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ORIGINAL ARTICLE

Sex-specific serum biomarker patterns in adults with Asperger's syndrome

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Autism spectrum conditions have been hypothesized to be an exaggeration of normal male low-empathizing and high-systemizing behaviors. We tested this hypothesis at the molecular level by performing comprehensive multi-analyte profiling of blood serum from adult subjects with Asperger's syndrome (AS) compared with controls. This led to identification of distinct sex-specific biomarker fingerprints for male and female subjects. Males with AS showed altered levels of 24 biomarkers including increased levels of cytokines and other inflammatory molecules. Multivariate statistical classification of males using this panel of 24 biomarkers revealed a marked separation between AS and controls with a sensitivity of 0.86 and specificity of 0.88. Testing this same panel in females did not result in a separation between the AS and control groups. In contrast, AS females showed altered levels of 17 biomarkers including growth factors and hormones such as androgens, growth hormone and insulin-related molecules. Classification of females using this biomarker panel resulted in a separation between AS and controls with sensitivities and specificities of 0.96 and 0.83, respectively, and testing this same panel in the male group did not result in a separation between the AS and control groups. The finding of elevated testosterone in AS females confirmed predictions from the 'extreme male brain' and androgen theories of autism spectrum conditions. We conclude that to understand the etiology and development of autism spectrum conditions, stratification by sex is essential.
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Introduction

Autism spectrum conditions are diagnosed on the basis of difficulties in communication and social development, in addition to unusually narrow interests, repetitive behavior and resistance to change.[1] The two major subgroups are classic autism and Asperger's syndrome (AS). AS differs from classic autism in that there is no history of language delay or learning difficulties.[2] The prevalence of autism spectrum conditions is 1% of the general population[3] and there is a marked sex difference, with males being 4–10 times more likely to be affected than females.[4] This suggests that sex-linked factors might have a role in the etiology of the condition. In the general population, males on average perform at lower levels on tests of empathy and social sensitivity and show stronger interest in systems. People with autism spectrum conditions show an exaggeration of this cognitive profile, which has led to the 'extreme male brain' (EMB) theory as an explanation of the psychological manifestations.[5] The EMB theory is supported by the finding of tomboyism and other masculine behaviors in females with autism spectrum conditions.[6] The EMB theory has been extended recently to the neurological level. For example, in typical males the overall brain volume and specific regions such as the amygdala are larger. In autism spectrum conditions, these differences are even more extreme.[7] However, little is known about the molecular pathways underlying these effects.

The fetal androgen theory of autism spectrum conditions[7] suggests that increased levels of fetal testosterone leads to typical sex differences in brain structure and function. Individual differences in amniotic fetal testosterone are positively correlated with number of autistic traits[8] and systemizing,[9] and negatively correlated with language development, social development and empathy.[10] This theory is

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also supported by the observation of an increased rate of testosterone-related disorders such as polycystic ovary syndrome, acne and hirsutism in women with autism spectrum conditions,[11] a lower ratio of the second (index) and fourth (ring) digit lengths (2D:4D) in both males and females with autism spectrum conditions,[12] and an association of autism spectrum conditions with CAG repeats in the androgen receptor gene,[13] single nucleotide polymorphisms in the *HOXD* gene,[14] and an association between autistic traits and/or autism spectrum conditions with single nucleotide polymorphisms in sex-steroid related genes.[15]

Other molecular factors in the etiology of autism spectrum conditions may not be sex-linked. Neurotrophins such as brain-derived neurotrophic factor (BDNF) are elevated in the blood of children with autism, which may be associated with early brain overgrowth[16] and single nucleotide polymorphisms in genes related to neural connectivity and neurotrophic pathways may also be involved.[15] In addition, several studies have identified an over-activation of mast[17] and natural killer[18] cells in autism spectrum conditions that may be associated with autoimmunity or adverse immune interactions during critical periods of brain development. Autism spectrum conditions are also associated with familial autoimmune diseases and the presence of asthma, allergies and psoriasis during pregnancy.[19] The interactions of these pathways in the nervous system are likely to be mediated by hormones and cytokines.

Here, we present the first systematic serum proteome profiling study of adults with the AS subtype of autism spectrum conditions. We had three major objectives: (1) to test the EMB theory at the molecular level using a multiplex analyte profiling approach, (2) to test the prediction from the fetal androgen theory that adult females with AS would have elevated testosterone levels and (3) to determine whether males and females with AS have distinct sex-specific serum biomarker fingerprints.

Patients and methods

Sample collection

Protocols for recruitment, collection of clinical samples and the test methods were carried out in compliance with the Standards for Reporting of Diagnostic Accuracy initiative.[20] People with AS ($n = 45$) were recruited via the Cambridge Autism Research Centre database of volunteers (www.autismresearchcentre.com), the National Autistic Society (UK) and local autism social groups in the UK. Controls ($n = 50$) were recruited from the general population using leaflets and advertising and these individuals were matched to the AS group with respect to age, intelligence quotient, educational level, socioeconomic status, handedness, sex and body mass index (Supplementary Table S2). AS was diagnosed by psychiatrists based on the Structured Clinical Interview for the Diagnostic and Statistical Manual-IV-Text Review Disorders and accepted international criteria (DSM-IV-TR). The Wechsler Abbreviated Scale of Intelligence was administered to all participants to measure intelligence quotient. All diagnoses and clinical tests were performed by psychiatrists under Good Clinical Practice compliance to minimize variability. Any patients whose clinical diagnosis required revision at a later stage were not included in the study. Controls with a family history of serious mental illness or other medical conditions such as type II diabetes, hypertension, cardiovascular or autoimmune diseases were excluded.

Psychological measures

All participants were tested on the autism-spectrum quotient (AQ),[21] as a measure of the number of autistic traits an individual possesses, the empathy quotient (EQ) and the systemizing quotient-revised (SQ-R)[22] in order to test the EMB theory at the cognitive level. Normally, 80% of people with autism spectrum conditions score above a cutoff value of 32 (maximum = 50) on the AQ compared with only 2% for controls, a finding that has been confirmed cross-culturally.[23,24] AQ predicts clinical diagnosis[25] and has also been shown to be elevated among parents of children with autism spectrum conditions,[26] suggesting a heritability component.

Immunoassay profiling

We carried out HumanMAP profiling of 147 analytes (see Supplementary Table S1) in serum samples from the AS and control subjects in a Clinical Laboratory Improved Amendments-certified laboratory at Rules Based Medicine (Austin, TX, USA). This technology has already been applied successfully in several clinical studies.[27] Analytes were measured in multiplexes in 25–50 μl of serum samples. The assays were calibrated using standard curves and raw intensity measurements converted to protein concentrations using proprietary software. Analyses were conducted under blind conditions with respect to sample identities and the samples were analyzed randomly to avoid any sequential bias because of the presence or absence of diagnosis, participant age, or age of material.

Statistics

Analysis of variance was carried out on log-transformed data to investigate any interactions between sex and diagnosis for each molecular analyte and psychometric characteristic (AQ, EQ and SQ). To account for potential deviations from normality and homoscedasticity calculated interaction effects were compared against aligned rank transform non-parametric analyses. For this purpose, Hodges–Lehmann estimates of the two main effects were subtracted from the raw scores, data ranked and analyzed using two-way Analysis of variance. *P*-values are stated without adjustment for multiple hypothesis

correction as the necessity of this procedure remains controversial.[28]

Sensitivity and specificity values were calculated based on a leave-one out cross validation procedure using the AdaBoost algorithm.[29]

Results

Psychological measures

In this study, all participants were tested on the AQ,[21] the EQ and the SQ-R.[22] The AS group had higher scores for AQ and SQ-R, and lower scores for EQ, replicating previous findings.[21,22] Analysis of variance testing showed a significant interaction between sex and diagnosis for all three measures (AQ, $P=0.004$; EQ, $P=0.014$; SQ, $P=0.016$) with females with AS having more 'masculinized' scores compared with control females and compared with males with AS (Supplementary Table S2, Figure 1). Interaction effects were comparable to results obtained form non-parametric analysis.

Immunoassay profiling

We carried out HumanMAP profiling of 147 analytes in serum samples from the AS and control subjects. As a first step of the analysis, male and female subjects were analyzed separately because of the marked gender difference in prevalence rates. For this purpose, male and female subjects were randomized separately into training and blinded test sets to facilitate initial validation of gender specific biomarker signature. This is important as AS subjects are rare, and the prevalence is ~10 times lower for females than for males, making an independent replication of results difficult. Statistical analysis of the male training set identified a panel of 9 analytes, which showed significant differences in expression between AS ($n=12$) and control ($n=16$) subjects (Supplementary Table S3). Application of this panel to the male test set resulted in correct classification of 70% of AS ($n=10$) and 100% of control ($n=10$) subjects ($P=0.003$) (Figure 2a). In contrast, application of the panel to the female test set could not distinguish AS subjects from controls ($P=0.350$). Separate analysis of the female training set resulted in identification of 15 analytes with significant differences in expression between AS subjects ($n=13$) and controls ($n=14$; Supplementary Table S3). Application of this panel to the female test set resulted in correct classification of 90% of AS ($n=10$) and 90% of control ($n=10$) subjects ($P=0.001$; Figure 2b). Application of the female-specific panel to the male test set could not discriminate AS subjects from controls ($P>0.99$), as above for the male–female cross testing.

As a next step, serum samples were compared between the total set of AS and control subjects. This led to identification 25 analytes, which showed significant differences in expression between the groups (Supplementary Table S4). Analysis of variance found that 15 of these analytes also showed sex differences in expression. In addition, 14 separate

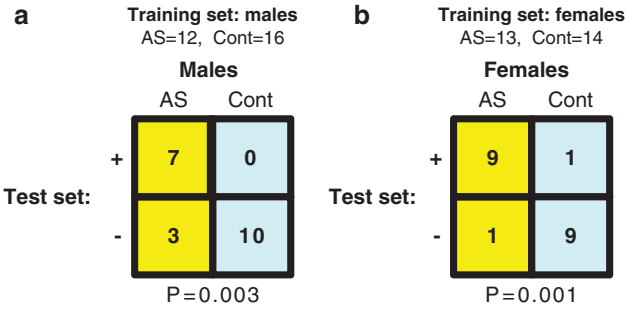

Figure 2 Classification and prediction of Asperger's syndrome (AS) and control (Cont) subjects using separate panels of (**a**) 10 male-specific analytes and (**b**) 15 female-specific analytes. The analytes were identified in separate training sets for male and female AS and control subjects. These were used for blinded prediction of male and female test subjects. The plus symbol indicate classification as AS and the minus symbol indicates classification as controls. *P*-values indicate the significance of the classification and were calculated using Fisher's exact test.

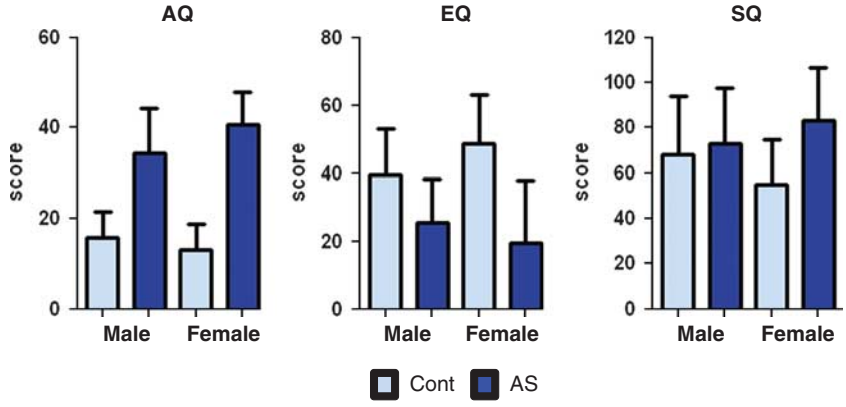

Figure 1 AQ, EQ and SQ in males and females with AS compared with controls. Abbreviations: AQ, autism-spectrum quotient; AS, Asperger's syndrome; EQ, empathy quotient; SQ, systemizing quotient.

analytes showed significant interactions between diagnosis and sex. Total testosterone showed no difference between AS and controls, but free testosterone levels showed a significant interaction between diagnosis and sex as determined by calculating the free androgen index, defined as the ratio of total testosterone to sex hormone-binding globulin.

The significant gender-diagnosis interactions support the presence of gender-specific biomarker signatures. Given the biological reproducibility observed in independent training and test sets, we repeated the gender-specific analysis using all subjects to increase the statistical power. This approach resulted in identification of 24 analytes that were altered significantly in AS males (see Table 1a) and 17 that were altered significantly in AS females (see Table 1b). Only 4 analytes (interleukin (IL)-12p40, tissue factor, IL-1B, glutamic oxaloacetic transaminase 1) were changed in the same direction in males and females, providing evidence for a minimal overlap of the molecular signatures (Figure 3a). One protein (tenascin-C) showed the opposite regulation (increased in males and decreased in females).

Multivariate classification revealed that the combined panel of 24 differentially expressed analytes in males produced a separation between AS and control subjects with a sensitivity of 0.86 and a specificity of 0.88 (visualized using partial least squares discriminant analysis in Figure 3b). Testing this same panel of 24 analytes in the female group did not result in a separation between AS and controls (Figure 3b). Likewise, the combined panel of 17 differentially expressed analytes in females produced a separation between AS and control subjects with a sensitivity of 0.96 and a specificity of 0.83, and testing this same panel in the male group did not result in a separation between AS and controls (Figure 3c).

Analytes that were altered in males with AS included several cytokines (IL-3, IL-4, IL-5, IL-10, IL-12p70, tumor necrosis factor-α, epithelial-derived neutrophil-activating protein-78), fatty acid binding protein, the neuroendocrine secreted protein chromogranin A, and the cardiovascular and blood cell-associated proteins thrombopoietin and erythropoietin.

The female-specific AS analytes showed a marked difference in nature and included growth factors, growth hormone, endothelin-1, BDNF, luteinizing hormone and free testosterone.

Because of the association of growth hormone deficiency in adult life and decreased insulin sensitivity,[30] we also measured the levels of insulin in AS compared with controls as this was part of the HumanMAP panel. There was no change for male AS subjects ($P = 0.469$) although AS females showed a non-significant 1.57-fold increase compared with levels observed in control females ($P = 0.208$; data not shown). As insulin is co-released with residual levels of unprocessed proinsulin and the des31, 32-proinsulin conversion intermediate, we also measured the levels these molecules specifically using sensitive two-site time resolved fluorometric assays.[31]

Table 1 Identification of differentially expressed analytes in serum of AS subjects

Analyte	P-*value*	*FC*
(a) Comparison of AS males and control males		
Interleukin-12p40 (IL-12p40)	0.0015	2.88
Fatty acid binding protein	0.0010	2.02
IL-3	0.0313	1.98
Erythropoietin	0.0093	1.92
Tissue factor (TF)	0.0001	1.70
IL-5	0.0011	1.59
Granulocyte colony-stimulating factor	0.0042	1.47
IL-1B	0.0034	1.44
Chromogranin A	0.0124	1.42
Neuronal cell adhesion molecule	0.0303	1.36
Tenascin-C	0.0300	1.32
Tumor necrosis factor-α (TNF-α)	0.0044	1.29
ENA-78	0.0493	1.26
IL-18	0.0186	1.24
Factor-VII	0.0316	1.21
Connective tissue growth factor	0.0090	1.19
IL-4	0.0267	1.19
Thrombopoietin	0.0022	1.19
Stem cell factor	0.0098	1.18
Sortilin 1	0.0078	1.14
IL-10	0.0333	1.12
IL-12p70	0.0466	1.10
Intercellular adhesion molecule 1 (ICAM-1)	0.0466	1.10
Serum glutamic oxaloacetic transaminase (GOT1)	0.0008	0.83
(b) Comparison of AS females and control females		
NARG1	0.0082	2.87
IL-12p40	0.0011	2.74
FAI	0.0001	2.01
IL-1B	0.0073	1.75
Luteinizing hormone	0.0471	1.56
IL-7	0.0224	1.47
TF	0.0328	1.42
BDNF	0.0150	1.20
GOT1	0.0238	0.84
Apolipoprotein-CIII (Apo-CIII)	0.0438	0.83
Immunoglobulin M	0.0130	0.76
sRAGE	0.0425	0.76
Apo-A1	0.0063	0.75
Tenascin-C	0.0021	0.68
Eotaxin-3	0.0266	0.53
Endothelin-1	0.0369	0.50
Growth hormone	0.0103	0.44
(c) Two-site TRF assays for insulin-related molecules in female AS subjects		
Insulin	0.0594	1.81
Proinsulin	0.2508	1.26
Des31,32-proinsulin (31,32-PI)	0.0228	1.89

Abbreviations: AS, Asperger's syndrome; BDNF, brain-derived neurotrophic factor; ENA-78, epithelial-derived neutrophil-activating protein-78; FAI, free androgen index; FC, fold change; NARG1, NMDA receptor regulated 1; sRAGE, receptor for advanced glycosylation end product.

Glucose levels showed a trend increase in AS females compared with control females (FC = 1.11; P = 0.0670). Glucose was determined spectrophotometrically using an adaptation of the hexokinase-glucose-6-phosphate dehydrogenase method in a Dimension RXL Clinical Chemistry System (Dade Behring; Milton Keynes, UK). Insulin was measured using a two-step time resolved fluorometric (TRF) assay from Perkin Elmer (Beaconsfield, Bucks, UK).[31] Proinsulin and des31,32-proinsulin (31,32-PI) were determined using two-site TRF assays employing combinations of monoclonal antibodies which can distinguish between the proinsulin forms.[31]

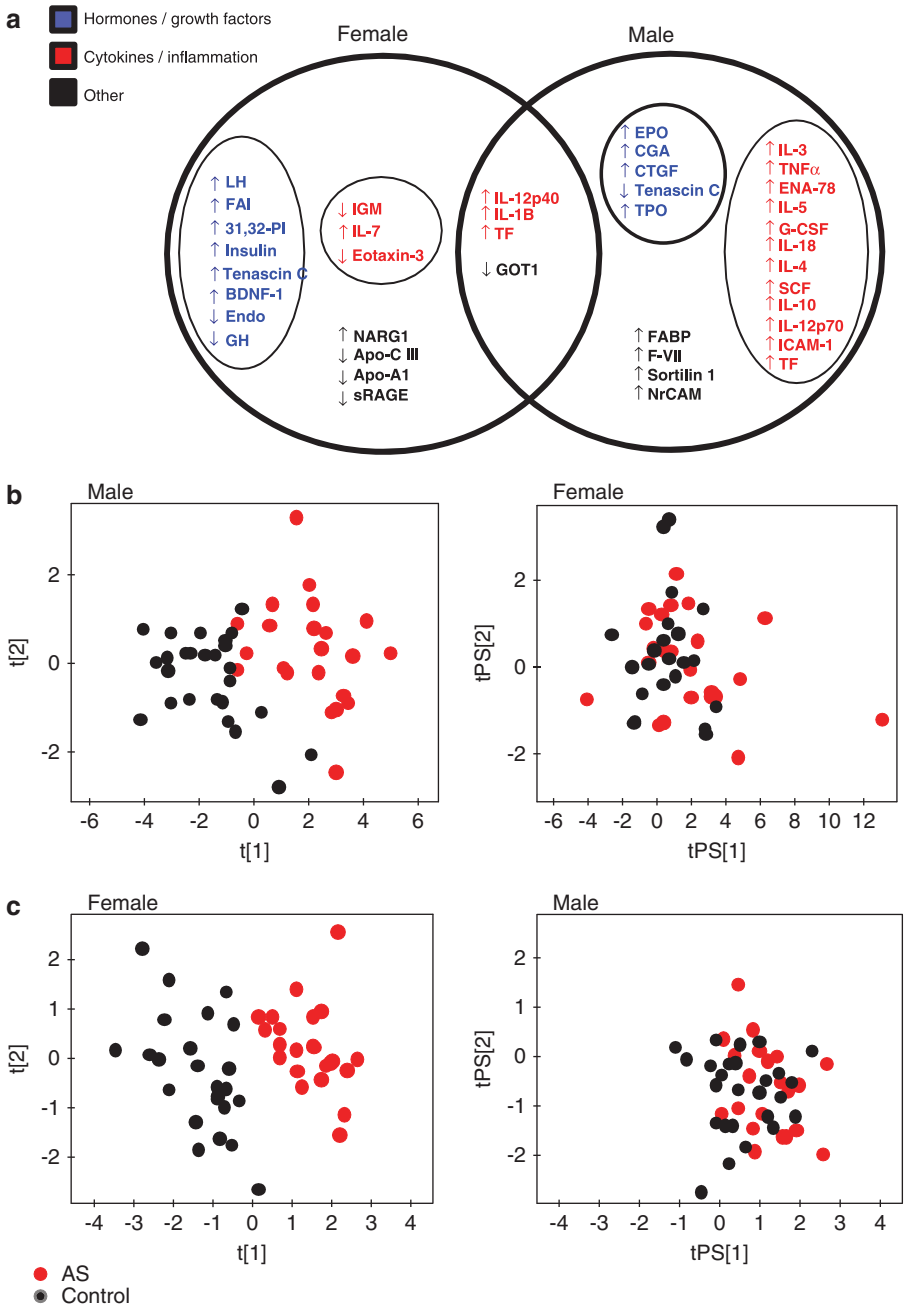

Figure 3 (**a**) Venn diagram showing distinct and common serum biomarker profiles for males and females with Asperger's syndrome (AS). Blue typeface and encircled area indicates hormones and growth factors. Red typeface and encircled area indicates cytokines and inflammatory factors. Black typeface indicates markers that fit into other (non-specified) categories. Up arrows indicate analytes that show increased expression and down arrows indicate analytes with decreased expression relative to the values seen in the respective controls. (**b**) Partial Least Squares Discriminant Analysis (PLSDA) showing the separation of AS and control subjects using differentially expressed analytes. Each spot represents an AS (red) or control (black) subject. The model built on males using 24 AS male-specific analytes (left) does not yield a separation in prediction of AS in females (right). (**c**) The model built on females with AS and female controls using 17 female specific analytes (left) does not yield a separation when predicting AS in males (right). This is a standard statistical technique for characterizing data variances. A simplified projection of multi-dimensional data is given such that the greatest variance lies on the first coordinate, the second greatest variance on the next coordinate, and so on. Abbreviations: Apo, apolipoprotein; BDNF, brain-derived neurotrophic factor; CTGF, connective tissue growth factor; ENA-78, epithelial-derived neutrophil-activating protein-78; EPO, erythropoietin; FABP, fatty acid binding protein; FAI, free androgen index; G-CSF, granulocyte colony-stimulating factor; GOT, glutamic oxaloacetic transaminase; ICAM, intercellular adhesion molecule; IGM, immunoglobulin M; IL, interleukin; NARG1, NMDA receptor regulated 1; NrCAM, neuronal cell adhesion molecule; SCF, stem cell factor; sRAGE, receptor for advanced glycosylation end product; TF, tissue factor; TNF-α, tumor necrosis factor-α; TPO, thrombopoietin.

For insulin, the results of the time resolved fluorometric assay showed a high correlation ($r^2 = 0.98$) with the multiplexed HumanMAP platform with an increase in AS females of 1.81-fold compared with control females although, again, this did not reach significance (Table 1c). The levels of proinsulin were not altered significantly although des31,32-proinsulin showed a significant 1.89-fold increase in females. The finding that glucose levels were relatively normal in the same subjects (1.11-fold; $P = 0.0670$) suggested that the relatively high levels of insulin-related molecules may be due to insulin resistance.

Discussion

This is the first study demonstrating that males and females with AS have distinct biomarker fingerprints, which can be detected in blood serum. For many of these molecules, this is the first description for their association with autism spectrum conditions. The remainder has been described previously thereby adding an extra layer of validity to the results. The finding that the biomarker fingerprint for AS males was comprised mostly of increased levels of cytokines is consistent with previous reports showing effects on immune function and autoimmune disorders in autism spectrum conditions.[17–19] The changes in testosterone and BDNF in females with AS are also consistent with the findings of other studies, which have suggested that these molecules may be elevated in adults with autism spectrum conditions.[11,16] The observation of high testosterone levels and relatively low cytokine signature in females aligns well with previous studies, which have suggested that testosterone may have protective effects on inflammatory disorders.[32]

Our results showing that free testosterone levels were elevated in adults with AS lends further support to the androgen theory of autism spectrum conditions.[7] This effect was specific for females, which underlines the importance of recognizing sex as a key variable in autism research. Also, the finding of higher levels of biologically active testosterone in AS females provides molecular evidence of the EMB theory.[5] Females with AS showed an extreme of the male brain phenotype as seen by the AQ, EQ and SQ-R psychological measures suggesting that the masculinization in cognition and androgens may be correlated. The increases in testosterone levels in AS females were paralleled by increases in the levels of luteinizing hormone in the same subjects. Previous studies have shown that luteinizing hormone pulsatility may predispose or cause hyperandrogenism in female adolescents[33] and has been associated with polycystic ovarian syndrome.[34] This is consistent with our earlier work showing elevated rates of testosterone-related disorders including polycystic ovarian syndrome in women with AS.[11]

The serum levels of BDNF were also increased specifically in females with AS. This is interesting as previous studies have shown increased levels of this growth factor in children with autism spectrum conditions in association with atypical cerebral development.[16] Females with AS also showed an approximate twofold decrease in growth hormone levels, supporting a previous studies that found a growth hormone deficit in autism.[35] The findings on growth hormone in autism spectrum conditions have not been consistent, with some studies reporting elevated levels of growth-related hormones,[36] whereas others have found low cerebrospinal fluid insulin-like growth factor-I concentrations in children with autism.[37] However, it should be noted that all these previous studies were conducted using children during the early developmental stages and not on adults as in the case of our study.

As growth hormone deficiency in adult life has been associated with decreased insulin sensitivity,[30] we also measured serum levels of insulin-related peptides and glucose. This showed that insulin and the conversion intermediate, des31,32-proinsulin, were both elevated even though glucose levels were relatively normal. This provided evidence suggesting that at least some of the females with AS may have insulin resistance. This is intriguing because of the link between hyperinsulinemia and hyperandrogenism as described in previous studies of polycystic ovarian syndrome and post-menopausal women.[38,39] Importantly, treatment with insulin sensitizing agents such as rosiglitazone has been shown to improve insulin sensitivity, leading to alleviation of hyperandrogenism and the associated symptoms.[40] This highlights the importance of further studies exploring the relationships between androgen abnormalities and BDNF, growth hormone and insulin signalling in autism spectrum conditions.

In AS males, the specific serum biomarker fingerprint showed only a small overlap with that in females with AS. In particular, the predominant feature of this signature in males was increased levels of several cytokines and other inflammatory molecules, consistent with the proposed role of these pathways in autism spectrum conditions.[41,42] In contrast, AS females showed altered levels of biomarkers including growth factors and hormones such as androgens, growth hormone and insulin-related molecules. The distinct nature of these sex-specific molecular fingerprints was exemplified by statistical classification analyses, which showed that these were only capable of providing good separation of AS and control subjects within the respective male and female groups. The male-specific biomarkers yielded good separation only of male AS and control subjects, and the female-specific panel was capable of separating only AS females from control females. Although the observed classification accuracy needs to be reproduced in an independent cohort, these differences underscore the importance of stratifying samples according to sex in biological studies of autism spectrum conditions. The sex-specific molecular profiles observed here lead to the suggestion that either different compensatory mechanisms occur in

males and females with autism spectrum conditions or that these conditions may develop through distinct sex-specific molecular pathways.

Assessing the specificity of the present fingerprints for AS against other autism spectrum conditions in young, pubertal and adult subjects could lead to the development of much-needed novel diagnostic strategies associated with a personalized medicine strategy. The analytes identified through the present study could also be assessed through comparison with the biomarker fingerprints obtained for other neuropsychiatric disorders. In addition, application of a convergent functional genomic strategy, such as that proposed by Le-Niculescu *et al.*[43] could be useful for improving the sensitivity and specificity of emerging biomarker panels. Further studies of these factors could provide greater insight into the molecular mechanisms involved in both the etiology and development of autism spectrum conditions.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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