

Solving for X: evidence for sex-specific autism biomarkers across multiple transcriptomic studies

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Abstract

Autism spectrum disorder (ASD) is a markedly heterogeneous condition with a varied phenotypic presentation. Its high concordance among siblings, as well as its clear association with specific genetic disorders, both point to a strong genetic etiology. However, the molecular basis of ASD is still poorly understood, although recent studies point to the existence of sex-specific ASD pathophysiologies and biomarkers. Despite this, little is known about how exactly sex influences the gene expression signatures of ASD probands. In an effort to identify sex-dependent biomarkers (and characterise their function), we present an analysis of a single paired-end post-mortem brain RNA-Seq data set and a meta-analysis of six blood-based microarray data sets. Here, we identify several genes with sex-dependent dysregulation, and many more with sex-independent dysregulation. Moreover, through pathway analysis, we find that these sex-independent biomarkers have substantially different biological roles than the sex-dependent biomarkers, and that some of these pathways are ubiquitously dysregulated in both post-mortem brain and blood. We conclude by synthesizing the discovered biomarker profiles with the extant literature, by highlighting the advantage of studying sex-specific dysregulation directly, and by making a call for new transcriptomic data that comprise large female cohorts.

1 Introduction

Autism Spectrum Disorder (ASD) is a markedly heterogeneous condition with a varied phenotypic presentation and a spectrum of disability for those affected. As a neurodevelopmental disorder, the ASD syndrome is characterised by social abnormalities, language abnormalities, and stereotyped behavioural patterns [3]. The presence of a genetic link in ASD etiology is well-established [38, 39], first evidenced by ASD concordance among siblings and by a clear association between ASD and specific genetic disorders (e.g., Fragile X mental retardation) [3]. This link has prompted a number of transcriptomic studies (e.g., [20, 17, 19]) to identify gene expression signatures (i.e., as a kind of biomarker) that might help elucidate the etiology of ASD and aid in its diagnosis (an important objective since early diagnosis and therapy is shown to improve outcomes in ASD [13]). However, despite the number of transcriptomic studies performed, the pathophysiology and biomarker profile of ASD are still not known. Rather, these studies have tended to produce inconsistent results, suggesting wide heterogeneity among both the individual patients and the study populations. Indeed, ASD may not have one signature at all, but instead multiple diverging signatures [53].

47 Transcriptomic studies of ASD probands typically use cells collected from either post-mortem
48 brains or blood in order to estimate the mRNA abundance for thousands of gene transcripts
49 (by way of microarray technology or massively parallel high-throughput sequencing (RNA-Seq)).
50 Since many expressed transcripts are a precursor to structural or functional proteins, these studies
51 can provide an insight into the functional state of a cell, capturing the common pathway for
52 hereditary predisposition and environmental exposure. Although post-mortem brain studies have
53 an advantage in that they look directly at the tissue of interest, blood-based studies can identify
54 clinically useful biomarkers while also serving as a reliable proxy for gene expression in the brain
55 [55] (though a complete understanding of ASD pathophysiology and its biomarker profile will likely
56 require careful consideration of both lines of evidence). To date, more than a dozen studies have
57 measured the transcriptomic profiles of ASD probands (and controls), the results of which have
58 been summarised by two separate meta-analyses [10, 42] and one “mega-analysis” [53].

59 Sex is often called a risk factor for ASD, and it is stated that the risk for a male to have ASD
60 is four to five times higher than that for females [56, 11] (although the magnitude of this difference
61 may be partly due to diagnostic biases [28]). A similar observation, that the increased male
62 risk is even higher among high-functioning ASD probands [16], likewise suggests that sex-specific
63 mechanisms could influence ASD pathophysiology and its biomarker profile. Further evidence
64 for sex-specific mechanisms is found in recent transcriptomic and functional-imaging studies. For
65 example, Tylee et al., using transformed lymphoblastoid cell lines, found evidence for sex-specific
66 differential regulation of genes and pathways among ASD probands [52]. Similarly, Trabzuni et
67 al. found sex-specific differences in alternative splicing in adult human brains, including for a
68 well-known ASD risk gene NRXN3 [50]. Functional brain connectivity studies using fMRI imaging
69 have also identified sexual heterogeneity among ASD probands, showing dysregulation in sexually
70 dimorphic brain regions across two large studies [15, 27]. Taken together, it seems plausible
71 that sex could interact with other genetic and environmental factors to create sex-specific ASD
72 pathophysiologies and biomarker profiles.

73 As ASD is more common in males, it suggests that females may have some underlying protection
74 whereby a higher risk load is required for them to become afflicted [44]. One hypothesis posits that
75 ASD itself reflects a shift towards “extreme maleness” such that males are necessarily predisposed
76 [4]. In support of this, females with ASD do harbour more (and larger) copy number variants than
77 males with ASDs [32], and moreover exhibit differential penetrance given the same genetic etiology
78 [36]. Unfortunately, however, the increased prevalence of ASD in males has led to the exclusion
79 of females from many transcriptomic studies (e.g., [21, 45, 1]), making it difficult to understand
80 the male skew in ASD prevalence. Indeed, individual studies are often underpowered to detect
81 subtle sex-specific differences (if they contain female subjects at all). When female subjects are
82 included, sex is typically modelled as a simple covariate rather than an interaction term (i.e. the
83 ASD-sex interaction), meaning that only sex-independent (and not sex-dependent) biomarkers are
84 discovered. When male ASD is contrasted with female ASD, it typically involves loosely comparing
85 simple sex-specific differences (e.g., differential expression present in males but not females, and
86 *vice versa*) in a statistically anticonservative manner. To our knowledge, no study has looked
87 at whether gene expression signatures show a sex-autism interaction across multiple studies and
88 human tissues.

89 Using a single paired-end post-mortem brain RNA-Seq data set and a meta-analysis of six blood-
90 based microarray data sets, we present a analysis of transcriptomic data that focuses on comparing
91 sex-dependent and sex-independent ASD biomarkers (and the functional profiles thereof) across
92 multiple tissues. By modelling the interaction of sex and ASD directly, we identify biomarkers (as
93 well as functional pathways) that show sex-differences in ASD probands that are different than
94 those in control subjects. Then, for those biomarkers that show no interaction, we pool male and
95 female probands for a secondary sex-independent analysis. Our results suggest that, despite low
96 power, some genes have FDR-adjusted significant sex-dependent interactions, while even more have
97 significant sex-independent main effects. Subsequent pathway analysis further shows that these
98 sex-independent biomarkers have substantially different biological roles than the sex-dependent
99 biomarkers, and that some of these pathways are ubiquitously dysregulated in both post-mortem
100 brain and blood.

2 Methods

2.1 Data acquisition

2.1.1 RNA-Seq data

We searched for relevant publicly available RNA-Seq data using the Gene Expression Omnibus (GEO) [5] with the term ("expression profiling by high throughput sequencing"[DataSet Type] AND ("autism spectrum disorder"[MeSH Terms] OR "autistic disorder"[MeSH Terms])) AND "homo sapiens"[Organism] (query made January 2018). We restricted eligible data sets to those sequenced with paired-end and non-poly-A-selected libraries. After excluding any data sets that used cell lines or did not have female cases, only one experiment, GSE107241 [57], remained. These data comprise a RiboZero Gold paired-end RNA-Seq data set from 52 postmortem dorsolateral prefrontal cortex tissue samples.

Prior to alignment and quantification, raw RNA-Seq reads were trimmed using Trimmomatic (docker image quay.io/biocontainers/trimmomatic:0.36-4) [9] and quality control metrics were recorded (before and after trimming) using FastQC (docker image biocontainers/fastqc:0.11.5) [2]. We aligned trimmed reads and quantified expression using Salmon (docker image combinelab/salmon:0.9.0) [43] as run in pseudo-quantification mode with a k-mer index of length 31. For the reference, we concatenated a human coding reference (i.e., GRCh38.90.cds) with the corresponding non-coding reference (i.e., GRCh38.90.ncrna).

2.1.2 Microarray data

We collected multiple microarray data sets to perform a meta-analysis of sex-autism interactions and main effects of ASD (i.e., sex-independent effects, where males and females are pooled). We referenced two prior meta-analyses [10, 42], and one "mega-analysis" [53], to prepare a list of data sets to study. Of these data sets, we excluded any study that (a) measured transcript expression from brain tissue, (b) had no female cases, (c) used cell lines (i.e., GSE37772 and GSE43076), or (d) treated cells with PPA (i.e., GSE32136). Six data sets remained after exclusion, as described in Table 1.

Data acquired from the Gene Expression Omnibus (GEO) [5] (i.e., GSE6575 [18] and GSE18123 [26]) were acquired already normalised and were not modified further. The other data sets (i.e., the Glatt et al. Wave I and Wave II data [17], the CHARGE study data [20], and the Kong et al. 2013 data [25]) each underwent RMA normalization, quantile normalization, and base-2 logarithm transformation. All subjects with a labelled condition other than typically developed (TD) were assigned to the autism spectrum disorder (ASD) group, except for the two Glatt et al. data sets where "Type-1 errors" were assigned to the TD group. Note that, in crafting this dichotomy, some subjects assigned to the ASD group have delays that fall outside of the "spectrum" *per se*.

2.2 Differential expression analysis of RNA-Seq data

We used DESeq2 (Version 3.6) [37] to test for differential transcript expression within the Salmon-generated counts. We applied a conservative expression filter (i.e., at least 10 estimated counts per-gene in every sample) to the raw count matrix to ensure that the high variability of lowly expressed transcripts did not bias results due to the small group sizes. For each transcript that passed the expression filter, a model was fit using the formula $\sim \text{ASD} * \text{Sex} + \text{Age}$ (where Age is the age of death). Interaction and sex-independent main effects (i.e., of the ASD condition) were then extracted from the model by specifying the relevant contrasts to the DESeq2::results function. We corrected for multiple testing using the Benjamini-Hochberg procedure [7].

2.3 Meta-analysis of microarray data

Before proceeding with the meta-analysis, we established a set of probes (i.e., for each microarray platform) that represent genes also represented by probes in the other platforms. In other words, we established a final probe set based on the intersection of unique gene symbols present in all microarray platforms under study. Note that we resolved one-to-many mapping ambiguities by excluding any probe that mapped to multiple gene symbols.

For each microarray data set, and for each probe (i.e., of those representing genes found in all data sets), we performed differential expression analysis using limma (Version 3.34) [46], applying

152 the following steps: (1) fit a model with the formula $\sim \text{ASD} * \text{Sex} + \text{Age}$ where ASD and Sex are
153 each two-level factors (except GSE6575, where the Age covariate is unknown), (2) define contrasts
154 for the sex-autism interaction and for the sex-independent main effects (i.e., of the ASD condition),
155 and (3) measure the differential expression for each contrast using the eBayes procedure.

156 Next, we transformed platform-specific probe p-values to HGNC symbol p-values using An-
157 notationDbi (available from Bioconductor [22]). We resolved many-to-one mapping ambiguities
158 by FDR-adjusting the minimum p-value of all probes for a given gene symbol (i.e., calculating a
159 within-gene FDR correction). We then used Fisher's method to perform a meta-analysis of the
160 p-values obtained from the differential expression analysis. For K studies, Fisher's method scores
161 each gene based on (negative two times) the sum of the logarithm of the p-values:

$$\chi_{2K}^2 = -2 \sum_i^K \log p_i \quad (1)$$

162 This score follows a χ^2 distribution with $2K$ degrees of freedom [41]. Thus, for each gene, we
163 computed a p-value directly from this score. We corrected for multiple testing using the Benjamini-
164 Hochberg procedure [7].

165 2.4 Adjustment of latent batch effects

166 To ensure that latent batch effects did not inflate the discovery of false positives, we performed
167 all analyses above with adjustment for batch effects using *sva* (Version 3.26) [31, 30], applying
168 the following steps: (1) estimate the number of surrogate variables while specifying the ASD
169 $* \text{Sex}$ interaction as the variable of interest and *Age* as an adjustment variable, (2) use the *sva*
170 function (or, in the case of *Salmon*-generated counts, the *svaseq* function) to estimate the surrogate
171 variables, and (3) include the surrogate variables in the differential expression model(s) described
172 above. Generally speaking, using *sva* yielded more conservative results than not using *sva*. All
173 tables and figures show results generated with *sva* except where otherwise noted.

174 2.5 Pathway analysis and knowledge integration

175 We performed pathway analysis using GSEA (Version 3.0) [48] in PreRanked mode with classic en-
176 richment and 1,000 permutations. Enrichment scores were calculated for specific MSigDB (Version
177 6.1) [47, 34] gene sets, including the curated KEGG (*c2.cp.kegg*[23]), Gene Ontology Biological
178 Process (*c5.bp*) [49], Reactome (*c2.cp.reactome*) [14], and MSigDB Hallmark (*h.all*) [35] sets.

179 Based on the nature of the analyses, input rank lists were prepared differently for the RNA-Seq
180 and microarray results. For the RNA-Seq analysis, we ranked transcripts based on the p-value, p ,
181 and the magnitude of the fold-change, FC:

$$\text{Rank} = -\log_{10}(p) \times \text{sign}(\log_2(\text{FC})) \quad (2)$$

182 Then, these transcript-level ranks were converted into gene-level ranks based on the top transcript-
183 level rank. For the microarray meta-analysis, we ranked genes using the χ^2 test statistic (as
184 calculated from Fisher's method). Note that since this latter metric is agnostic to the direction of
185 expression changes, we focused here on pathways enriched with a positive score (effectively making
186 this pathway enrichment test one-tailed).

187 3 Results

188 3.1 Evidence for sex-dependent autism biomarkers

189 By modelling the sex-autism interaction directly, we can detect gene expression signatures that
190 have differential dysregulation in male ASD probands when compared with female ASD probands.
191 In other words, we can find sexually dimorphic ASD biomarkers (e.g., a gene up-regulated in male
192 ASD but not in female ASD, or *vice versa*). Despite small study sizes (and disproportionately
193 fewer females), we find some evidence for a sex-autism interaction among biomarkers, especially
194 throughout the microarray meta-analysis data.

195 From the analysis of the RNA-Seq data derived from post-mortem brain tissue, we find no tran-
196 scripts with significant (FDR-adjusted p-value < 0.05) sex-dependent dysregulation, although one

197 of these transcripts showed a significant interaction prior to batch correction with *sva*. To illustrate
198 what a sex-autism interaction might look like, Figure 1 shows the per-group expression profiles
199 for the two transcripts with the largest interaction effect (i.e., those with the smallest corrected
200 p-value). Table 2 characterises those transcripts with the most sex-dependent dysregulation.

201 From the meta-analysis of the blood-based microarray data, we find two genes with significant
202 (FDR-adjusted) sex-dependent dysregulation: *TTF2* and *UTY*. Table 3 characterises those genes
203 with the most sex-dependent dysregulation. Since for a meta-analysis by Fisher's method, a large
204 departure from the null (i.e., a very small p-value) in only one of several studies could cause
205 the meta-analysis to post a significant result (i.e., even after FDR-adjustment) [51], it is useful
206 to inspect visually how each study contributed to the results of the meta-analysis. For this,
207 Figure 2 shows how each study contributed to the meta-analysis findings by plotting the aggregate
208 Fisher score for each gene (of those with large sex-dependent dysregulation) along with the study-
209 wise nominal significance (unadjusted p-value < 0.05). Notably, several of the most significantly
210 dysregulated genes are at least nominally significant in more than one study.

211 3.2 Evidence for sex-independent autism biomarkers

212 In situations where a sex-autism interaction is not detectable, we can proceed to measure main
213 condition (i.e., sex-independent) effects by pooling male ASD probands with female ASD probands
214 (and male controls with female controls), without having to model sex as a covariate. Genes with
215 significant sex-independent main effects (i.e., of the ASD condition) have large unidirectional effect
216 sizes in male ASD probands, female ASD probands, or both. Yet, because the interaction is tested
217 first, we can interpret the main condition effects as sex-independent.

218 From the analysis of the RNA-Seq data derived from post-mortem brain tissue, we find seven
219 transcripts with significant (FDR-adjusted p-value < 0.05) sex-independent differential expression.
220 Of these, only one transcript showed significant up-regulation in ASD. Figure 3 shows the expres-
221 sion profile for the two transcripts with the most significant sex-independent main effects (i.e., of
222 the ASD condition). Table 4 characterises those transcripts with significant sex-independent dys-
223 regulation. Interestingly, several of the transcripts called differentially expressed by the analysis
224 are annotated as non-coding RNA species.

225 From the meta-analysis of blood-based microarray data, we find 21 genes with significant (FDR-
226 adjusted) sex-independent dysregulation. Table 5 characterises those genes with the most sex-
227 independent dysregulation. As in Figure 2, Figure 4 shows how each study contributed to the
228 meta-analysis findings by plotting the aggregate Fisher score for each gene (i.e., of those with large
229 sex-independent dysregulation) along with the study-wise nominal significance (unadjusted p-value
230 < 0.05). Again, most genes selected as statistically significant by the meta-analysis are at least
231 nominally significant in more than one study.

232 3.3 Pathway enrichment of ASD biomarkers

233 In an effort to summarise the biological relevance of the biomarker profiles generated above, we used
234 the complete ranked lists of the differentially expressed transcripts (and genes) in four separate
235 gene set enrichment analyses to identify common differentially regulated pathways. Four enrich-
236 ment profiles were generated using the sex-dependent RNA-Seq (brain) biomarkers, sex-independent
237 RNA-Seq (brain) biomarkers, sex-dependent microarray (blood) biomarkers, and sex-independent
238 microarray (blood) biomarkers.

239 Figure 5 shows the KEGG pathways enriched by the biomarkers as ranked by the analysis of
240 the RNA-Seq data. For the sex-dependent biomarkers, nine pathways showed significant (FDR-
241 adjusted p-value < 0.15) enrichment. For the sex-independent biomarkers, five pathways showed
242 significant enrichment. Interestingly, all significant enrichment occurred in the same direction.

243 Figure 6 shows the KEGG pathways enriched by the biomarkers as ranked by the analysis of
244 the microarray data. For the sex-dependent biomarkers, one pathway (i.e., Alanine Aspartate and
245 Glutamate Metabolism) showed significant (FDR-adjusted p-value < 0.30) enrichment. For the
246 sex-independent biomarkers, thirty-six pathways showed significant enrichment. Note that because
247 only positive (i.e., one-tailed) enrichments are considered for these data, an FDR-adjusted p-value
248 < 0.30 is used here (see Methods for more details).

249 Figure 7 compares the overlap between these significant pathways. For the sex-dependent
250 analyses, no pathways are enriched in both the RNA-Seq and microarray data. However, for the
251 sex-independent analyses, two pathways are enriched in both data. Interestingly, this agreement

252 exists despite differences in the ranked lists, suggesting that ASD biomarker profiles may show
253 some degree of higher-order conservation at the pathway-level that exists not only across multiple
254 studies, but across multiple tissues (as well as multiple transcript quantification assays). Note
255 that we also tested for enrichment among the Gene Ontology Biological Process, Reactome, and
256 MSigDB Hallmarks gene sets, all of which show more examples of overlap between the separate
257 sex-independent analyses (see the Supplementary Information for more details).

258 4 Discussion

259 In this report, we present an analysis of several ASD transcriptomic studies, including an analysis of
260 RNA-Seq data derived from post-mortem brain and a meta-analysis of six blood-based microarray
261 data sets. Specifically, we focus on identifying both sex-dependent and sex-independent biomarker
262 profiles for ASD by modelling the sex-autism interaction directly and secondarily measuring main
263 effects of the ASD condition (i.e., sex-independent effects where males and females are pooled). In
264 addition to identifying transcript (and gene) biomarkers, we use gene set enrichment analysis to
265 summarise the observed dysregulation at the pathway level, contrasting sex-dependent pathway
266 enrichment with sex-independent pathway enrichment. In doing so, we find evidence that ASD
267 biomarker profiles may show some degree of higher-order conservation at the pathway level that
268 exists not only across multiple studies, but across multiple tissues (and across multiple transcript
269 quantification assays).

270 Despite small sample sizes in all studies, we found evidence for the existence of some sex-
271 dependent biomarkers in human tissue. The meta-analysis identified two genes, *TTF2* and *UTY*,
272 with sexually dimorphic expression in the blood. One of these, *TTF2*, plays an important role in
273 normal thyroid development [12]. Interestingly, a loss of thyroid hormone homeostasis has been
274 linked to ASD [8, 24]. Since it is well-known that thyroid diseases have a sex-specific presentation
275 [6], it seems plausible that thyroid abnormalities could contribute to a sexually dimorphic ASD
276 signature. The other, *UTY*, is a Y-chromosome gene, making any interpretation of its differential
277 dysregulation difficult. Two other genes, *KCNJ8* and *MAP1B*, had FDR-adjusted p-values very
278 close to the pre-defined significance cutoff, warranting follow-up in another study. Although the
279 RNA-Seq analysis did not yield any significant interactions, it is not surprising considering this
280 data set contained only three female ASD probands. Nevertheless, the large (albeit non-significant)
281 effect sizes warrant repeat studies with bigger cohorts and more female ASD probands.

282 By modelling the sex-autism interaction directly, we are able to follow-up the sex-dependent
283 analysis with a secondary sex-independent analysis for any transcript (or gene) whose expression
284 did not significantly interact with sex. In this scenario, we contrast the pooled male ASD probands
285 and female ASD probands against the pooled male controls and female controls to calculate the
286 main effects (which we can thus interpret as sex-independent biomarkers). Here, over twenty
287 transcripts and genes exceeded the threshold for FDR-adjusted significance. Interestingly, for the
288 RNA-Seq data, several of the significant biomarkers are not protein-coding genes (highlighting the
289 value of using non-poly-A-selected libraries to quantify both coding and non-coding transcripts).
290 For the microarray meta-analysis, several of the sex-independent biomarkers are associated with
291 key neurodevelopmental processes, including some X-chromosome genes. For example, *MAGED2*,
292 differentially expressed in ASD probands, is located on an X-linked intellectual disability hotspot
293 (i.e., Xp11.2) [29, 40] (which, if causally relevant, could contribute to the male risk bias).

294 For both the RNA-Seq analysis and the microarray meta-analysis, we tested the ranked sex-
295 dependent and sex-independent biomarker profiles separately for pathway-level enrichment. We
296 found some pathway enrichment for the sex-dependent profiles, and even more for the sex-independent
297 profiles. Importantly, very few of the enriched pathways were the same for both the interaction
298 and main effects. This suggests that males and females exhibit unique pathway-level signatures
299 that, if causally relevant, might further suggest the existence of both sex-specific and common ASD
300 pathophysiologies. Although few KEGG pathways are enriched among the sex-dependent results,
301 there are dozens of significantly enriched sex-dependent pathways across other tested gene sets
302 (see Supplementary Information for more details). Among the sex-independent enriched pathways
303 (for the meta-analysis results), there are a number of pathways for known neurodevelopmental
304 and neurodegenerative diseases, including Huntingtons, Parkinsons, Alzheimers, and amyotrophic
305 lateral sclerosis (ALS), suggesting that at least some of these ASD biomarkers may have functions
306 important to general brain health. Considering that both unique and shared signatures (i.e., at the
307 biomarker-level and pathway-level) exist among ASD probands, it seems plausible that molecular

308 diagnostics could benefit from modelling sex-specific processes directly.

309 Although we found pathway enrichment to differ considerably between the sex-dependent and
310 sex-independent biomarker profiles, we found that several sex-independent pathways (i.e., based
311 on KEGG and other genes sets) were enriched across both the RNA-Seq and microarray data.
312 Interestingly, this overlap exists despite the fact that analyses were performed on different human
313 tissues (and with different transcript quantification assays). In fact, more than fifty Gene Ontology
314 pathways were enriched among both sets of ranked sex-independent biomarkers (even though no
315 gene products showed significant differential expression in both data). This overlap supports the
316 hypothesis that there may exist common diagnostic (and perhaps etiological) signatures across the
317 widely heterogeneous population of ASD probands. If true, it seems plausible that molecular diag-
318 nostics could further benefit from modelling pathway-level dysregulation directly (i.e., in addition
319 to modelling conventional transcriptomic biomarkers).

320 When we compare our pathway enrichments to the previous ASD “mega-analysis” pathway
321 enrichments [54], we observe several complementary results. First, we found positive enrichment
322 of the MAPK pathway in our sex-dependent RNA-Seq results, agreeing with the male-specific
323 enrichment of Mek targets found in the Tylee et al. study [54]. Second, we found an enrichment of
324 the ribosome-related pathway in both of our sex-independent analyses, agreeing with the ribosome-
325 related pathway enrichment identified by the sex-independent “mega-analysis” [54]. Third, we
326 found an enrichment of the Toll-like receptor (TLR) signalling pathway in our sex-independent
327 meta-analysis results, agreeing with the TLR 3 and 4 signalling pathway enrichment identified by
328 the sex-independent “mega-analysis” [54]. Importantly, these complementary results exist despite
329 considerable differences in statistical methodology and data set inclusion.

330 Our analysis is not without limitations. First, although we used *sva* to adjust for latent batch
331 effects, it is still possible that any number of remaining factors (or batch effects) could coincide with
332 the diagnostic label (e.g., undocumented co-morbidities or medication use), thereby confounding
333 the discovered biomarker profile. Second, as with any observational study, it is impossible to con-
334 clude whether the gene expression signatures (and their biological pathways) are causally related
335 to ASD (or, likewise, the sex-autism interaction). Third, this analysis is likely under-powered to
336 detect both sex-autism interactions and main effects, owing to the small sample sizes and dispro-
337 portionately smaller female cohorts. Yet, based on the extant literature (which clearly highlights
338 sex as an ASD risk factor) and the results published here, we believe that modelling the sex-autism
339 interaction should become a mainstay of ASD transcriptomic research. Advantageously, as shown
340 here, interaction modelling is compatible with the most commonly used softwares for batch-effect
341 correction [31], RNA-Seq analysis [37], and microarray analysis [46]. Yet, this analytical technique
342 cannot offer any benefit if transcriptomic studies continue to systematically exclude female subjects
343 ([21, 45, 1]). Although there seems to exist a strong skew in the prevalence of male ASD, this very
344 fact underlies the importance of studying female ASD at equal proportions: a complete under-
345 standing of the molecular basis of ASD will require the intentional study of both sex-dependent
346 and sex-independent mechanisms, as well as their differences and commonalities.

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569 List of Figures

570	1	These violin plots show the base-2 logarithm-transformed expression for the two transcripts with the largest interaction effect from the RNA-Seq data (i.e., those with the smallest corrected p-value). The solid lines show sex-specific mean expression differences. The dashed line shows the sex-independent (i.e., pooled) mean expression difference.	14
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575	2	This figure shows the genes with the most significant sex-dependent dysregulation (i.e., a sex-autism interaction) according to the meta-analysis of the microarray data. Above, the bar plot shows the χ^2 score for each gene as calculated using Fisher's method (where the dark bars indicate that the gene has an FDR-adjusted p-value < 0.05). Below, the dot plot shows whether a gene showed a nominally significant sex-dependent dysregulation at an unadjusted p-value < 0.05 for a given study. Note that most genes selected for by the meta-analysis show at least nominal significance across multiple studies.	15
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607	7	This UpSet plot [33] shows set intersections (and their sizes) from a GSEA of four results against the MSigDB KEGG pathways. Set identity is indicated by the joined lines. Set size is indicated by the top bar chart. The bar chart on the left shows the total set size for each individual GSEA run. Results are filtered using a liberal FDR threshold of FDR < 0.15 for the RNA-Seq data and FDR < 0.3 for the meta-analysis data (see Methods).	20
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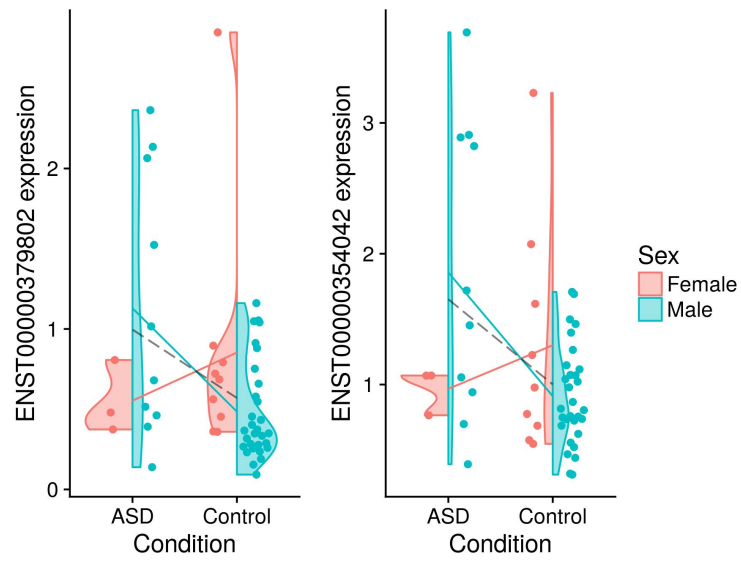


Figure 1: These violin plots show the base-2 logarithm-transformed expression for the two transcripts with the largest interaction effect from the RNA-Seq data (i.e., those with the smallest corrected p-value). The solid lines show sex-specific mean expression differences. The dashed line shows the sex-independent (i.e., pooled) mean expression difference.

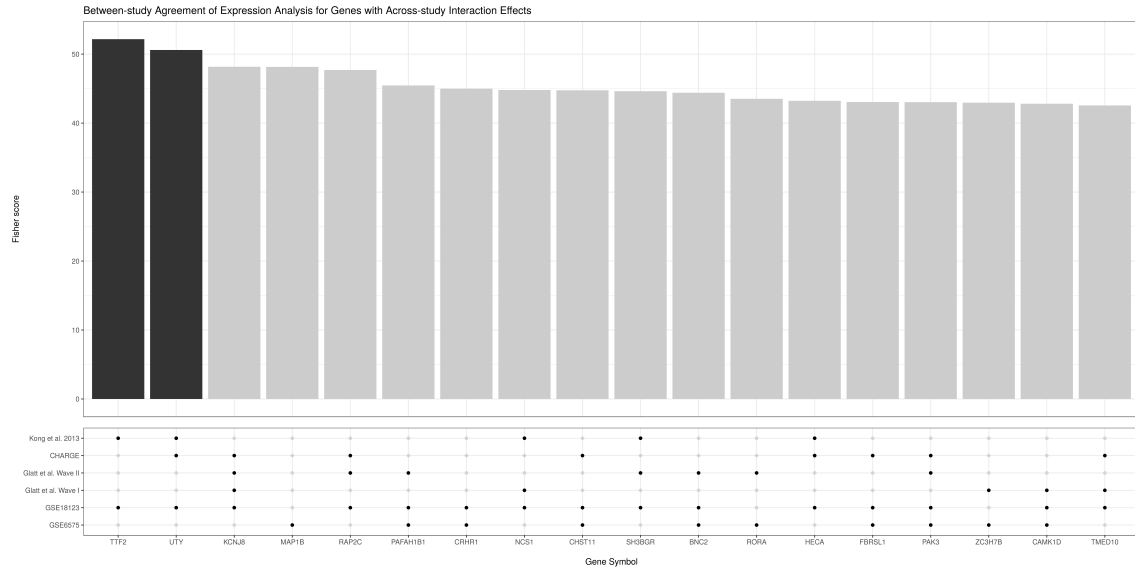


Figure 2: This figure shows the genes with the most significant sex-dependent dysregulation (i.e., a sex-autism interaction) according to the meta-analysis of the microarray data. Above, the bar plot shows the χ^2 score for each gene as calculated using Fisher's method (where the dark bars indicate that the gene has an FDR-adjusted p-value < 0.05). Below, the dot plot shows whether a gene showed a nominally significant sex-dependent dysregulation at an unadjusted p-value < 0.05 for a given study. Note that most genes selected for by the meta-analysis show at least nominal significance across multiple studies.

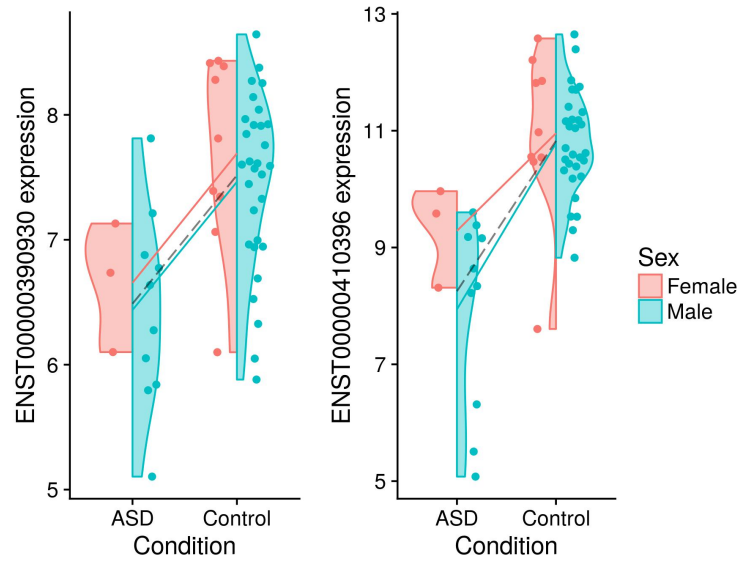


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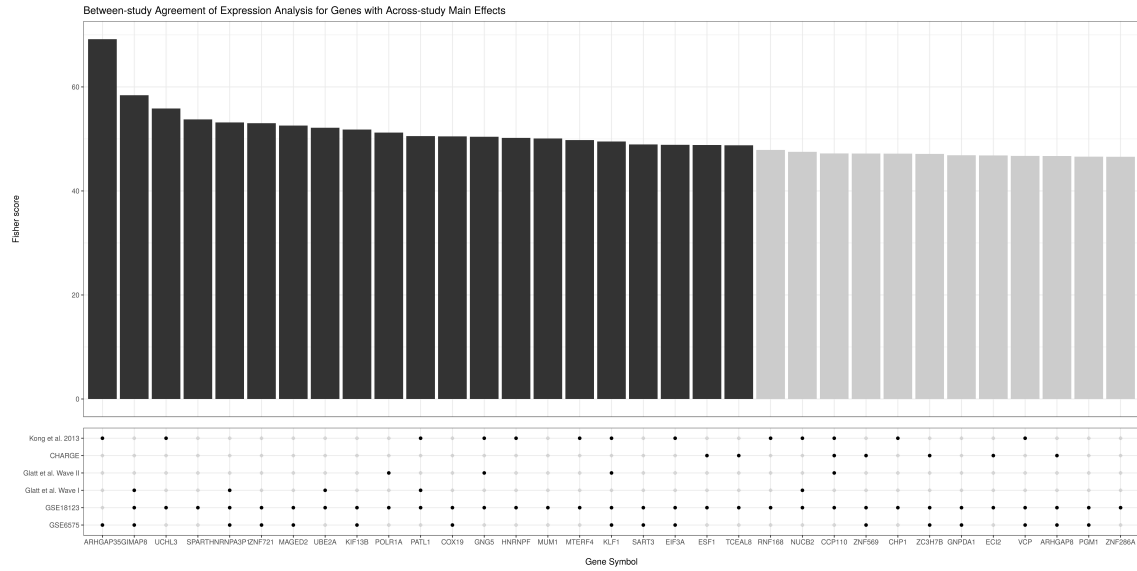


Figure 4: This figure shows the genes with the most significant sex-independent main effects (i.e., of the ASD condition) according to the meta-analysis of the microarray data. Above, the bar plot shows the χ^2 score for each gene as calculated using Fisher's method (where the dark bars indicate that the gene has an FDR-adjusted p-value < 0.05). Below, the dot plot shows whether a gene showed a nominally significant sex-independent main effect at an unadjusted p-value < 0.05 for a given study. Note that most genes selected for by the meta-analysis show at least nominal significance across multiple studies.

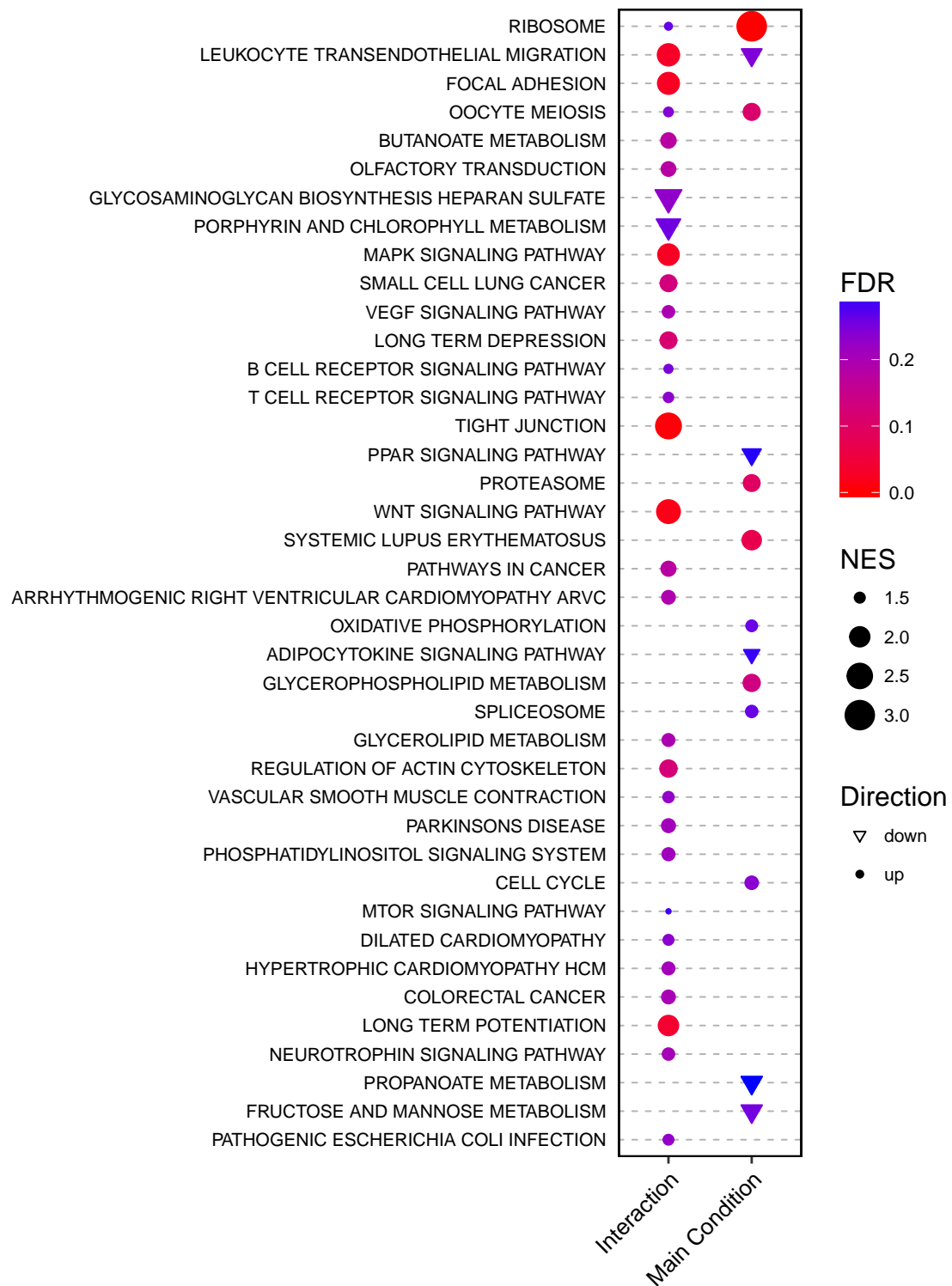


Figure 5: This dot plot shows results from a GSEA of the RNA-Seq data against the MSigDB KEGG pathways. For the two sets of results (i.e., the sex-autism interaction and the main effect), a KEGG pathway (y-axis) has a circle (or triangle) if it is enriched (or depleted). The size of the points indicates the absolute normalised enrichment score. The colour indicates the FDR. Note that only points with an FDR < 0.3 are plotted (see Methods).

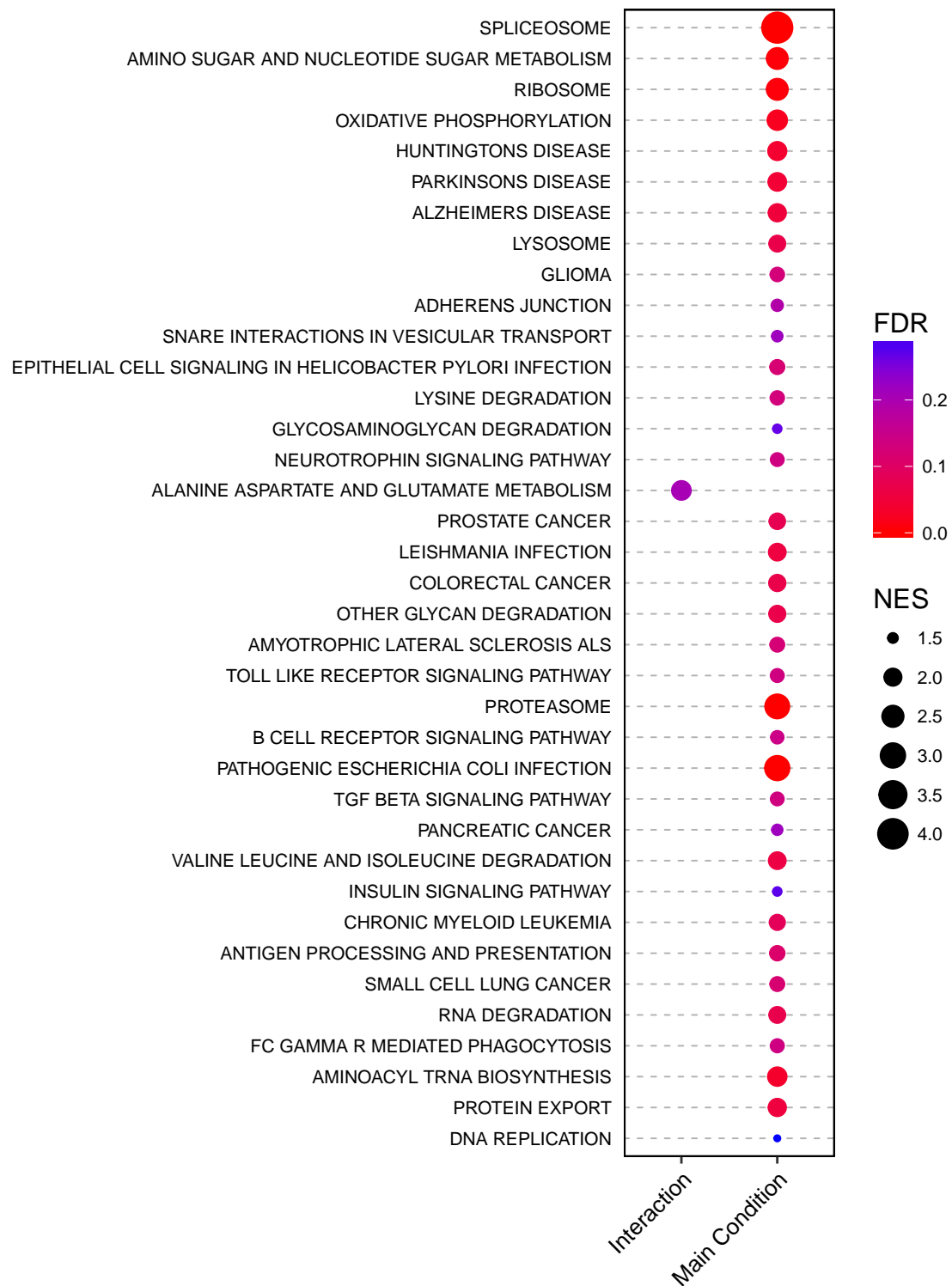


Figure 6: This dot plot shows results from a GSEA of the meta-analysis data against the MSigDB KEGG pathways. For the two sets of results (i.e., the sex-autism interaction and the main effect), a KEGG pathway (y-axis) has a circle if it is enriched. The size of the points indicates the absolute normalised enrichment score. The colour indicates the FDR. Note that only points with an FDR < 0.3 are plotted (see Methods).

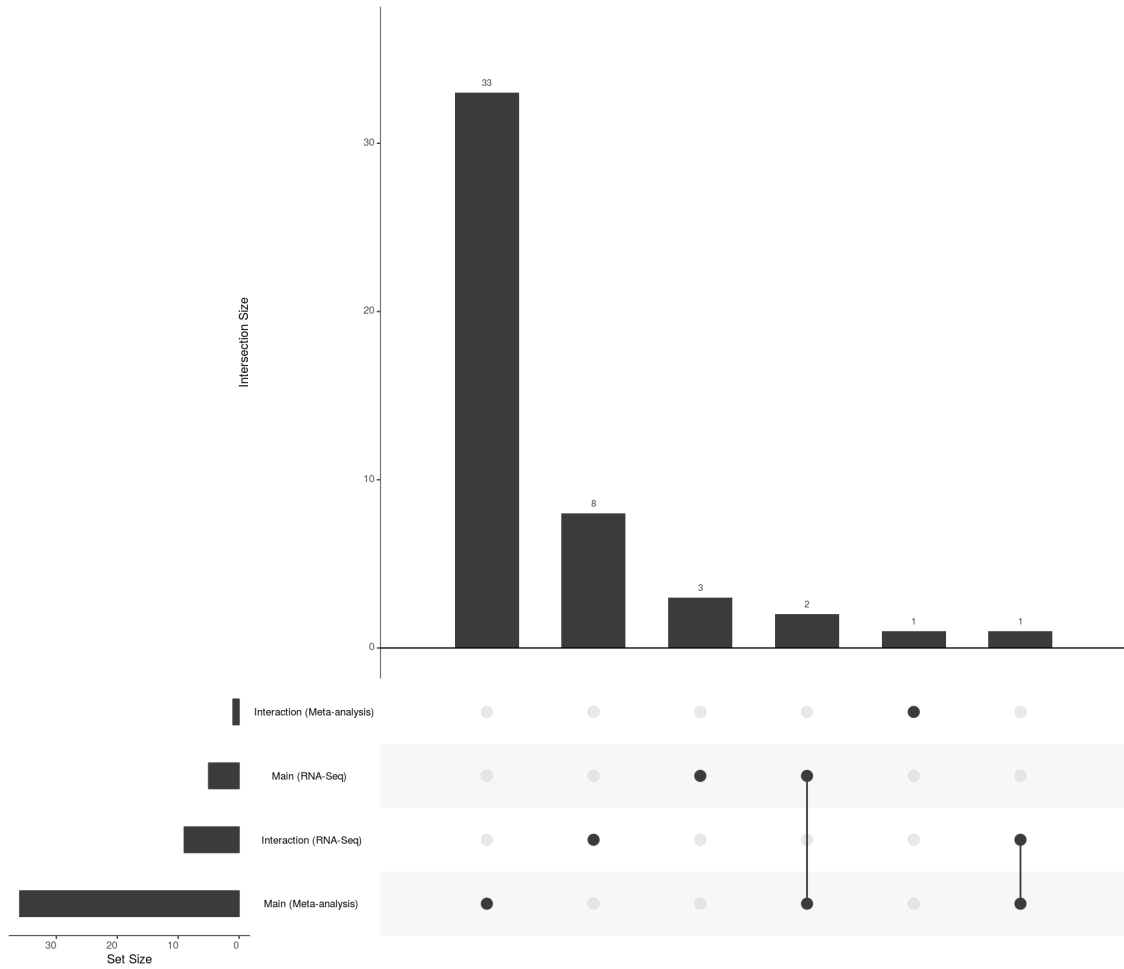


Figure 7: This UpSet plot [33] shows set intersections (and their sizes) from a GSEA of four results against the MSigDB KEGG pathways. Set identity is indicated by the joined lines. Set size is indicated by the top bar chart. The bar chart on the left shows the total set size for each individual GSEA run. Results are filtered using a liberal FDR threshold of $FDR < 0.15$ for the RNA-Seq data and $FDR < 0.3$ for the meta-analysis data (see Methods).

613 List of Tables

614	1	This table details all studies included in the meta-analysis, and the number of probes available after establishing a final probe set. All subjects with a labelled condition other than typically developed (TD) were assigned to the autism spectrum disorder (ASD) group, except for the two Glatt et al. data sets where “Type-1 errors” were assigned to the TD group.	22
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626	4	This table shows SVA-adjusted results for the main effects (i.e., of the ASD condition) for the RNA-Seq data (sorted by FDR-adjusted p-value). Note that FDR-adjusted p-values are also shown for an analysis performed without the adjustment of latent batch effects.	25
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Study ID	Probes (Intersect)	Females (TD)	Males (TD)	Females (ASD)	Males (ASD)
GSE6575	39561	3	9	8	36
GSE18123	19532	34	48	24	80
Glatt et al. Wave I	28424	28	40	23	88
Glatt et al. Wave II	28424	35	56	28	85
CHARGE	39561	15	75	15	103
Kong et al. 2013	19532	7	10	7	46

Table 1: This table details all studies included in the meta-analysis, and the number of probes available after establishing a final probe set. All subjects with a labelled condition other than typically developed (TD) were assigned to the autism spectrum disorder (ASD) group, except for the two Glatt et al. data sets where “Type-1 errors” were assigned to the TD group.

Transcript ID	Gene symbol	Transcript biotype	Log 2 FC	P-adj SVA	P-adj (no SVA)
ENST00000354042	SLC13A4	protein_coding	3.27	0.293	0.1136846
ENST00000379802	DSP	protein_coding	3.19	0.293	0.6534814
ENST00000262551	OGN	protein_coding	2.97	0.299	0.8169099
ENST00000371625	PTGDS	protein_coding	1.74	0.299	0.0329544
ENST00000223357	AEBP1	protein_coding	1.85	0.529	0.8713166

Table 2: This table shows SVA-adjusted results for the sex-autism interaction for the RNA-Seq data (sorted by FDR-adjusted p-value). Note that FDR-adjusted p-values are also shown for an analysis performed without the adjustment of latent batch effects.

	Location	Fisher	Fisher p-adj	Fisher (no SVA)	Fisher p-adj (noSVA)
TTF2	1p13.1	52.16404	0.0105053	28.88686	1.0000000
UTY	Yq11.221	50.59543	0.0198876	45.76688	0.1378710
KCNJ8	12p12.1	48.17048	0.0528841	38.16932	1.0000000
MAP1B	5q13.2	48.15632	0.0531822	47.94878	0.0578051
RAP2C	Xq26.2	47.70446	0.0637312	24.82099	1.0000000
PAFAH1B1	17p13.3	45.45517	0.1559409	17.84249	1.0000000
CRHR1	17q21.31	44.98624	0.1876599	43.46097	0.3416423
NCS1	9q34.11	44.79693	0.2021903	30.46521	1.0000000
CHST11	12q23.3	44.75342	0.2056750	21.19593	1.0000000
SH3BGR	21q22.2	44.61154	0.2174809	32.59585	1.0000000
BNC2	9p22.3-p22.2	44.40363	0.2360031	39.81245	1.0000000
RORA	15q22.2	43.52113	0.3335702	34.11125	1.0000000
HECA	6q24.1	43.22311	0.3747481	33.12178	1.0000000
FBRSL1	12q24.33	43.04625	0.4015007	35.53452	1.0000000
PAK3	Xq23	43.03339	0.4034965	43.20181	0.3780235
ZC3H7B	22q13.2	42.95711	0.4156536	35.03776	1.0000000
CAMK1D	10p13	42.80430	0.4411269	24.56439	1.0000000
TMED10	14q24.3	42.55614	0.4858196	17.45529	1.0000000

Table 3: This table shows genes with the most sex-dependent dysregulation (and their chromosomal position), sorted by Fisher score and adjusted p-value. In addition, this table shows the Fisher score and adjusted p-value calculated for an analysis repeated without the adjustment of latent batch effects.

Transcript ID	Gene symbol	Transcript biotype	Log 2 FC	P-adj (SVA)	P-adj (no SVA)
ENST00000390930	SNORD17	snoRNA	-2.98	1.54e-05	0.0000102
ENST00000410396	RNU2-2P	snRNA	-4.76	4.04e-05	0.0000000
ENST00000613119		snRNA	-3.23	9.18e-05	0.0000000
ENST00000258526	PLXNC1	protein_coding	0.48	0.00468	0.4273372
ENST00000393775	IGSF11	protein_coding	-1.18	0.00468	1.0000000
ENST00000459255	SCARNA10	snoRNA	-1.71	0.00468	0.0014803
ENST00000618786	RN7SL1	misc_RNA	-1.35	0.0124	0.0026454

Table 4: This table shows SVA-adjusted results for the main effects (i.e., of the ASD condition) for the RNA-Seq data (sorted by FDR-adjusted p-value). Note that FDR-adjusted p-values are also shown for an analysis performed without the adjustment of latent batch effects.

	Location	Fisher	Fisher p-adj	Fisher (no SVA)	Fisher p-adj (noSVA)
ARHGAP35	19q13.32	69.17663	0.0000083	59.97651	0.0004125
GIMAP8	7q36.1	58.39735	0.0008000	52.71485	0.0083436
UCHL3	13q22.2	55.85012	0.0023073	31.88589	1.0000000
SPART	13q13.3	53.75888	0.0054659	43.79029	0.2920570
HNRNPA3P1	10q11.21	53.16493	0.0069742	54.55326	0.0039291
ZNF721	4p16.3	53.02620	0.0073817	45.32102	0.1608751
MAGED2	Xp11.21	52.57098	0.0088931	31.43801	1.0000000
UBE2A	Xq24	52.15816	0.0105264	24.84369	1.0000000
KIF13B	8p12	51.80723	0.0121459	44.99172	0.1830060
POLR1A	2p11.2	51.21815	0.0154371	35.12970	1.0000000
PATL1	11q12.1	50.53892	0.0203385	37.55012	1.0000000
COX19	7p22.3	50.48910	0.0207524	51.68452	0.0126954
GNG5	1p22.3	50.42442	0.0213024	21.04799	1.0000000
HNRNPF	10q11.21	50.20526	0.0232786	52.19956	0.0102957
MUM1	19p13.3	50.09134	0.0243757	38.59229	1.0000000
MTERF4	2q37.3	49.77445	0.0277066	40.36576	1.0000000
KLF1	19p13.13	49.50019	0.0309497	35.07655	1.0000000
SART3	12q23.3	48.93549	0.0388576	51.89275	0.0116656
EIF3A	10q26.11	48.86929	0.0399046	48.66280	0.0429001
ESF1	20p12.1	48.82351	0.0406442	40.26756	1.0000000
TCEAL8	Xq22.1	48.76924	0.0415389	30.32699	1.0000000
RNF168	3q29	47.89156	0.0590766	40.39014	1.0000000
NUCB2	11p15.1	47.52251	0.0684739	46.57846	0.0981743
CCP110	16p12.3	47.21328	0.0774723	30.63996	1.0000000
ZNF569	19q13.12	47.18319	0.0784042	35.01402	1.0000000
CHP1	15q15.1	47.17381	0.0786939	46.71912	0.0928712
ZC3H7B	22q13.2	47.11959	0.0804103	31.75604	1.0000000
GNPDA1	5q31.3	46.86648	0.0889439	39.70348	1.0000000
ECI2	6p25.2	46.83204	0.0901676	54.27612	0.0044030
VCP	9p13.3	46.73363	0.0937667	33.68338	1.0000000
ARHGAP8	22q13.31	46.70772	0.0947338	50.13461	0.0237714
PGM1	1p31.3	46.58133	0.0996154	36.39139	1.0000000
ZNF286A	17p12	46.57586	0.0998268	31.41283	1.0000000

Table 5: This table shows genes with the most sex-independent dysregulation (and their chromosomal position), sorted by Fisher score and adjusted p-value. In addition, this table shows the Fisher score and adjusted p-value calculated for an analysis repeated without the adjustment of latent batch effects.