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# Thyroid dysfunction in children with autism spectrum disorder is associated with folate receptor $\alpha$ autoimmune disorder

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Folate receptor  $\alpha$  (FR $\alpha$ ) autoantibodies (FRAAs) are prevalent in autism spectrum disorder (ASD). FRAAs disrupt folate transport across the blood-brain barrier by binding to the FR $\alpha$ . Thyroid dysfunction is frequently found in children with ASD. We measured blocking and binding FRAAs and thyroid-stimulating hormone (TSH), free thyroxine (T4) (FT4), total triiodothyronine (T3) (TT3), reverse T3 (rT3), thyroid-releasing hormone (TRH) and other metabolites in 87 children with ASD, 84 of whom also underwent behaviour and cognition testing and in 42 of whom FRAAs, TSH and FT4 were measured at two time points. To better understand the significance of the FR $\alpha$  in relation to thyroid development, we examined FR $\alpha$  expression on prenatal and postnatal thyroid. TSH, TT3 and rT3 were above the normal range in 7%, 33% and 51% of the participants and TRH was below the normal range in 13% of the participants. FT4 was rarely outside the normal range. TSH concentration was positively and the FT4/TSH, TT3/TSH and rT3/TSH ratios were inversely related to blocking FRAA titres. On repeated measurements, changes in TSH and FT4/TSH ratio were found to correspond to changes in blocking FRAA titres. TSH and the FT4/TSH, TT3/TSH and rT3/TSH ratios were related to irritability on the Aberrant Behavior Checklist and several scales of the Social Responsiveness Scale (SRS), whereas TT3 was associated with SRS subscales and TRH was related to Vineland Adaptive Behavior Scale subscales. The thyroid showed significant FR $\alpha$  expression during the early prenatal period, although expression decreased significantly in later gestation and postnatal thyroid tissue. The results of the present study suggest that thyroid dysfunction in ASD may be related to blocking FRAA. The high expression of FR $\alpha$  in the early foetal thyroid suggests that foetal and neonatal exposure to maternal FRAAs could affect the development of the thyroid and may contribute to the pathology in ASD.

## KEYWORDS

autism spectrum disorders, folate receptor autoantibody, free T4, thyroid function, thyroid-stimulating hormone

## 1 | INTRODUCTION

There is increasing recognition that thyroid dysfunction is associated with neurological and psychiatric disease, including neurodevelopmental disorders such as intellectual disability<sup>1</sup> and autism spectrum

disorder (ASD),<sup>2–7</sup> focal and generalised neurological abnormalities,<sup>8</sup> as well as psychiatric manifestations such as psychosis,<sup>9,10</sup> panic attacks,<sup>11</sup> anxiety disorders,<sup>11,12</sup> bipolar disorder,<sup>13–15</sup> depression<sup>12,14</sup> and schizophrenia.<sup>16,17</sup> In addition, normal thyroid function is essential for normal cognition.<sup>18</sup> However, the exact nature of the relationship

between thyroid dysfunction and neurological and psychiatric disease is unclear.

Early uncontrolled treatment studies suggested that children with ASD showed a positive clinical response to triiodothyronine (T3).<sup>2,19</sup> A small controlled cross-over study found that T3 resulted in limited symptomatic improvement in children with ASD, especially in those with a lower intellectual quotient.<sup>20</sup> Other studies examining thyroid hormones have been inconsistent. A letter to editor in 1970 indicated a high rate of hypothyroidism (73%) in 62 children with ASD using T3 uptake testing,<sup>21</sup> although two other small studies could not confirm thyroid dysfunction in ASD, finding no difference in thyroid-stimulating hormone (TSH), thyroxine (T4) or T3 between ASD and control children.<sup>22,23</sup> Other studies suggest that ASD has been associated with a family history of autoimmune thyroid disorders<sup>24</sup> and that deficiencies in thyroid function may be present before or at birth in children with ASD.<sup>5,6</sup> More recent studies have examined thyroid regulation in the context of the hypothalamic-pituitary-axis (HPA) in ASD. TSH at baseline and following thyrotrophin-releasing hormone (TRH) stimulation was lower in 41 Japanese autistic boys compared to controls with mental retardation, minimal brain dysfunction and typical development, suggesting a blunted HPA response.<sup>3</sup> Diurnal variation in TSH levels was found to be larger in young adults with ASD compared to controls, again indicating differences in HPA function.<sup>4</sup>

One of the reasons for inconsistent findings could be explained if only subgroups of children with ASD had abnormal thyroid function. A recent study reported that folate receptor  $\alpha$  (FR $\alpha$ ) autoantibodies (FRAA), which are present in up to 75% of children with ASD,<sup>25</sup> may contribute to thyroid dysfunction in ASD.<sup>7</sup> In 32 children with ASD, those positive for the blocking FRAAs had a higher TSH compared to those negative for the blocking FRAAs, and the blocking FRAA was found to correlate positively with TSH.<sup>7</sup>

The relationship between thyroid function and FRAA titres was investigated because of the likelihood that autoantibodies could bind to the FR $\alpha$  on the thyroid and affect its function as a result of monoclonal antibodies to the two different epitopes of the glycosyl-phosphatidylinositol linked folate-binding membrane gp38 protein being found on normal thyroid tissue<sup>26</sup> with limited focal reactivity in some studies.<sup>27,28</sup> Binding of FRAAs to thyroid cells could affect thyroid function in several ways. When FRAAs bind to epithelial cells of the choroid plexus<sup>29</sup> they can disrupt FR $\alpha$  function, resulting in reduced transport of folate across the blood-brain barrier and causing a disorder known as cerebral folate deficiency syndrome.<sup>30</sup> Thus, it is possible that FRAA binding on the thyroid could decrease folate entry into thyroid cells. Folate is not a prominent cofactor in the synthesis of thyroid hormones. However, tyrosine, which is derived from phenylalanine using phenylalanine hydroxylase, is essential for the production of thyroid hormones. Importantly, an essential cofactor for phenylalanine hydroxylase is tetrahydrobiopterin, which is ultimately derived from the folate cycle.<sup>31–33</sup>

Alternatively, FRAAs binding to thyroid cells could activate the immune system, resulting in inflammation. Additionally, the folate pathway is intimately interconnected with methylation and redox regulation pathways, both of which are abnormal in children with ASD.<sup>34</sup> Methylation regulates gene expression and enzyme function, and so

alterations in methylation can affect cellular function. The reduced form of glutathione (GSH) is the major intracellular anti-oxidant that not only protects the cell from damage, but also regulates enzyme function; thus, decreased GSH can result in both cellular damage and deficits in enzyme activity.

Although the previous study<sup>7</sup> only examined the relationship between FRAAs and TSH, in the present study, free T4 (FT4), total T3 (TT3), reverse T3 (rT3) and TRH were also examined. We hypothesise that FRAAs may be disrupting the HPA by blunting the sensitivity of the thyroid to TSH or, in other words, making the thyroid resistant to the effects of TSH. This would result in a higher TSH concentration to produce a particular thyroid hormone concentration. Quantitatively, such an effect should be detectable by measuring the ratio of a thyroid hormone to TSH and determining whether this ratio is altered by FRAAs. We investigated this by examining the relationships between FRAAs and TSH, FT4, TT3, rT3, TRH and the ratio of thyroid hormones (FT4/TT3/rT3) to TSH. In addition, we determined whether markers of glutathione and methylation and blood concentrations of folate are related to thyroid function aiming to ensure that these physiological processes are not confounders in the thyroid function-FRAAs relationship. We also examined the relationship between thyroid function and behaviour and cognition.

Lastly, we also investigated the developmental timing of the potential relationship between FRAAs and thyroid function. Because thyroid metabolism is critical prenatally<sup>35,36</sup> and some mothers of children with ASD are positive for FRAAs,<sup>25,37</sup> we examined the developmental aspect of the FR $\alpha$  on both foetal and post-natal thyroid tissue to determine whether the FR $\alpha$  is more significantly represented during a specific developmental time period. We also examined the change in FRAAs, TSH and FT4 over a 12-week period during a clinical trial.<sup>38</sup> The aim was to determine whether FRAAs and thyroid function change together, in support of the notion that FRAAs and thyroid function are dynamically linked.

## 2 | MATERIALS AND METHODS

Eighty-seven children with ASD (mean $\pm$ SD age 6 years 10 months $\pm$ 3 years 1 month) and twelve typically developing controls (mean $\pm$ SD age 8 years 2 months $\pm$ 5 years 2 months) participated in the present study. Controls were used to obtain measures of normal FT4 and TSH in the same population, independent of established control ranges. The samples used in the present study were obtained from two research protocols approved by the Institutional Review Board at the University of Arkansas for Medical Science (Little Rock, AR, USA). For both studies, written informed consent was obtained from parents of participants; participant assent was waived. For both studies, fasting blood samples before breakfast were required as one of the first procedures after consent. The inclusion and exclusion criteria remained broad, aiming to include a representative sample of children with idiopathic ASD.

A diagnosis of ASD was required for entry into the study. A diagnosis was defined by one of the following: (i) a gold-standard diagnostic

instrument such as the Autism Diagnostic Observation Schedule and/or Autism Diagnostic Interview-Revised (ADI-R); (ii) the State of Arkansas diagnostic standard, defined as agreement of a physician, psychologist and speech therapist; and/or (iii) Diagnostic Statistical Manual diagnosis by a physician along with standardised validated questionnaires and diagnosis confirmation by the principal investigator (R. E. Frye). Reconfirmation of the diagnosis using the ADI-R by an independent research reliable rater was requested for a portion of participants to confirm that the criteria used for including the participants was equivalent to this gold-standard instrument.<sup>39</sup>

Excluded from the study were those children on medication interfering with thyroid function, including thyroid supplements, steroids,  $\beta$ -blockers, antipsychotics and lithium, as well as children with well-defined genetic syndromes.

## 2.1 | Folate autoantibody assay

Approximately 1 mL of serum was collected and sent to the laboratory of Dr Edward Quadros at the State University of New York, Downstate (Brooklyn, NY, USA). The assay for both the blocking and binding FRAAs has been described previously.<sup>30,40</sup> Blocking FRAAs were expressed as pmol of folic acid blocked from binding to FR $\alpha$  per mL of serum, and binding FRAAs were expressed as pmol of immunoglobulin (Ig)G antibody per mL of serum.

## 2.2 | Thyroid function assays

Plasma TSH, FT4, TT3, TRH, rT3 and cortisol were measured using enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's instructions (ab1000660, ab108686, ab108685 from Abcam, Cambridge, MA, USA; CEA839Hu from Cloud-Clone Corp, Houston, TX, USA; MBS2510365 from MyBioSource, San Diego, CA, USA; 11-CORHU-E01 from ALPCO, Salem, NH, USA). TSH, TT3 and FT4 were interpreted using the National Academy of Clinical Biochemistry standard for children.<sup>41</sup> TSH reference range was 0.4–5.0 mIU/L, FT4 reference range was 9–20 pmol/L and TT3 reference range was 80–180 ng/dL. Because the FT4 to TSH ratio is known to be log distributed, the thyroid hormone (FT4, TT3, rT3) to TSH ratios were log-transformed before analysis.<sup>41</sup> Normative values for TRH, rT3 and cortisol were obtained from the manufacturers as being in the range 35.7–167.8 pg/mL, 2.4–45.8 ng/dL and 7–25  $\mu$ g/dL, respectively. Cortisol was measured because thyroid hormones can be modulated by cortisol.

### 2.2.1 | Immunohistochemical analysis of FR $\alpha$ expression in the thyroid

Foetal thyroid tissue from 15, 17, 18, 19 and 20 weeks old fetuses preserved in formalin were obtained from the University of Maryland Brain and Tissue Bank, which is a Brain and Tissue Repository of the NIH NeuroBioBank. These were embedded in paraffin and sections (6  $\mu$ m thick) were cut. Thyroid sections from a 5-month-old infant, 3-year-old child and a 27-year-old adult were purchased from US

Biomax Inc. (Rockville, MD, USA). All sections were deparaffinised and incubated with 100  $\mu$ L of goat serum for 1 hour followed by incubation with a 1 : 500 dilution of rabbit antihuman FR $\alpha$  in 100  $\mu$ L of goat serum overnight. The sections were washed and incubated with a 1 : 600 dilution of goat anti rabbit IgG-peroxidase conjugate (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 hour, washed and incubated with diaminobenzidine as the chromagen. The sections were stained with haematoxylin (Vector Laboratories, Inc.) to visualise the nuclei.

The polyclonal antiserum used was prepared by immunising rabbits with affinity purified FR $\alpha$  from human epidermoid carcinoma KB cells conditioned to grow in low folate that up-regulates FR $\alpha$  expression.<sup>42</sup> The purity of the antigen was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The titre of the antiserum was checked by direct immunoprecipitation of <sup>3</sup>HPGA-FR $\alpha$  and by ELISA and the specificity of the antiserum was checked by blocking the titre with excess purified FR $\alpha$ . The specificity of immunostaining was established by absorbing out the antibody on to FR $\alpha$ -sepharose matrix and by blocking of immunostaining when the antiserum is pre-incubated with a ten-fold molar excess of purified FR $\alpha$ .

## 2.3 | Redox, methylation, immune and vitamin biomarkers

Redox and methylation potential was measured by the free reduced-to-oxidised glutathione redox ratio (GSH/GSSG) and SAM to SAH ratio (SAM/SAH), respectively. Fasting blood (4 mL) was collected into an ethylenediaminetetraacetic acid-Vacutainer tube, chilled on ice and centrifuged at 1500 g for 15 minutes at 4°C. Plasma was stored at –80°C and analysed by high-performance liquid chromatography with electrochemical detection within 2 weeks of collection as described previously.<sup>43</sup> Plasma total folate and vitamin B<sub>12</sub> was measured using a SimulTRAC-SNB Radioassay Kit (catalogue number 06B264806; MP Diagnostics, Santa Ana, CA, USA).

## 2.4 | Cognitive and behavioural assessments

The Preschool Language Scale-4 and two versions of the Clinical Evaluations of Language Fundamentals were used to assess language ability.<sup>38</sup> Both instruments provide a standardised core language score, thus providing an index of language ability independent of the instrument used. For each participant, the most ability appropriate instrument was used to prevent floor and ceiling effects. Language testing was available on 84 participants.

Adaptive behaviour was assessed using the Vineland Adaptive Behavior Scales (2nd edition), Interview Edition, Survey Form (VABS), an instrument that has demonstrated good reliability and validity.<sup>44</sup> Standardised scores for summary scales examined were communication, daily living skills, social skills, motor skills and adaptive behaviour composite. VABS testing was available on 84 participants.

The Aberrant Behavior Checklist (ABC) was designed to measure disruptive behaviours in individuals with developmental disabilities.<sup>45</sup> The ABC has been shown to have convergent and divergent validity

in ASD<sup>46</sup> and has been used in multiple autism clinical trials.<sup>47</sup> ABC scores were available on 82 participants.

The Social Responsiveness Scale (SRS) measures the severity of social skill deficits.<sup>48</sup> It has been validated and shown to be reliable and to have good correspondence to the gold-standard ADI-R, at the same time as being more time efficient and cost effective.<sup>49</sup> SRS scores were available on 81 participants.

## 2.5 | Folinic acid treatment

Forty-two participants were part of a double-blind, placebo-controlled trial on the effect of folinic acid on children with ASD.<sup>38</sup> In these participants, FRAA titres, TSH and T4 were measured prior to starting treatment and at the end of 12 weeks of treatment with either high-dose folinic acid (2 mg/kg/day in two divided doses; maximum 50 mg/day) or placebo in most of the participants in the trial.

## 2.6 | Statistical analysis

The 'glm' and 'glimmix' procedure of SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA) were used for the cross-sectional analysis and repeated-measures analysis, respectively, and a two-tailed  $\alpha$  of 0.05 was used. We dichotomised blocking and binding FRAA status separately, as well as the overall FRAA status (positive vs negative). We also examined the relationship between thyroid hormones and blocking and binding FRAA titres separately. A mixed-model was used for the repeated measures analysis with the participant variable as the random-effect.

# 3 | RESULTS

## 3.1 | Participants

Participants were recruited from our research registry (40%), autism clinic (23%), community advertisement and social media (10%), word-of-mouth (15%) and physician referrals (13%). The basic participant characteristics did not differ across the FRAA status (Table 1). All participants evaluated by an independent research reliable rater exceeded the threshold for autism diagnosis.

## 3.2 | Overall thyroid function

For the overall ASD population, the mean $\pm$ SD TSH concentration was 2.76 $\pm$ 1.82 mIU/L (range 0.63–11.55 mIU/L) and was below and above the standard reference range in 0% and 7% of ASD participants, respectively, and no different from controls (4.14 $\pm$ 2.13 mIU/L).

For the overall ASD population, FT4 concentration was 14.38 $\pm$ 2.27 pmol/L (range 8.66–21.12 pmol/L) and was below and above the standard reference range in 1% and 2% of ASD children, respectively, and slightly higher than controls (13.77 $\pm$ 3.00 pmol/L) ( $F_{1,94}=5.19$ ,  $P=.03$ ).

For the overall ASD population, the FT4/TSH ratio was 0.78 $\pm$ 0.26 and was not significantly different from controls (0.56 $\pm$ 0.29).

TT3 concentration was 170.5 $\pm$ 24.4 ng/dL (range 117.4–224.6 ng/dL) and was abnormally low and high in 0% and 33% of the ASD children, respectively, using the standard reference range. The TT3/TSH ratio was 1.86 $\pm$ 0.28 (range 1.12–2.50).

rT3 concentration was 49.8 $\pm$ 13.5 ng/dL (range 28.8–95.3 ng/dL) and was abnormally low and high in 0% and 51% of the ASD children, respectively, using the manufacturer's reference range. The rT3/TSH ratio was 1.32 $\pm$ 0.28 (range 0.65–1.92).

Thyroid-releasing hormone concentration was 61.0 $\pm$ 24.0 pg/mL (range 19.7–127.9 pg/mL) and was abnormally low and high in 13% and 0% of the ASD children, respectively, using the manufacturer's reference range.

Thyroid hormones were not significantly correlated with cortisol levels.

## 3.3 | Thyroid function and folate-related metabolism

FT4 was related to the SAM/SAH ratio, an index of methylation abnormalities, such that a higher SAM/SAH ratio (better methylation) was related to a lower FT4 ( $F_{1,84}=8.30$ ,  $P=.005$ ). TSH was found to be positively related to the glutathione redox ratio, such that higher (better) glutathione redox ratio was related to higher TSH concentrations ( $F_{1,84}=4.48$ ,  $P=.05$ ).

None of the other thyroid function measures were linearly related to glutathione, SAM/SAH, folate or B<sub>12</sub>.

## 3.4 | Thyroid function and folate receptor $\alpha$ autoantibodies

Thyroid-stimulating hormone was significantly higher in participants positive (3.94 $\pm$ 2.94 mIU/L) for the blocking FRAA compared to those negative (2.49 $\pm$ 1.38 mIU/L) for the blocking FRAA ( $F_{1,83}=10.85$ ,  $P=.001$ ) and the TSH concentration was found to increase as the blocking FRAA titres increased ( $F_{1,84}=7.85$ ,  $P=.006$ ) (Figure 1A).

Participants who were positive for the blocking FRAA demonstrated a lower FT4/TSH ratio (0.66 $\pm$ 0.30) compared to participants negative for the blocking FRAA (0.82 $\pm$ 0.25) ( $F_{1,83}=6.12$ ,  $P=.02$ ). In addition, the blocking FRAA titre demonstrated an inverse relationship with the T4/TSH ratio ( $F_{1,84}=5.27$ ,  $P=.02$ ) (Figure 1B).

Participants who were positive for the blocking FRAA demonstrated a lower TT3/TSH ratio (1.71 $\pm$ 0.32) compared to participants negative for the blocking FRAA (1.90 $\pm$ 0.27) ( $F_{1,83}=6.09$ ,  $P=.02$ ). In addition, the blocking FRAA titre demonstrated an inverse relationship with the TT3/TSH ratio ( $F_{1,84}=7.58$ ,  $P<0.01$ ) (Figure 1C).

Participants who were positive for the blocking FRAA demonstrated a lower rT3/TSH ratio (1.36 $\pm$ 0.27) compared to participants negative for the blocking FRAA (1.67 $\pm$ 0.28) ( $F_{1,83}=6.09$ ,  $P=.02$ ). In addition, the blocking FRAA titre demonstrated an inverse relationship with the rT3/TSH ratio ( $F_{1,84}=4.95$ ,  $P=.03$ ) (Figure 1D).

Thyroid-stimulating hormone and the ratios of thyroid hormones (FT4, TT3, rT3) to TSH were not significantly different across positive vs negative binding FRAA participants, nor were they significantly linearly related to binding FRAA titres.

**TABLE 1** Demographic and clinical characteristics by folate receptor  $\alpha$  autoantibody groups

Variable	FRAA negative (n=35)	FRAA positive (n=52)
Age (years, months), mean $\pm$ SD	6 years 10 months $\pm$ 3 years 6 months	6 years 11 months $\pm$ 2 years 11 months
Males, n (%)	28 (80%)	42 (81%)
Vineland adaptive behaviour composite, mean (SD)	64.2 (11.7)	64.8 (9.8)
TSH (mIU/L)	2.44 (1.36)	3.00 (2.06)
High TSH (<0.4 mIU/L)	2 (5%)	5 (9%)
Low TSH (>5.0 mIU/L)	0 (0%)	0 (0%)
Free T4 (pmol/L)	14.47 (1.92)	14.33 (2.51)
High T4 (<9 pmol/L)	0 (0%)	2 (4%)
Low T4 (>20 pmol/L)	0 (0%)	1 (2%)
Free T4 to TSH ratio (log-transformed)	0.84 (0.26)	0.75 (0.27)
Total T3 (ng/dL)	170.41 (25.1)	170.5 (24.2)
High T3 (>180 ng/dL)	0 (0%)	0 (0%)
Low T3 (<80 ng/dL)	13 (37%)	16 (31%)
Total T3 to TSH ratio (log-transformed)	1.91 (0.28)	1.73 (0.23)
Reverse T3 (ng/dL)	48.9 (8.3)	57.9 (23.4)
High rT3 (>45.77 ng/dL)	0 (0%)	0 (0%)
Low rT3 (<2.39 ng/dL)	20 (57%)	24 (46%)
Reserve T4 to TSH ratio (log transformed)	1.36 (0.27)	1.18 (0.22)
Thyroid-releasing hormone (pg/mL)	65.6 (24.6)	50.4 (16.2)
High TRH (>167.8 pg/mL)	3 (9%)	8 (15%)
Low TRH (<35.7 pg/mL)	0%	0%
Cortisol ( $\mu$ g/dL)	18.45 (8.9)	20.4 (13.0)
Folate (ng/mL) (normal 5-21)	16.9 (3.9)	18.0 (4.5)
B <sub>12</sub> (pg/mL) (normal 200-900)	786 (395)	1313 (1391)
Language testing, n (%)		
Preschool Language Scales	13 (37%)	16 (30%)
Clinical evaluation of language fundamentals 2	14 (40%)	17 (32%)
Clinical evaluation of language fundamentals 4	7 (20%)	20 (38%)
Diagnostic documentation, n (%)		
Autism diagnostic observation schedule	18 (51%)	25 (47%)
3 Practitioner agreement	27 (77%)	35 (66%)
Single practitioner with standardised questionnaires	5 (14%)	13 (25%)
Autism diagnostic interview-revised		
Participated in confirmation testing, n (%)	28 (80%)	41 (77%)
Social interaction score, mean $\pm$ SD (range)	21.57 $\pm$ 5.41 (10-30)	22.68 $\pm$ 4.85 (11-30)
Communication score: verbal, mean $\pm$ SD (range)	16.67 $\pm$ 4.24 (10-24)	19.38 $\pm$ 3.67 (7-25)
Communication score: nonverbal, mean $\pm$ SD (range)	12.69 $\pm$ 2.36 (7-14)	13.07 $\pm$ 1.53 (9-14)
Restricted & repetitive play score, mean $\pm$ SD (range)	5.79 $\pm$ 1.83 (2-10)	5.95 $\pm$ 2.47 (2-12)
Summary score, mean $\pm$ SD (range)	4.43 $\pm$ 0.92 (2-5)	4.39 $\pm$ 0.89 (2-5)
Medications (concurrent treatments), n (%)		
Melatonin	15 (43%)	11 (21%)
Allergy/asthma medications	11 (31%)	11 (21%)
Gastrointestinal medications	11 (31%)	9 (17%)
$\alpha$ -Adrenergic agonists	6 (17%)	10 (19%)
Stimulant	6 (17%)	8 (15%)

(Continues)



**TABLE 1** (Continued)

Variable	FRAA negative (n=35)	FRAA positive (n=52)
Anti-epileptic medication	1 (3%)	7 (13%)
Selective serotonin reuptake inhibitors	0 (0%)	7 (13%)
Antimicrobial medications	2 (6%)	3 (6%)
Immunomodulatory medications	1 (3%)	2 (4%)
Other psychotropic medications	0 (0%)	1 (2%)
Supplements (concurrent treatments), n (%)		
Multivitamin	14 (40%)	16 (30%)
Minerals	5 (14%)	11 (21%)
Fatty acids	4 (11%)	9 (17%)
Vitamin B <sub>12</sub>	1 (3%)	10 (19%)
Other B vitamins	2 (6%)	8 (15%)
Folate	4 (11%)	6 (11%)
Other antioxidants	2 (6%)	7 (13%)
Carnitine	2 (6%)	6 (11%)
Other vitamins	4 (11%)	4 (8%)
Coenzyme Q10	2 (6%)	3 (6%)
Other supplements	0 (0%)	2 (4%)
Amino acids	0 (0%)	1 (2%)
Comorbid medical conditions, n (%)		
Allergic disorders	15 (43%)	21 (40%)
Gastrointestinal disorders	14 (40%)	22 (42%)
Neurological disorders	6 (17%)	17 (32%)
Nondiagnostic copy number variants	8 (23%)	15 (28%)
Psychiatric disorders	2 (6%)	15 (28%)
Immune abnormality	6 (17%)	9 (17%)

FRAA, folate receptor  $\alpha$  autoantibody; T3, triiodothyronine; T4, thyroxine; TRH, thyroid-releasing hormone; TSH, thyroid-stimulating hormone.

Thyroid-releasing hormone, FT4, T3 and rT3 were not significantly different across FRAA groups, nor were they significantly linearly related to blocking or blinding FRAA titres.

### 3.5 | Relationship between thyroid function and behavior and cognition

Higher TSH was related to lower (better) ABC Irritability ( $F_{1,80}=5.94$ ,  $P=.02$ ) (Figure 2A) and SRS Awareness ( $F_{1,79}=5.01$ ,  $P=.03$ ) (Figure 2B), Cognition ( $F_{1,79}=5.97$ ,  $P=.03$ ) (Figure 2C), Motivation ( $F_{1,79}=4.46$ ,  $P=.04$ ) (Figure 2D), Mannerisms ( $F_{1,79}=6.87$ ,  $P=.01$ ) (Figure 2E) and Total score ( $F_{1,79}=5.72$ ,  $P=.02$ ) (Figure 2F).

A higher FT4/TSH ratio was related to higher (worse) ABC Irritability ( $F_{1,80}=7.56$ ,  $P=.01$ ) (Figure 2G) and Hyperactivity ( $F_{1,80}=5.29$ ,  $P=.02$ ) (Figure 2H) and SRS Awareness ( $F_{1,79}=4.70$ ,  $P=.03$ ) (Figure 2I), Cognition ( $F_{1,79}=5.82$ ,  $P=.02$ ) (Figure 2J), Communication ( $F_{1,79}=4.00$ ,  $P=.05$ ) (Figure 2K), Motivation ( $F_{1,79}=4.89$ ,  $P=.03$ ) (Figure 2L), Mannerisms ( $F_{1,79}=5.35$ ,  $P=.02$ ) (Figure 2M) and Total score ( $F_{1,79}=5.61$ ,  $P=.02$ ) (Figure 2N).

Higher T3 was related to higher (worse) SRS Cognition ( $F_{1,79}=4.83$ ,  $P=.03$ ) (Figure 2O), Motivation ( $F_{1,79}=6.98$ ,  $P=.01$ ) (Figure 2P),

Mannerisms ( $F_{1,79}=4.55$ ,  $P=.04$ ) (Figure 2Q) and Total score ( $F_{1,79}=5.14$ ,  $P=.03$ ) (Figure 2R).

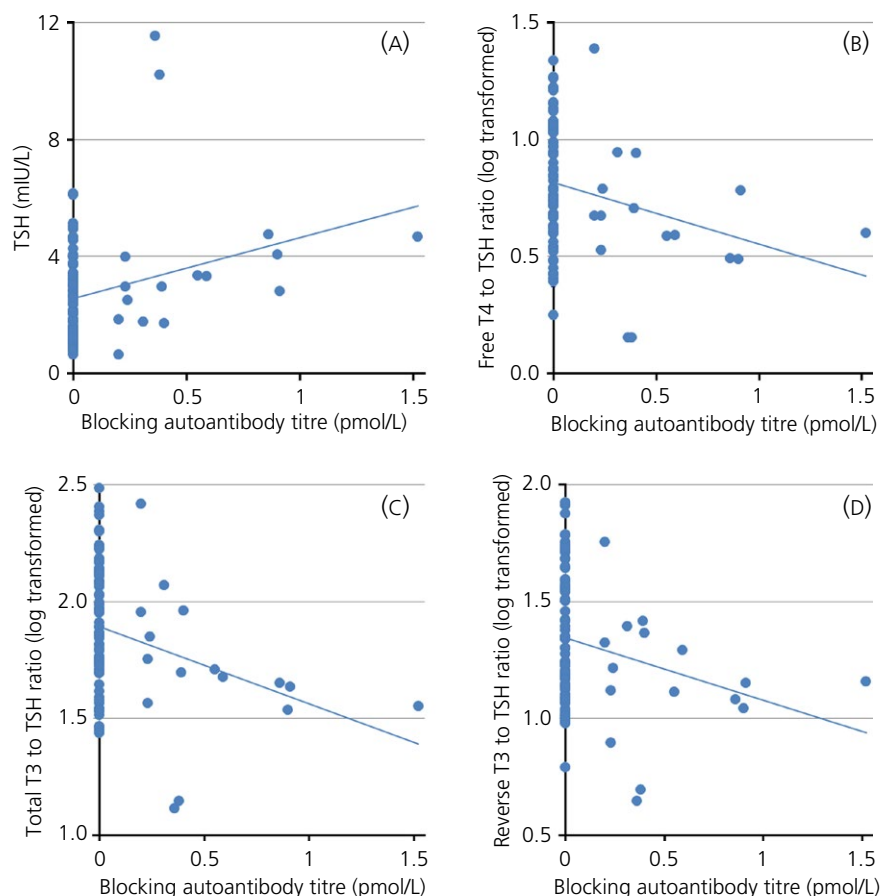
A higher TT3/TSH ratio was related to higher (worse) ABC Irritability ( $F_{1,80}=8.40$ ,  $P=.005$ ) (Figure 2S) and SRS Awareness ( $F_{1,79}=6.45$ ,  $P=.01$ ) (Figure 2T), Cognition ( $F_{1,79}=6.87$ ,  $P=.01$ ) (Figure 2U), Communication ( $F_{1,79}=3.83$ ,  $P=.05$ ) (Figure 2V), Motivation ( $F_{1,79}=4.90$ ,  $P=.03$ ) (Figure 2W), Mannerisms ( $F_{1,79}=6.35$ ,  $P=.01$ ) (Figure 2X) and Total score ( $F_{1,79}=7.50$ ,  $P<.01$ ) (Figure 2Y).

A higher rT3/TSH ratio was related to higher (worse) ABC Irritability ( $F_{1,80}=10.26$ ,  $P=.002$ ) (Figure 2AA) and SRS Awareness ( $F_{1,79}=5.14$ ,  $P=.03$ ) (Figure 2AB), Cognition ( $F_{1,79}=4.35$ ,  $P=.04$ ) (Figure 2AC) and Total score ( $F_{1,79}=5.30$ ,  $P=.02$ ) (Figure 2AD).

Higher TRH was related to lower (worse) VABS Daily Living Skills ( $F_{1,82}=5.36$ ,  $P=.02$ ) (Figure 2AE) and Social Skills ( $F_{1,82}=4.25$ ,  $P=.04$ ) (Figure 2AF).

### 3.6 | Repeated thyroid and autoantibody measurements

Forty-two participants were part of a clinical trial in which they were blindly randomised to receive either placebo or high-dose folinic acid



**FIGURE 1** In children with autism spectrum disorder higher blocking folate receptor  $\alpha$  autoantibody (FRAA) titres are associated with (A) higher thyroid-stimulating hormone (TSH), (B) lower thyroxine (T4) to TSH ratio, (C) lower total triiodothyronine (T3) to TSH ratio and (D) lower reserve T3 to TSH ratio

and had both thyroid function and FRAA measured at the beginning and end of the trial. This allowed us to examine the correspondence between the change in thyroid function and FRAA titres and to determine whether high-dose folinic acid had any systematic effect on thyroid function.

The change in TSH and FT4/TSH ratio (but not FT4) was significantly related to the change in blocking FRAA, but not the binding FRAA. The change in TSH had a positive relationship with the blocking FRAA titres ( $F_{1,40}=6.22$ ,  $P=.02$ ) (Figure 3A), whereas the change in the FT4/TSH ratio had an inverse relationship to the blocking FRAA titres ( $F_{1,40}=4.39$ ,  $P=.04$ ) (Figure 3B). Neither TSH and FT4, nor FT4/TSH ratios were related to whether or not the patient received high-dose folinic acid or placebo.

### 3.7 | FR $\alpha$ expression in the thyroid

To examine the developmental aspect of the FR $\alpha$  with respect to thyroid development, FR $\alpha$  expression was measured on the thyroid gland during various ages of life. Figure 4 shows that FR $\alpha$  is highly expressed at 15 and 17 weeks of gestation, whereas FR $\alpha$  is not expressed in the foetal tissue at 18, 19 or 20 weeks of gestation, nor was it expressed in the 5-month postpartum thyroid tissue, suggesting that FR $\alpha$  expression is only present in early gestation and lost in later gestation as the thyroid matures. We have also examined thyroid from a 3-year-old child and a 27-year-old adult and found no FR $\alpha$  expression (data not shown).

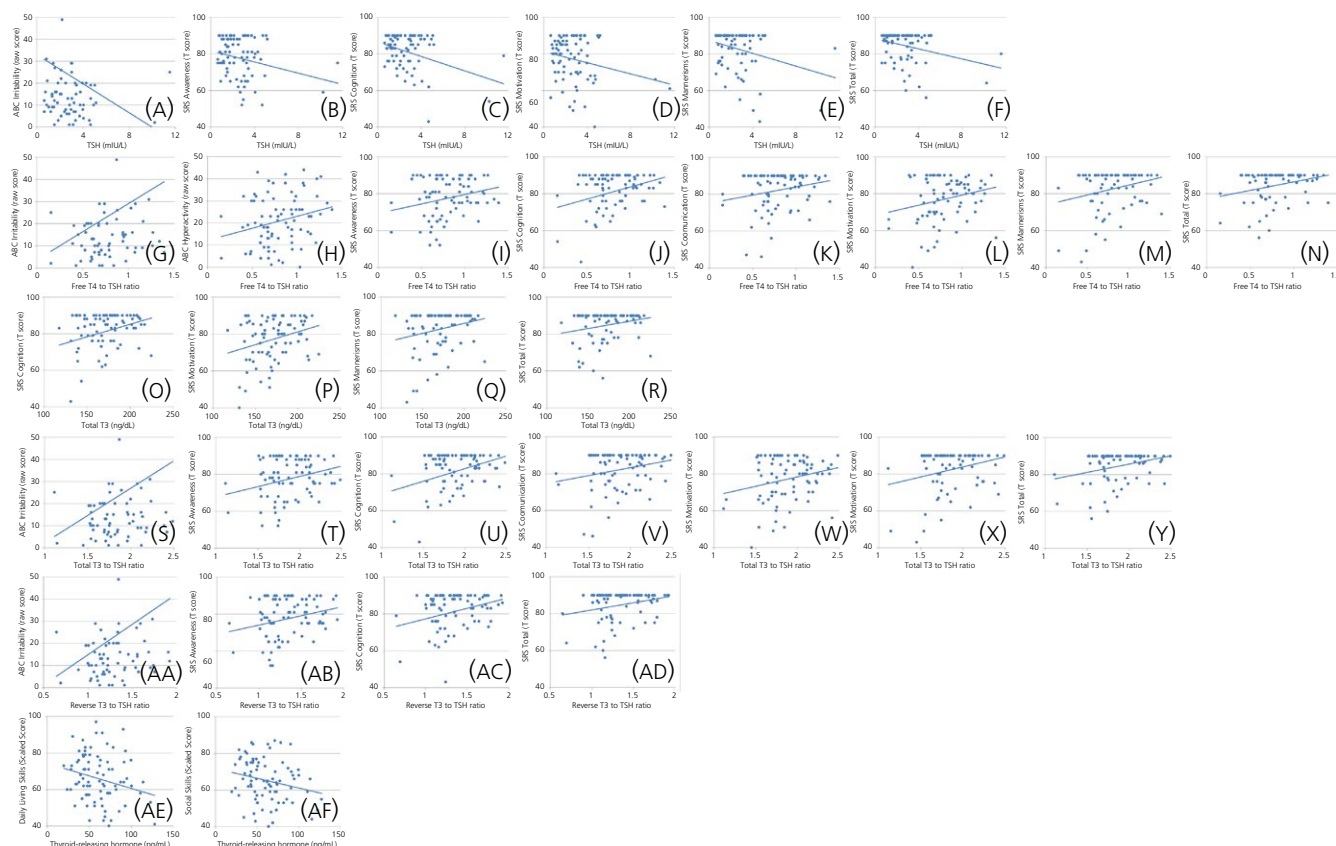
## 4 | DISCUSSION

Both blocking and binding FRAAs are considered to have pathological consequences in ASD because they bind to the FR $\alpha$  where they can interfere with function of the FR $\alpha$  and reduce the transportation of folate into the central nervous system<sup>25</sup> and they may also activate the immune system.<sup>50</sup> There are reports of the FR $\alpha$  being expressed on normal thyroid tissue,<sup>26</sup> although sometimes only to a limited extent.<sup>27,28</sup> In the present study, we investigated the significance of FRAAs in conjunction with thyroid function in children with ASD to follow-up on our previous report.<sup>7</sup> We also examined FR $\alpha$  expression on the thyroid tissue at different stages of human development. Several interesting relationships were identified.

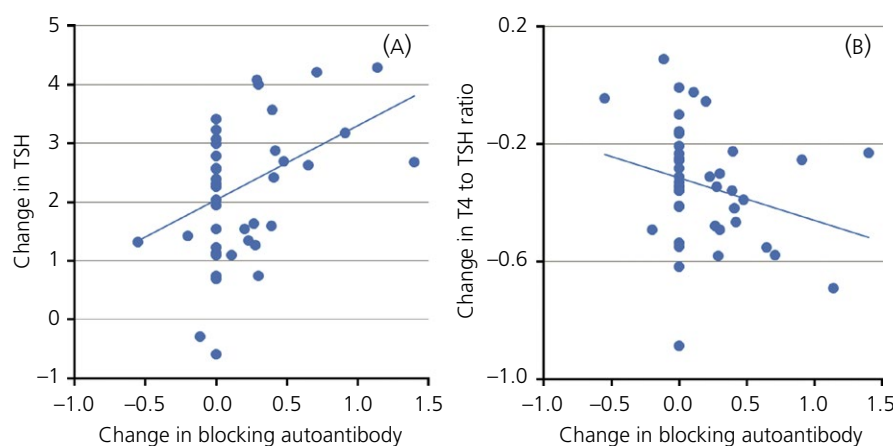
Autism spectrum disorder children positive for blocking FRAAs had higher TSH and lower FT4/TSH, TT3/TSH and rT3/TSH ratios than those negative for blocking FRAAs. Additionally, blocking FRAA titres were linearly related to TSH concentrations and FT4/TSH, TT3/TSH and rT3/TSH ratios. Furthermore, when we examined the changes in FRAA titres and thyroid function (TSH, FT4) over a 12-week period, we found a strong relationship between a change in blocking (but not binding) FRAA titres and a change in the TSH and FT4/TSH ratio, consistent with the cross-section data. The blocking FRAA did not appear to have significant influence on FT4, TT3 or rT3 specifically.

It appears that the blocking FRAA has an influence on TSH and the FT4/TSH, TT3/TSH and rT3/TSH ratios by increasing the TSH relative





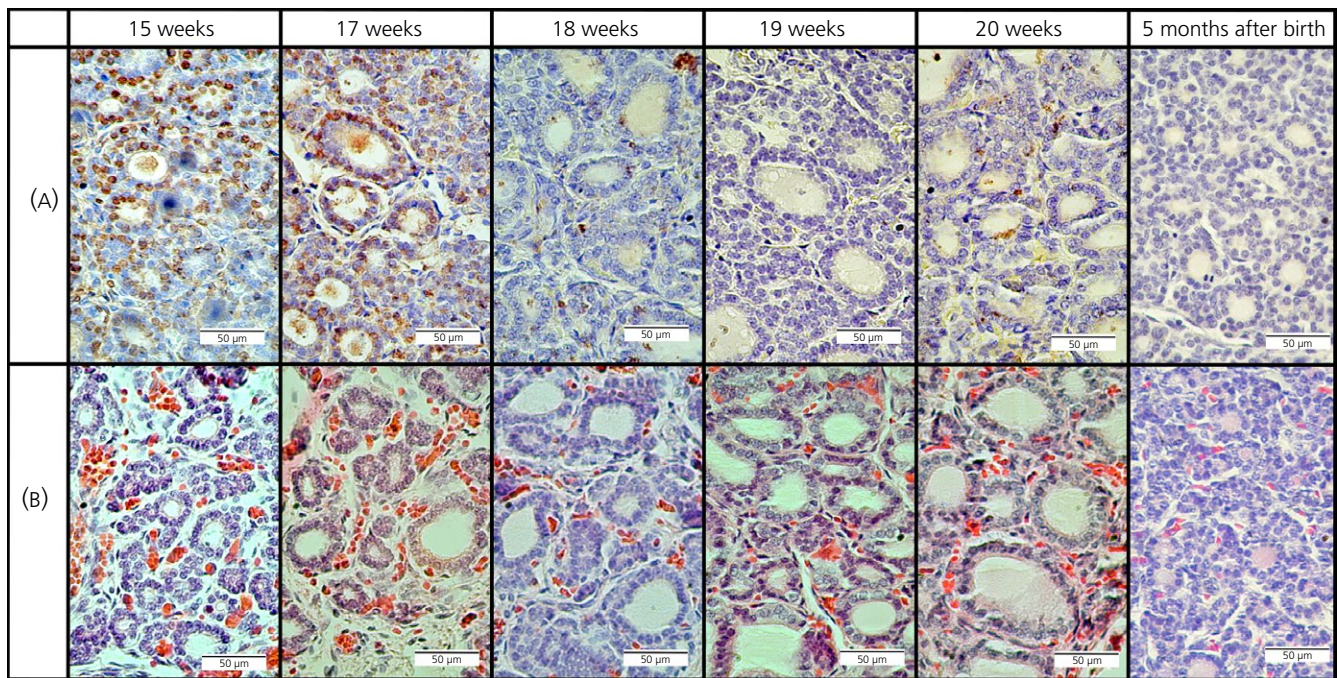
**FIGURE 2** The relationship between thyroid function and behaviour in children with autism spectrum disorder. Higher thyroid-stimulating hormone (TSH) was associated with lower (better) (A) Aberrant Behavior Checklist (ABC) Irritability, (B) Social Responsiveness Scale (SRS) Awareness, (C) SRS Cognition, (D) SRS Motivation, (E) SRS Mannerisms (F) SRS Total Score; Higher free thyroxine (T4) to TSH ratio was associated with higher (worse) (G) ABC Irritability, (H) ABC Hyperactivity, (I) SRS Awareness, (J) SRS Cognition, (K) SRS Communication, (L) SRS Motivation, (M) SRS Mannerisms and (N) SRS Total Score; Higher total T3 was associated with higher (worse) (O) SRS Cognition, (P) SRS Motivation, (Q) SRS Mannerisms and (R) SRS Total Score. Higher total triiodothyronine (T3) to TSH ratio was associated with higher (worse) (S) ABC Irritability, (T) SRS Awareness, (U) SRS Cognition, (V) SRS Communication, (W) SRS Motivation, (X) SRS Mannerisms and (Y) SRS Total Score; Higher reverse T3 to TSH ratio was associated with higher (worse) (AA) ABC Irritability, (AB) SRS Awareness, (AD) SRS Cognition, and (AD) SRS Total Score; Higher Thyroid Releasing Hormone was related to worse (lower) (AE) VABS Daily Living Skills and (AF) VABS Social Skills



**FIGURE 3** Change in (A) thyroid-stimulating hormone (TSH) and (B) the free thyroxine (T4) to TSH ratio is associated with a change in the blocking folate receptor  $\alpha$  autoantibody

to the production of thyroid hormones. This suggests that the effect of the blocking FRAA on thyroid function may occur at the level of the thyroid by making the thyroid less sensitive to TSH or altering the sensitivity of the HPA. Specifically, a lower FT4/TSH, TT3/TSH, rT3/TSH ratios would suggest that a higher TSH concentration is needed for the

production of thyroid hormones, implying a decreased sensitivity of the thyroid or HPA. The idea that children with ASD may have differences in HPA dynamics and/or thyroid sensitivity to TSH is consistent with both research and clinical studies. Studies have shown a blunted pituitary response to TRH stimulation in ASD boys<sup>3</sup> and the diurnal



**FIGURE 4** Folate receptor  $\alpha$  expression in the thyroid tissue during embryonic development and following birth. (A) Folate receptor  $\alpha$  expression in the thyroid gland. Counterstained samples with haematoxylin to visualise nuclei. (B) Tissue stained with haematoxylin and eosin.

variation of TSH appears to be larger in young adults with ASD.<sup>4</sup> TRH concentrations were not related to blocking FRAA titres, which suggests that the effect is at the level of the thyroid and not the pituitary.

The effect of thyroid hormones on the brain is complex, especially during foetal and neonatal development.<sup>35,36</sup> FR $\alpha$  is highly expressed in foetal thyroid tissue only early in gestation, with no observable expression in late gestation or postnatal thyroid tissue. This is based on a limited number of thyroid samples and therefore this finding should be considered preliminary. Exposure to maternal FRAAs and folate deficiency *in utero* may contribute to poor development of the gland and dysfunction in later life. The reported histological data suggests that the influence of FRAAs could occur during foetal development. Because several studies have demonstrated that mothers of children positive for FRAAs also are positive for FRAAs,<sup>25,37</sup> it is possible that maternal FRAAs present during gestation could disrupt thyroid development and/or alter the developmental expression of the FR $\alpha$  on the thyroid.

There is evidence that maternal FRAAs can disrupt foetal development. Maternal FRAAs result in neurodevelopmental abnormalities in rodent offspring<sup>51</sup> and maternal FRAAs have been linked to neural tube defects and subfertility in humans,<sup>52</sup> as well as preterm birth.<sup>53</sup> It is possible that disruption of foetal thyroid development by maternal FRAAs could have resulted in altered regulation of the thyroid during childhood. However, the change in TSH with change in FRAA demonstrated on repeated measurements suggest that FRAAs may have a more direct effect on regulation of the thyroid during childhood. For example, it is possible that foetal exposure to FRAAs may have altered developmental FR $\alpha$  expression so that the FR $\alpha$  has increased expression on the thyroid during childhood. Examining thyroid tissue from

children exposed to FRAAs during gestation would help clarify this possibility. However, to our knowledge, no such tissue is available at present.

Behaviour appears to be related to thyroid function in ASD children such that higher levels of TSH is associated with better ASD behavioural characteristics using the ABC as well as the SRS scales, an instrument that has high correspondence with a gold-standard ASD assessment, the ADI-R. The FT4/TSH, TT3/TSH and rT3/TSH ratios also appear to be similarly related to ABC and SRS scales, such that a lower ratio is associated with better overall behaviour. The relationship between thyroid hormones and behaviour should not be surprising because thyroid hormones can both positively and negatively modulate neurotransmitter systems as, well as have a direct effect on gene expression.<sup>54</sup> However, the exact mechanism by which thyroid hormones influence behaviour and cognition in ASD remains to be clarified.

Thyroid-stimulating hormone is related to behaviour even though, for the most part, it is within the normal range in our sample, and both TSH and the FT4/TSH are related to behaviour even though they are not significantly different than the control values in our sample. This suggests that the effect is not related to absolute thyroid hormone levels but rather to HPA sensitivity, such that some children with ASD may be more sensitive to a small variation in thyroid hormone levels. This would suggest that the relationship between TSH and thyroid hormones produced by the thyroid (ie, FT4, TT3, rT3) is more important than the absolute concentrations of these hormones. This could explain why some studies have demonstrated normal thyroid hormone levels in children with ASD,<sup>22,23</sup> whereas others have shown differences in dynamic changes in thyroid hormones in children with ASD,

such as a blunted response to TRH stimulation<sup>3</sup> and differences in the diurnal variation of thyroid hormone levels.<sup>4</sup> These data suggest that simply using normal ranges to interpret levels of thyroid hormones in children with ASD may not capture subtle abnormalities in thyroid function.

Several preliminary clinical studies that have suggested a favourable response to thyroid hormone supplementation in children with ASD<sup>2,19,20</sup> and animal models have demonstrated an improvement in ASD behaviours with T4 supplementation.<sup>55</sup> Although thyroid hormone supplementation would appear to be counter to our behavioural data and the fact that TT3 and rT3 was found to be above normal in a high percentage of children with ASD, it is possible that such supplementation could reduce variations in the HPA, leading to a more stable regulation of thyroid hormones. Interestingly, clinical studies in ASD individuals show a positive effect of propranolol, a medication that blocks the conversion of T4 to T3, thereby potentially providing benefit in those with high TT3 or rT3 concentrations. Indeed, in individuals with ASD, propranolol has been shown to improve language,<sup>56,57</sup> cognitive flexibility,<sup>58</sup> working memory,<sup>59</sup> facial scanning<sup>60</sup> and behaviour.<sup>61,62</sup> Lithium, a medication that inhibits thyroid function, has been shown to have positive effect on ASD behaviours in several clinical<sup>63-65</sup> and animal studies,<sup>66</sup> thereby also indicating a relative overactivity of the thyroid as the culprit.

The findings of the present study could guide future research studies aiming to better understand the importance of thyroid function in ASD, such that the clinical implications of the thyroid in ASD can be better understood. First, it does appear that there is a relationship between FRAAs and the thyroid, with FRAAs most likely affecting the sensitivity of the HPA, probably at the level of the thyroid. There also appears to be a relatively increased expression of the FR $\alpha$  during prenatal development, suggesting that the effects of FRAAs may be most significant before birth. Additionally, data reported within the present study suggest that variations in thyroid function do indeed have an effect on the behaviour of children with ASD, although the exact mechanism for this influence is not clear. We consider that it might be wise for further research to concentrate on the HPA with respect to the thyroid and focus on the dynamic changes in thyroid hormones at baseline, as well as on treatments that affect the thyroid.

In conclusion, the present study has helped define the importance of thyroid function in ASD and the effect of the FRAA on thyroid function in ASD. It appears that foetal exposure to blocking FRAAs could affect the development of the thyroid, potentially making the thyroid less sensitive to TSH. TSH and the ratio of hormones produced by the thyroid to TSH appear to be related to ASD behaviour, implicating dysregulation of the HPA, although the exact significance of these findings will require further clinical study with larger populations. Overall the present study highlights the importance of the FRAAs and thyroid function, both together and separately, in ASD and the need for further clinical studies to better understand how targeted therapeutic interventions can help improve the lives of individuals with ASD and their families. These findings may also be applicable to other disorders associated with FRAAs, such as schizophrenia<sup>67</sup> and subfertility.<sup>68</sup>

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## CONFLICT OF INTERESTS

Drs Frye and Quadros are on the Scientific Advisory Board of Iliad Neurosciences Inc. (Plymouth Meeting, PA). Two of the authors (JMS and EVQ) are inventors on a US patent for the detection of FR autoantibodies issued to the Research Foundation of the State University of New York. The other authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

REF, RW, SR, JS, LD, MT, SCB, SM, JMS and EVQ performed the research. REF, SR, JS, LD and EVQ designed the research study. REF, LD and EVQ analysed the data. REF, SR, JS, LD, SGK, JMS and EVQ wrote the paper.

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