASD. Moreover, clinicians should be aware that the increased EA-CSF may not be benign, and its presence may signal the need for careful monitoring and developmental screening. Earlier detection may ultimately lead to earlier behavioral intervention, which appreciably improves a child's long-term outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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RESEARCH IN CONTEXT

Evidence before this study:

We searched PubMed for published articles, using combinations of search terms that included: “extra-axial fluid”, “benign extra-axial fluid”, “extra-axial fluid of infancy”, “extra-axial cerebrospinal fluid”, “subarachnoid cerebrospinal fluid”, “enlargement of subarachnoid spaces”, “benign effusions of infancy”, “external hydrocephalus”, and “communicating hydrocephalus”. Our search did not have any language or date restrictions. We also searched the reference lists of selected articles for other relevant publications. In addition, we identified a review paper on extra-axial CSF (Zahl et al., 2011) and reviewed the articles listed in the reference list. None of the selected articles mentioned an association between the search terms and autism spectrum disorder (ASD), with the exception of our previous two publications that reported that high-risk infants (i.e., with an older sibling with autism), who later developed ASD themselves, had increased extra-axial CSF volume from 6–24 months of age (Shen et al., 2013; Shen et al., 2017).

Added value of this study:

It was previously unknown whether extra-axial CSF remains elevated beyond infancy and whether it occurs in normal-risk children with ASD. Preschool- aged children with ASD from both normal-risk and high-risk familial backgrounds had significantly increased EA-CSF (15%) at 2–3 5 years of age compared to control children. Normal-risk and high-risk children with ASD had nearly identical amounts of extra-axial CSF. Both extra-axial CSF volume and brain volume uniquely contributed to enlarged head circumference in the ASD group. Elevated extra-axial CSF was associated with greater sleep disturbances. EA-CSF volume correctly predicted ASD diagnosis with a positive predictive value of 83%.

Implications of all the available evidence:

Increased extra-axial CSF is a reliable brain anomaly that has now been found in three independent cohorts, comprised of both high-risk and normal-risk children. Increased extra-axial CSF is detectable using conventional structural MRI scans from infancy through preschool age. These results suggest that increased extra-axial CSF could be a potential early stratification biomarker of a biologically-homogenous subtype of autism that potentially shares a common underlying biology. The clinical utility of this potential stratification biomarker would be to parse the etiologic heterogeneity of autism into clinically significant subtypes that may map on to mechanistically-targeted treatments.

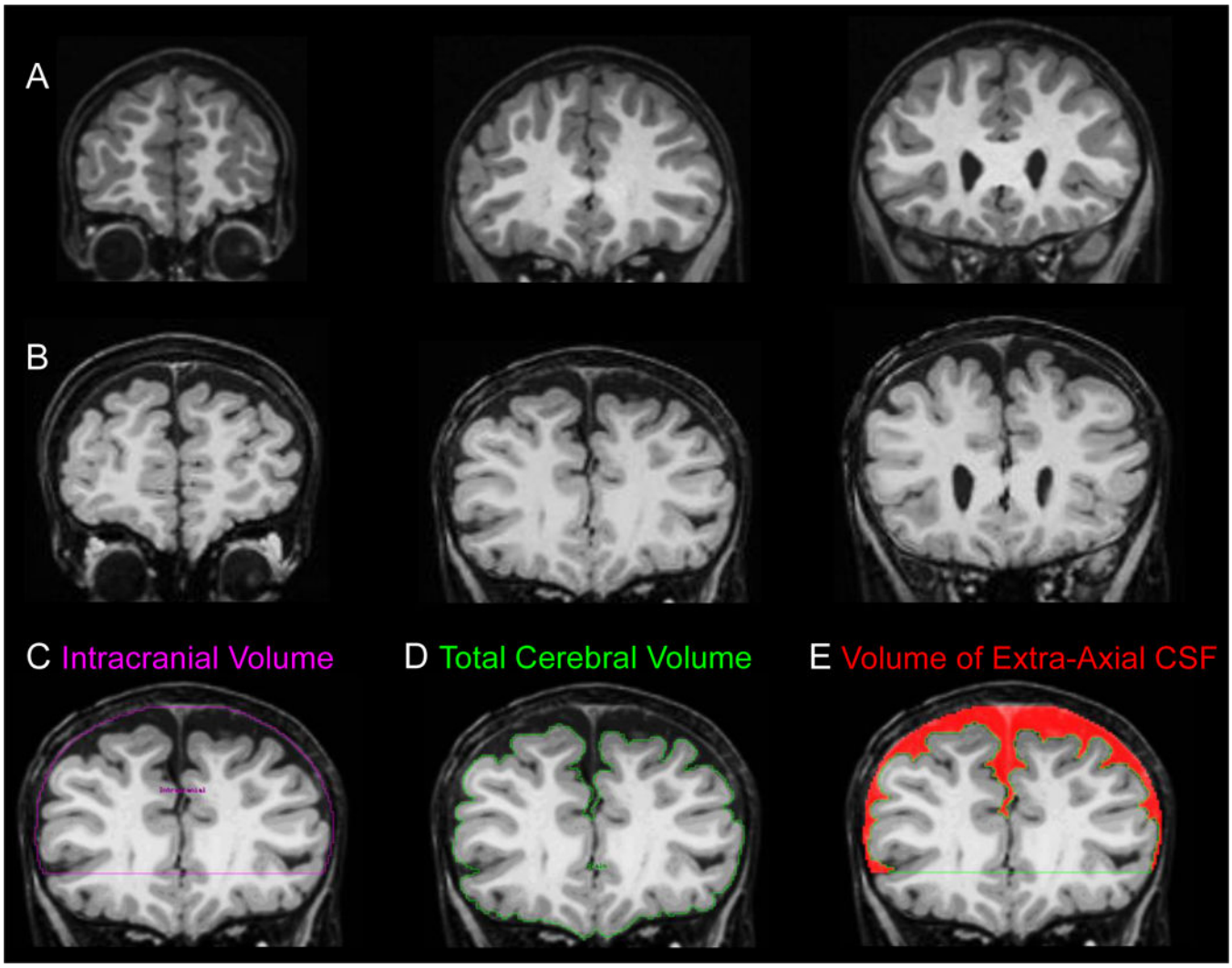

Figure 1. Brain images illustrating the appearance and quantification of extra-axial CSF.

(A) T1-weighted coronal images of a typically developing child with a normal MRI at 3 years of age. (B) T1-weighted coronal images of a child with ASD and increased extra-axial CSF at 3 years of age. (C-E) Quantification of extra-axial CSF. (C) Manual tracing of dura on successive coronal slices are summed to yield intracranial volume; (D) semi-automated tissue segmentation yields total cerebral volume; (E) resulting space between the dura and cortical surface yields extra-axial CSF volume. (See Supplementary Methods for more details.)

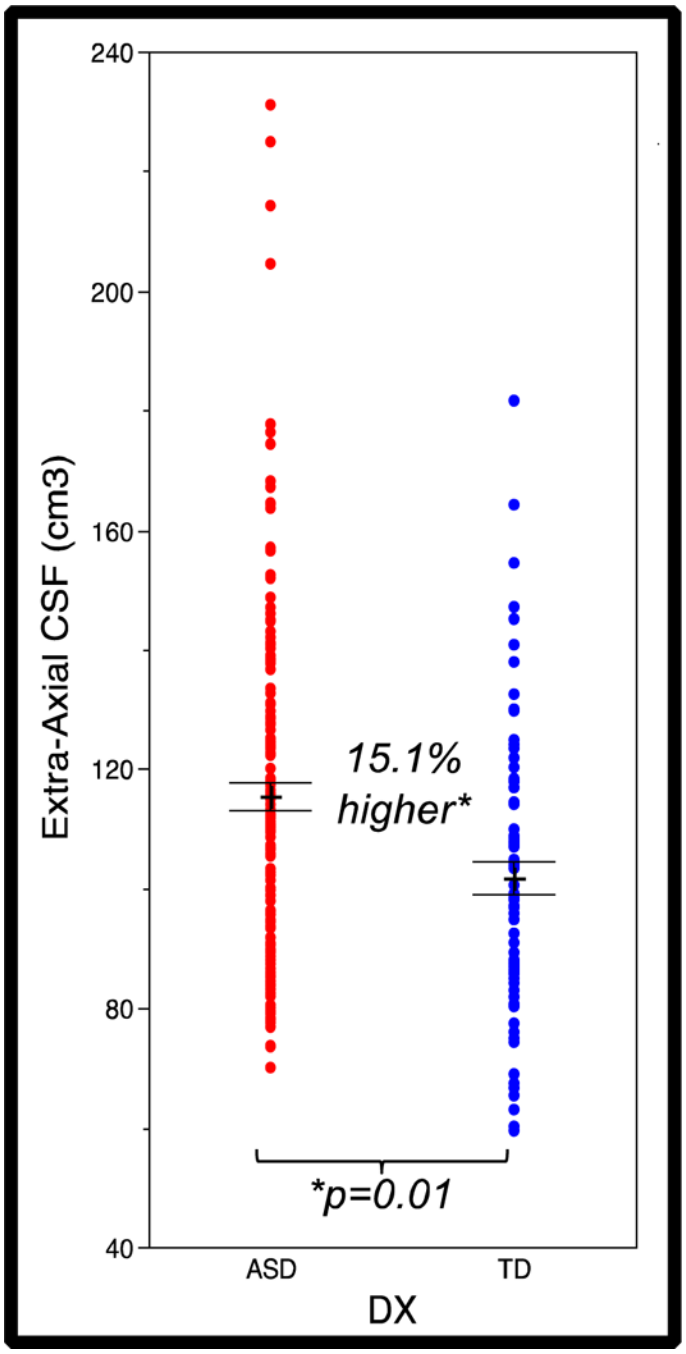

Figure 2. The ASD group had significantly greater extra-axial CSF volume compared to TD group.

A plot of the raw values of EA-CSF volume for each individual and the modeladjusted least squares means (LSM) for each group are illustrated. The least squares mean of the ASD group was 15·1% greater than the TD group. (Least squares means and p-value are from the model with age, sex, body weight, and TCV as covariates. *Error bars* = ±*1 SEM*.)

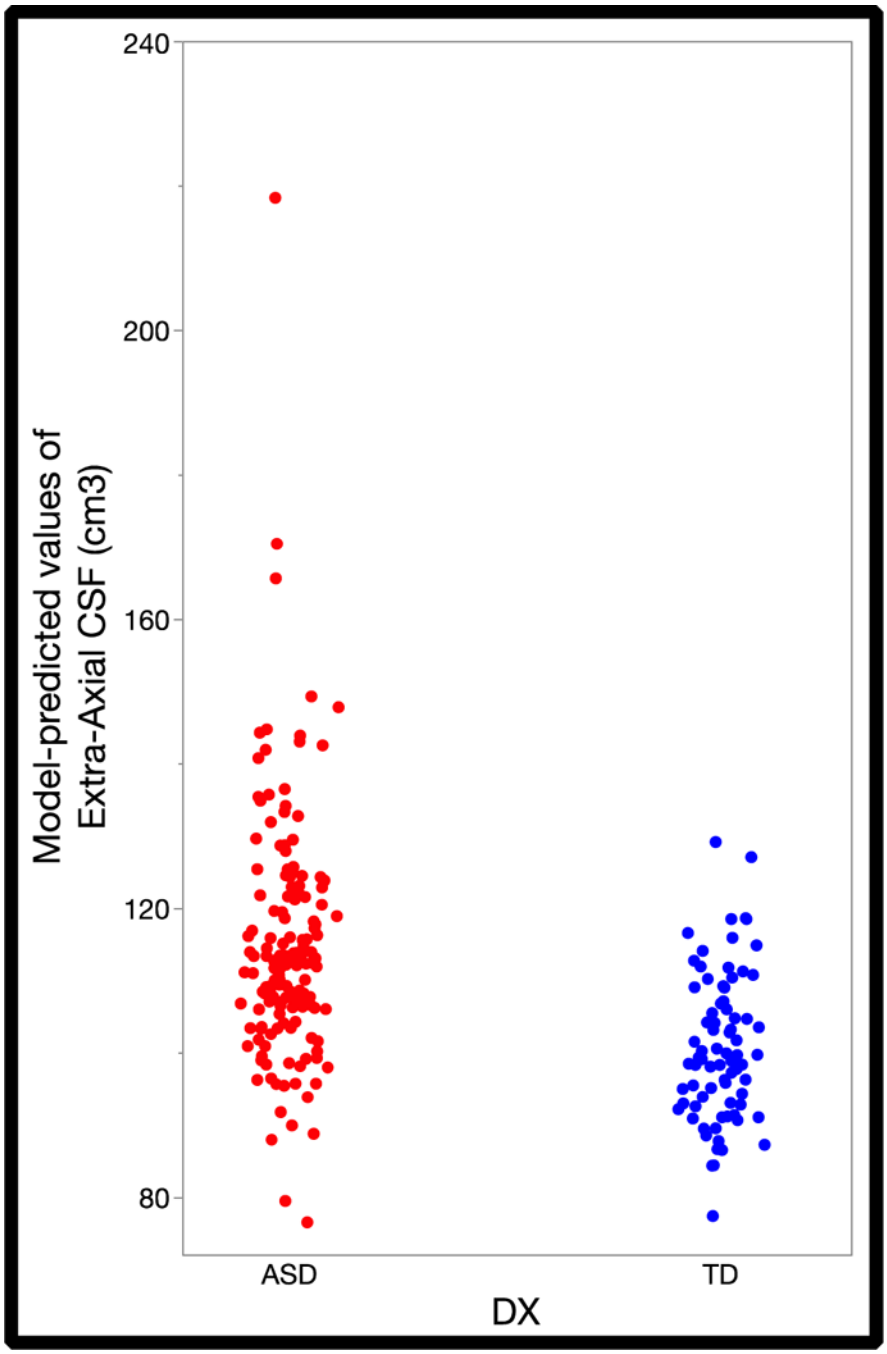

Figure 3. A plot of the model-predicted values of EA-CSF for each individual.
(These modelpredicted values were computed from the beta weights of all the variables in the full statistical model.)

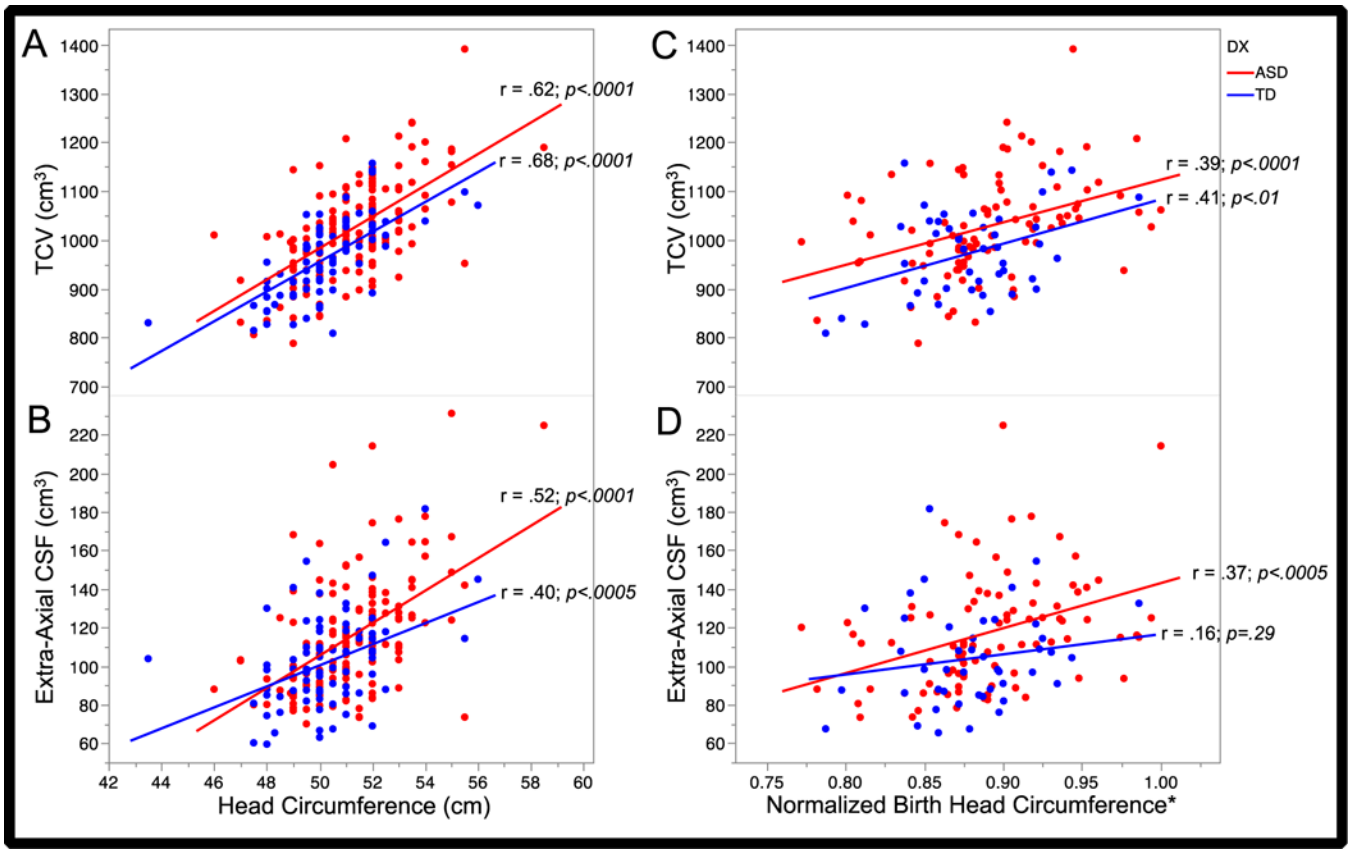

Figure 4. Relationship with head circumference at 3 years and at birth.

(A) Head circumference was significantly correlated with TCV in both groups. (B) Head circumference was also significantly correlated with extra-axial CSF volume in both groups. (C) Head circumference at birth was significantly correlated with later TCV at 3 years of age in both groups. (D) Head circumference at birth was significantly correlated with volume of extra-axial CSF at 3 years of age in the ASD group, but not TD group. (*Normalized head circumference at birth is corrected by gestational age.)

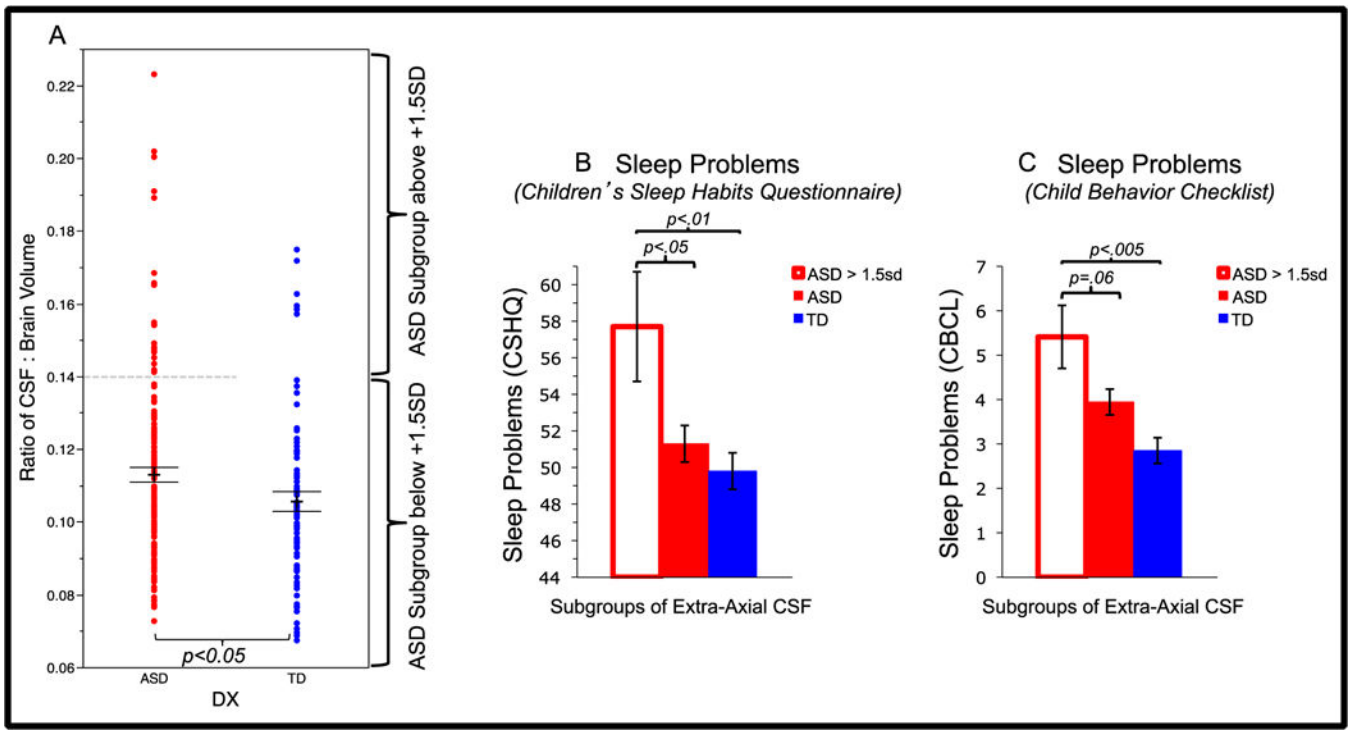

Figure 5. Subgroups of high Extra-axial CSF have more sleep problems.

(A) A ratio of CSF-to-Brain volume was derived by dividing extra-axial CSF by total cerebral volume. The ASD group had greater extra-axial CSF even when accounting for brain size in this manner (covariates: age, sex, weight). ASD subgroups of CSF:Brain Volume were then determined using a cutoff of +1·5 SD above the TD mean. (B and C) The ASD group with higher levels of extra-axial CSF (>1·5 SD above TD mean) had more sleep problems indicated on two sleep assessments: the Children's Sleep Habits Questionnaire (B) and the Child Behavior Checklist (C). (*Error bars* = ±*1 SEM*.)

Table 1:

Diagnostic group comparison of participant characteristics

	Mean(SD)		
	ASD	**TD**	**p-value**
N	159	77	
Sex	132M; 27 F	49 M; 28 F	
Age (years)	3·1 (0·48)	3·0 (0·42)	ns
Overall Cognitive Ability	63·3 (21·7)	106·5 (11·6)	<0·0001 *
Verbal Ability	55·7 (27·0)	107·8 (12·3)	<0·0001 *
Nonverbal Ability	70·7 (19·0)	105·1 (14·7)	<0·0001 *
ADOS Severity	7·9 (1·8)	n/a	n/a

Standard scores for overall cognitive, verbal, nonverbal ability have Mean=100, SD=15

* p-value of 2-tailed t-test between groups

Table 2:

ASD normal-risk and ASD high-risk subgroup comparison of participant characteristics

	Mean (SD)		
	ASD normal-risk subgroup	**ASD high-risk subgroup**	**p-value**
N	132	27	
Sex	114M; 18 F	18 M; 9 F	
Age (years)	3·1 (0·49)	3·1 (0·45)	ns
Overall Cognitive Ability	62·7 (21·7)	65·9 (21·9)	.49
Verbal Ability	55·3 (27·2)	58·3 (26·4)	.59
Nonverbal Ability	70·2 (18·8)	73·4 (19·9)	.42
ADOS Severity	8·0 (1·7)	7·6 (2·1)	.27

Standard scores for overall cognitive, verbal, nonverbal ability have Mean=100, SD=15

* p-value of 2-tailed t-test between groups