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Sex-Specific Serum Biomarker Patterns in Adults with Asperger Syndrome

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Abbreviations: Analysis of Variance, ANOVA; Autism-Spectrum Quotient, AQ; Asperger syndrome, AS; Brain Derived Neurotrophic Factor, BDNF; Diagnostic and Statistical Manual of Mental Disorders, DSM; Empathy Quotient, EQ; Extreme Male Brain, EMB; Fetal Testosterone, FT; PolyCystic Ovarian Syndrome, PCOS; Systemizing Quotient-revised, SQ-R;

Keywords: Asperger syndrome, serum, gender, sexual dimorphism, profiling, biomarker

37 **Abstract**

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

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65 Introduction

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

84 The fetal androgen theory of autism spectrum conditions (7) suggests that increased
85 levels of fetal testosterone (FT) leads to typical sex differences in brain structure and
86 function. Individual differences in amniotic FT are positively-correlated with number of
87 autistic traits (8) and systemizing (9), and negatively-correlated with language development,
88 social development and empathy (10). This theory is also supported by the observation of an
89 increased rate of testosterone-related disorders such as polycystic ovary syndrome (PCOS),
90 acne and hirsutism in women with autism spectrum conditions (11), a lower ratio of the 2nd
91 (index) and 4th (ring) digit lengths (2D:4D) in both males and females with autism spectrum
92 conditions (12), and an association of autism spectrum conditions with CAG repeats in the

93 androgen receptor gene (13), single nucleotide polymorphisms (SNPs) in the HOXD gene
94 (14), and an association between autistic traits and/or autism spectrum conditions with SNPs
95 in sex-steroid related genes (15).

96 Other molecular factors in the aetiology of autism spectrum conditions may not be
97 sex-linked. Neurotrophins such as brain-derived neurotrophic factor (BDNF) are elevated in
98 the blood of children with autism, which may be associated with early brain overgrowth (16)
99 and SNPs in genes related to neural connectivity and neurotrophic pathways may also be
100 involved (15). In addition, several studies have identified an over-activation of mast (17) and
101 natural killer (18) cells in autism spectrum conditions that may be associated with
102 autoimmunity or adverse immune interactions during critical periods of brain development.
103 Autism spectrum conditions are also associated with familial autoimmune diseases and the
104 presence of asthma, allergies and psoriasis during pregnancy (19). The interactions of these
105 pathways in the nervous system are likely to be mediated by hormones and cytokines.
106 Here we present the first systematic serum proteome profiling study of adults with the AS
107 subtype of autism spectrum conditions. We had three major objectives: 1) to test the EMB
108 theory at the molecular level using a multiplex analyte profiling approach, 2) to test the
109 prediction from the fetal androgen theory that adult females with AS would have elevated
110 testosterone levels and 3) to determine whether males and females with AS have distinct
111 sex-specific serum biomarker fingerprints.

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118 **Methods**

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120 *Sample collection*

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

137

138 *Psychological measures*

139 All participants were tested on the Autism-Spectrum Quotient (AQ) (21), as a measure of the
140 number of autistic traits an individual possesses, the Empathy Quotient (EQ) and the
141 Systemizing Quotient-revised (SQ-R) (22) in order to test the EMB theory at the cognitive
142 level. Normally, 80% of people with autism spectrum conditions score above a cut-off value
143 of 32 (maximum = 50) on the AQ compared to only 2% for controls, a finding that has been

144 confirmed cross-culturally (23, 24). AQ predicts clinical diagnosis (25) and has also been
145 shown to be elevated among parents of children with autism spectrum conditions (26),
146 suggesting a heritability component.

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150 *Immunoassay profiling*

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

161

162 *Statistics*

163 ANOVA was carried out on log-transformed data to investigate any interactions
164 between sex and diagnosis for each molecular analyte and psychometric characteristic (AQ,
165 EQ and SQ). To account for potential deviations from normality and homoscedasticity
166 calculated interaction effects were compared against Aligned Rank Transform (ART) non-
167 parametric analyses. For this purpose, Hodges-Lehmann estimates of the two main effects
168 were subtracted from the raw scores, data ranked and analyzed using two-way ANOVA. P-

169 values are stated without adjustment for multiple hypothesis correction as the necessity of
170 this procedure remains to be controversial (28).

171 Sensitivity and specificity values were calculated based on a leave-one out cross validation
172 procedure using the AdaBoost algorithm (29).

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175 **Results**

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177 *Psychological measures*

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179 In this study, all participants were tested on the Autism-Spectrum Quotient (AQ) (21), the
180 Empathy Quotient (EQ) and the Systemizing Quotient-revised (SQ-R) (22). The AS group
181 had higher scores for AQ and SQ-R, and lower scores for EQ, replicating previous findings
182 (21, 22). Analysis of variance (ANOVA) testing showed a significant interaction between sex
183 and diagnosis for all three measures (AQ, $p=0.004$; EQ, $p=0.014$; SQ, $p=0.016$) with females
184 with AS having more “masculinized” scores compared to control females and compared to
185 males with AS (Supplementary Table S2, Fig. 1). Interaction effects were comparable to
186 results obtained from non-parametric analysis.

187

188

189 *Immunoassay profiling*

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191 We carried out HumanMAP® profiling of 147 analytes in serum samples from the AS and
192 control subjects. As a first step of the analysis, male and female subjects were analyzed
193 separately due to the marked gender difference in prevalence rates. For this purpose, male

194 and female subjects were randomized separately into training and blinded test sets to
195 facilitate initial validation of gender specific biomarker signature. This is important since
196 AS subjects are rare, and the prevalence is approximately 10 times lower for females than
197 for males, making an independent replication of results difficult. Statistical analysis of the
198 male training set identified a panel of 9 analytes which showed significant differences in
199 expression between AS (n=12) and control (n=16) subjects (Supplementary Table S3).
200 Application of this panel to the male test set resulted in correct classification of 70% of AS
201 (n=10) and 100% of control (n=10) subjects (P=0.003) (Fig. 2a). In contrast, application of
202 the panel to the female test set could not distinguish AS subjects from controls (P=0.350).
203 Separate analysis of the female training set resulted in identification of 15 analytes with
204 significant differences in expression between AS subjects (n=13) and controls (n=14)
205 (Supplementary Table S3). Application of this panel to the female test set resulted in correct
206 classification of 90% of AS (n=10) and 90% of control (n=10) subjects (P=0.001) (Fig. 2b).
207 Application of the female-specific panel to the male test set could not discriminate AS
208 subjects from controls (P>0.99), as above for the male-female cross-testing.

209 As a next step, serum samples from were compared between the total set of AS and
210 control subjects. This led to identification 25 analytes which showed significant differences in
211 expression between the groups (Supplementary Table S4). ANOVA found that 15 of these
212 analytes also showed sex differences in expression. In addition, 14 separate analytes
213 showed significant interactions between diagnosis and sex. Total testosterone showed no
214 difference between AS and controls but free testosterone levels showed a significant
215 interaction between diagnosis and sex as determined by calculating the free androgen index
216 (FAI), defined as the ratio of total testosterone to sex hormone binding globulin (SHBG).

217 The significant gender-diagnosis interactions support the presence of gender-specific
218 biomarker signatures. Given the biological reproducibility observed in independent training
219 and test sets, we repeated the gender-specific analysis using all subjects to increase the

220 statistical power. This approach resulted in identification of 24 analytes that were altered
221 significantly in AS males (see Table 1a) and 17 that were altered significantly in AS females
222 (see Table 1b). Only 4 analytes (IL-12p40, tissue factor, IL-1B, GOT1) were changed in the
223 same direction in males and females, providing evidence for a minimal overlap of the
224 molecular signatures (Fig. 3a). One protein (tenascin-C) showed the opposite regulation
225 (increased in males and decreased in females).

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

234 Analytes that were altered in males with AS included several cytokines (IL-3, IL-4, IL-5, IL-
235 10, IL-12p70, TNF α , ENA-78), fatty acid binding protein (FABP), the neuroendocrine
236 secreted protein chromogranin A, and the cardiovascular and blood cell-associated proteins
237 thrombopoietin (TPO) and erythropoietin (EPO).

238 The female-specific AS analytes showed a marked difference in nature and included growth
239 factors, growth hormone, endothelin-1, brain derived neurotrophic factor (BDNF), luteinizing
240 hormone and free testosterone.

241 Due to the association of growth hormone deficiency in adult life and decreased insulin
242 sensitivity (30), we also measured the levels of insulin in AS compared to controls as this
243 was part of the HumanMAP $\text{\textcircled{R}}$ panel. There was no change for male AS subjects ($p=0.469$)
244 although AS females showed a non-significant 1.57-fold increase compared to levels
245 observed in control females ($p=0.208$) (data not shown). As insulin is co-released with

246 residual levels of unprocessed proinsulin and the des31,32-proinsulin (31,32-PI) conversion
247 intermediate, we also measured the levels these molecules specifically using sensitive two-
248 site time resolved fluorometric (TRF) assays (31). For insulin, the results of the TRF assay
249 showed a high correlation ($r^2=0.98$) with the multiplexed xMAP[®] platform with an increase in
250 AS females of 1.81-fold compared to control females although, again, this did not reach
251 significance (Table 1c). The levels of proinsulin were not altered significantly although
252 des31,32-proinsulin showed a significant 1.89-fold increase in females. The finding that
253 glucose levels were relatively normal in the same subjects (1.11-fold; $p=0.0670$) suggested
254 that the relatively high levels of insulin-related molecules may be due to insulin resistance.

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262 **Discussion**

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264 This is the first study demonstrating that males and females with AS have distinct biomarker
265 fingerprints which can be detected in blood serum. For many of these molecules, this is the
266 first description for their association with autism spectrum conditions. The remainder have
267 been described previously thereby adding an extra layer of validity to the results. The finding
268 that the biomarker fingerprint for AS males was comprised mostly of increased levels of
269 cytokines is consistent with previous reports showing effects on immune function and

270 autoimmune disorders in autism spectrum conditions (17-19). The changes in testosterone
271 and BDNF in females with AS are also consistent with the findings of other studies which
272 have suggested that these molecules may be elevated in adults with autism spectrum
273 conditions (11, 16). The observation of high testosterone levels and relatively low cytokine
274 signature in females aligns well with previous studies which have suggested that
275 testosterone may have protective effects on inflammatory disorders (32).

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

289

290 The serum levels of BDNF were also increased specifically in females with AS. This is
291 interesting since previous studies have shown increased levels of this growth factor in
292 children with autism spectrum conditions in association with atypical cerebral development
293 (16). Females with AS also showed an approximate two-fold decrease in growth hormone
294 levels, supporting a previous studies that found a growth hormone deficit in autism (35). The
295 findings on growth hormone in autism spectrum conditions have not been consistent, with
296 some studies reporting elevated levels of growth-related hormones (36) whereas others

297 have found low cerebrospinal fluid insulin-like growth factor-I concentrations in children with
298 autism (37). However, it should be noted that all these previous studies were conducted
299 using children during the early developmental stages and not on adults as in the case of our
300 study.

301 Since growth hormone deficiency in adult life has been associated with decreased insulin
302 sensitivity (30), we also measured serum levels of insulin-related peptides and glucose. This
303 showed that insulin and the conversion intermediate, des^{31,32}-proinsulin, were both
304 elevated even though glucose levels were relatively normal. This provided evidence
305 suggesting that at least some of the females with AS may have insulin resistance. This is
306 intriguing because of the link between hyperinsulinemia and hyperandrogenism as described
307 in previous studies of PCOS and post-menopausal women (38, 39). Importantly, treatment
308 with insulin sensitizing agents such as rosiglitazone has been shown to improve insulin
309 sensitivity, leading to alleviation of hyperandrogenism and the associated symptoms (40).
310 This highlights the importance of further studies exploring the relationships between
311 androgen abnormalities and BDNF, growth hormone and insulin signalling in autism
312 spectrum conditions.

313

314 In AS males, the specific serum biomarker fingerprint showed only a small overlap with that
315 in females with AS. In particular, the predominant feature of this signature in males was
316 increased levels of several cytokines and other inflammatory molecules, consistent with the
317 proposed role of these pathways in autism spectrum conditions (41, 42). In contrast, AS
318 females showed altered levels of biomarkers including growth factors and hormones such as
319 androgens, growth hormone and insulin-related molecules. The distinct nature of these sex-
320 specific molecular fingerprints was exemplified by statistical classification analyses which
321 showed that these were only capable of providing good separation of AS and control
322 subjects within the respective male and female groups. The male-specific biomarkers
323 yielded good separation only of male AS and control subjects and the female-specific panel
324 was capable of separating only AS females from control females. Although the observed

325 classification accuracy needs to be reproduced in an independent cohort, these differences
326 underscore the importance of stratifying samples according to sex in biological studies of
327 autism spectrum conditions. The sex-specific molecular profiles observed here lead to the
328 suggestion that either different compensatory mechanisms occur in males and females with
329 autism spectrum conditions or that these conditions may develop through distinct sex-
330 specific molecular pathways.

331 Assessing the specificity of the present fingerprints for AS against other autism spectrum
332 conditions in young, pubertal and adult subjects could lead to the development of much-
333 needed novel diagnostic strategies associated with a personalised medicine strategy. The
334 analytes identified through the present study could also be assessed through comparison
335 with the biomarker fingerprints obtained for other neuropsychiatric disorders. In addition,
336 application of a convergent functional genomic strategy, such as that proposed by Le-
337 Niculescu and colleagues (43) could be useful for improving the sensitivity and specificity of
338 emerging biomarker panels. Further studies of these factors could provide greater insight
339 into the molecular mechanisms involved in both the aetiology and development of autism
340 spectrum conditions.

341

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343

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346 assays were performed by Keith Burling at the NIHR Cambridge Biomedical Research Centre, Core
347 Biochemistry Assay Laboratory, Addenbrookes Hospital, Cambridge, UK.

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350 **Conflict of interest**

351 The study has been funded by Psynova Neurotech Ltd. There are no conflicts of interest.

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359 **Tables**

360
361 **Table 1.**

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364 **a) Comparison of AS males and control males**

365

366 Analyte	p-value	FC
367 Interleukin-12p40 (IL-12p40)	0.0015	2.88
368 Fatty acid binding protein	0.0010	2.02
369 IL-3	0.0313	1.98
370 Erythropoietin (EPO)	0.0093	1.92
371 Tissue factor (TF)	0.0001	1.70
372 IL-5	0.0011	1.59
373 Granulocyte colony stimulating factor (G-CSF)	0.0042	1.47
374 IL-1B	0.0034	1.44
375 Chromogranin A (CGA)	0.0124	1.42
376 Neuronal cell adhesion molecule (NrcAM)	0.0303	1.36
377 Tenascin-C	0.0300	1.32
378 Tumour necrosis factor alpha (TNF α)	0.0044	1.29
379 ENA-78	0.0493	1.26
380 IL-18	0.0186	1.24
381 Factor-VII	0.0316	1.21
382 Connective tissue growth factor (CTGF)	0.0090	1.19
383 IL-4	0.0267	1.19
384 Thrombopoietin (TPO)	0.0022	1.19
385 Stem cell factor	0.0098	1.18
386 Sortilin 1	0.0078	1.14
387 IL-10	0.0333	1.12
388 IL-12p70	0.0466	1.10
389 Intercellular adhesion molecule 1 (ICAM-1)	0.0466	1.10
390 Serum Glutamic Oxaloacetic Transaminase (GOT1)	0.0008	0.83
391 ENA-78= epithelial-derived neutrophil-activating protein 78		

392
393 **b) Comparison of AS females and control females**

394

395 Analyte	p-value	FC
396 NARG1	0.0082	2.87
397 IL-12p40	0.0011	2.74
398 FAI	0.0001	2.01
399 IL-1B	0.0073	1.75
400 Luteinizing hormone	0.0471	1.56
401 IL-7	0.0224	1.47
402 TF	0.0328	1.42
403 BDNF	0.0150	1.20
404 GOT1	0.0238	0.84
405 Apolipoprotein-CIII (Apo-CIII)	0.0438	0.83
406 Immunoglobulin M (IGM)	0.0130	0.76
407 sRAGE	0.0425	0.76

408	Apo-A1	0.0063	0.75
409	Tenascin-C	0.0021	0.68
410	Eotaxin-3	0.0266	0.53
411	Endothelin-1	0.0369	0.50
412	Growth hormone	0.0103	0.44

413 NARG1=NMDA receptor regulated 1; sRAGE=receptor for advanced glycosylation end products

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419 **c) Two-site TRF assays for insulin-related molecules in female AS subjects**

420

421	Analyte	p-value	FC
422	Insulin	0.0594	1.81
423	Proinsulin	0.2508	1.26
424	<u>Des31,32-proinsulin (31,32-PI)</u>	<u>0.0228</u>	<u>1.89</u>

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

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599 **Figure legends**

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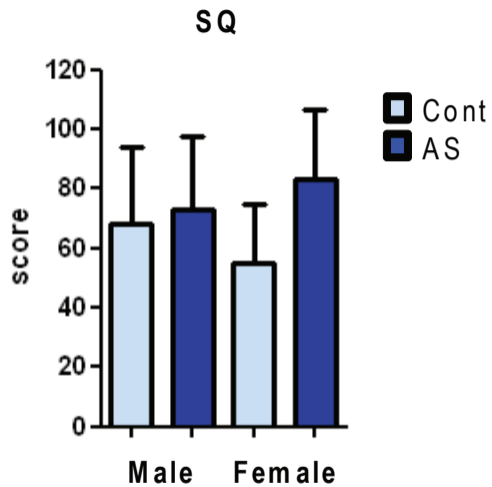
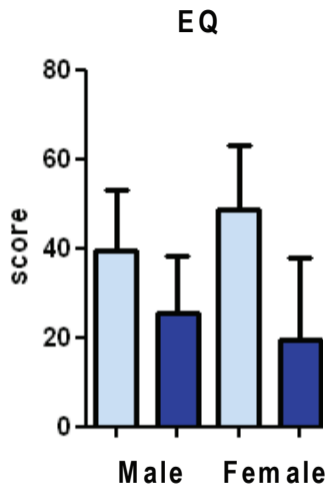
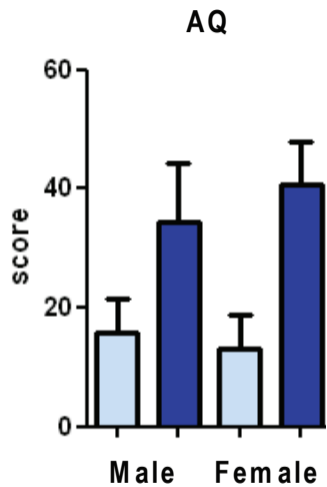
602 **Fig. 1.** AQ, EQ and SQ in males and females with AS compared to controls.

603 **Fig. 2.** Classification and prediction of AS and control (Cont) subjects using separate panels of a) 10
604 male-specific analytes and b) 15 female-specific analytes. The analytes were identified in separate
605 training sets for male and female AS and control subjects. These were used for blinded prediction of
606 male and female test subjects. The plus symbol indicate classification as AS and the minus symbol
607 indicates classification as controls. P values indicate the significance of the classification and were
608 calculated using Fisher's exact test.
609

610 **Fig. 3.** a) Venn diagram showing distinct and common serum biomarker profiles for males and
611 females with AS. Blue typeface and encircled area indicates hormones and growth factors. Red type
612 face and encircled area indicates cytokines and inflammatory factors. Black typeface indicates

613 markers that fit into other (non-specified) categories. Up arrows indicate analytes that show increased
614 expression and down arrows indicate analytes with decreased expression relative to the values seen
615 in the respective controls. **b)** Partial Least Squares Discriminant Analysis (PLSDA) showing the
616 separation of AS and control subjects using differentially expressed analytes. Each spot represents
617 an AS (red) or control (black) subject. The model built on males using 24 AS male specific analytes
618 (left) does not yield a separation in prediction of AS in females (right). **c)** The model built on females
619 with AS and female controls using 17 female specific analytes (left) does not yield a separation when
620 predicting AS in males (right). This is a standard statistical technique for characterizing data
621 variances. A simplified projection of multi-dimensional data is given such that the greatest variance
622 lies on the first coordinate, the second greatest variance on the next coordinate, and so on.

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a)

Training set: males
AS=12, Cont=16

		Males	
		AS	Cont
Test set:	+	7	0
	-	3	10

P=0.003

b)

Training set: females
AS=13, Cont=14

		Females	
		AS	Cont
Test set:	+	9	1
	-	1	9

P=0.001

