



ORIGINAL ARTICLE

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

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Funding information

Foundation for the National Institutes of Health, Grant/Award Number: R01MH107205UG3OD023365; National Institute of Environmental Health Sciences, Grant/Award Numbers: 1P30ES0235131R01ES0153592R01ES0153593P01ES011269, 3P01ES011269, 1P30ES023513, 2R01ES015359, 1R01ES015359; U.S. Environmental Protection Agency, Grant/Award Number: RD83543201; United States Environmental Protection Agency, Grant/Award Number: RD83543201; National Institute of Health, Grant/Award Numbers: UG3OD023365, R01MH107205; Australian Research Council, Grant/Award Number: LP140100240

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 characterize 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 postmortem 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.

KEYWORDS

ASD, autism, biomarkers, sex-specific, transcriptome

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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 characterized by social abnormalities, language abnormalities, and stereotyped behavioral patterns (Bailey, Phillips, & Rutter, 1996). The presence of a genetic link in ASD etiology is well-established (Miles, 2011; Miyauchi & Voineagu, 2013), as first evidenced by ASD concordance among siblings and by a clear association between ASD and specific genetic disorders (e.g., Fragile X mental retardation; Bailey et al., 1996). This link has prompted a number of transcriptomic studies (e.g., Glatt et al., 2012; Gupta et al., 2014; Hertz-Picciotto et al., 2006) to identify gene expression signatures as a 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 (Elder, Kreider, Brasher, & Ansell, 2017). 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, although several studies have converged to find associations among neuron-specific genes (Gupta et al., 2014; Parikshak et al., 2016; Voineagu et al., 2011). Indeed, ASD may not have one signature at all, but instead multiple diverging signatures (Tylee, Hess, et al., 2017).

Transcriptomic studies of ASD probands typically use cells collected from either postmortem brains or blood in order to estimate the mRNA abundance for thousands of gene transcripts, by way of microarray technology or massively parallel high-throughput sequencing (RNA-Seq). As many expressed transcripts are a precursor to structural or functional proteins, these studies can provide an insight into the functional state of a cell, capturing the common pathway for hereditary predisposition and environmental exposure. Although post-mortem brain studies have an advantage in that they look directly at the tissue of interest, blood-based studies can identify clinically useful biomarkers while also serving as a reliable proxy for gene expression in the brain (Tylee, Kawaguchi, & Glatt, 2013), though a complete understanding of ASD pathophysiology and its biomarker profile will likely require careful consideration of both lines of evidence. To date, more than a dozen studies have measured the transcriptomic profiles of ASD probands and controls, the results of which have been summarized by two separate meta-analyses (Ch'ng, Kwok, Rogic, & Pavlidis, 2015; Ning et al., 2015) and one mega-analysis (Tylee, Hess, et al., 2017).

Sex is often called a risk factor for ASD, and it is stated that the risk for a male to have ASD is four to five times higher than that for females (Christensen et al., 2016; Werling, Parikshak, & Geschwind, 2016), although the magnitude of this difference may be partly due to diagnostic biases (Lai, Lombardo, Auyeung, Chakrabarti, & Baron-Cohen, 2015). A similar observation, that the increased male risk is even higher among high-functioning ASD probands (Fombonne, 1999), likewise suggests that sex-specific mechanisms could influence ASD pathophysiology and its biomarker profile. Further evidence for

sex-specific mechanisms is found in recent transcriptomic and functional-imaging studies. For example, Tylee et al., using transformed lymphoblastoid cell lines, found evidence for sex-specific differential regulation of genes and pathways among ASD probands (Tylee, Espinoza, et al., 2017). Similarly, Trabzuni et al. found sex-specific differences in alternative splicing in adult human brains, including for a well-known ASD risk gene NRXN3 (Trabzuni et al., 2013). Functional brain connectivity studies using fMRI imaging have also identified sexual heterogeneity among ASD probands, showing dysregulation in sexually dimorphic brain regions across two large studies (Floris, Lai, Nath, Milham, & Di Martino, 2018; Lai et al., 2013). Moreover, recent work by Mitra et al. found evidence for pleiotropy between common single nucleotide polymorphisms (SNPs) for secondary sex characteristics and ASD risk, as well as sex heterogeneity on the X-chromosome, through a comprehensive SNP mega-analysis combining 12 individual data sets from diverse genetic backgrounds (Mitra et al., 2016). Taken together, it seems plausible that sex could interact with other genetic and environmental factors to create sex-specific ASD pathophysiologies and biomarker profiles.

As ASD is more common in males, it suggests that females may have some underlying protection whereby a higher risk load is required for them to become afflicted (Robinson, Lichtenstein, Anckarsäter, Happé, & Ronald, 2013). One hypothesis posits that ASD itself reflects a shift towards "extreme maleness" such that males are necessarily predisposed (Baron-Cohen, 2002). In support of this, females with ASD do harbor more and larger copy number variants than males with ASD (Levy et al., 2011), and moreover exhibit differential penetrance given the same genetic etiology (Lionel et al., 2014), although Mitra et al. found no evidence for an increased SNP load in females (Mitra et al., 2016). Unfortunately, however, the increased prevalence of ASD in males has led to the exclusion of females from many transcriptomic studies (e.g., Alter et al., 2011; Hu et al., 2009; Sarachana, Zhou, Chen, Manji, & Hu, 2010), making it difficult to understand the male skew in ASD prevalence. Indeed, individual studies are often underpowered to detect subtle sex-specific differences, if they contain female subjects at all. When female subjects are included, sex is typically modeled as a simple covariate rather than an ASD-sex interaction, meaning that only sex-independent, and not sex-dependent, biomarkers are discovered. When male ASD is contrasted with female ASD, it typically involves loosely comparing simple sex-specific differences in a statistically anticonservative manner (e.g., noting differential expression in males but not females, or vice versa). To the best of our knowledge, no study has looked at whether gene expression signatures show a sex-autism interaction across multiple studies and human tissues.

Using a meta-analysis of six blood-based microarray data sets and an exploratory analysis of a single paired-end postmortem brain RNA-Seq data set, we present an analysis of transcriptomic data that focuses on comparing sex-dependent and sex-independent ASD biomarkers across multiple tissues. By modeling the interaction of sex and ASD directly, we identify biomarkers and functional pathways that show sex-differences in ASD probands that are different than those in control subjects. Then, for those biomarkers that show no interaction, we pool male and female probands to look for sex-independent biomarkers. Our results suggest that, despite low power,

some genes have FDR-adjusted significant sex-dependent interactions, while even more have significant sex-independent main effects. Subsequent pathway analysis further shows that these sex-independent biomarkers have substantially different biological roles than the sex-dependent biomarkers, and that some of these pathways are dysregulated in both postmortem brain and blood.

2 | METHODS

2.1 | Data acquisition

2.1.1 | 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 (Ch'ng et al., 2015; Ning et al., 2015), and one mega-analysis (Tylee, Hess, et al., 2017), 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 (i.e., GSE32136, treated with PPA). Six data sets remained after exclusion, as described in Table 1.

Data acquired from the Gene Expression Omnibus (GEO; Barrett & Edgar, 2006) including, GSE6575 (Gregg et al., 2008) and GSE18123 (Kong et al., 2012) were acquired already normalized and were not modified further. The other data sets (i.e., the Glatt et al. Wave I and Wave II data (Glatt et al., 2012), the CHARGE study data (Hertz-Picciotto et al., 2006), and the Kong et al., 2013 data (Kong et al., 2013) each underwent RMA normalization, quantile normalization, and base-2 logarithm transformation. We filtered each data set so that all subjects in the ASD group had a diagnosis of autism spectrum disorder. Some typically developing subjects from the Glatt et al. data sets were considered "Type-1 errors".

2.1.2 | RNA-Seq data

We searched for relevant publicly available RNA-Seq data using the Gene Expression Omnibus (GEO; Barrett & Edgar, 2006) 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 nonpoly-A-selected libraries. After excluding any

data sets that used cell lines or did not have female cases, only one experiment, GSE107241 (Wright et al., 2017), remained. These data comprise a RiboZero Gold paired-end RNA-Seq data set from 52 post-mortem dorsolateral prefrontal cortex tissue samples (10 ASD males, 3 ASD females, 30 control males, and 9 control females). No other data met this search criteria.

Prior to alignment and quantification, raw RNA-Seq reads were trimmed using Trimmomatic (docker image quay.io/biocontainers/trimmomatic:0.36-4; Bolger, Lohse, & Usadel, 2014) and quality control metrics were recorded (before and after trimming) using FastQC (docker image biocontainers/fastqc:0.11.5; Andrews, 2010). We aligned trimmed reads and quantified expression using Salmon (docker image combinelab/salmon:0.9.0; Patro, Duggal, Love, Irizarry, & Kingsford, 2017) 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 noncoding reference (i.e., GRCh38.90.ncrna).

2.2 | Meta-analysis of microarray data

Before proceeding with the meta-analysis, we established a set of probes 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.

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; Smyth, 2004), applying the following steps: (1) fit a single model with the formula $\sim \text{ASD} + \text{Sex} + \text{ASD}:\text{Sex} + \text{Age}$ where ASD and Sex are each two-level factors (except GSE6575, where the Age covariate is unknown), (2) define contrasts for the sex-autism interaction and for the sex-independent main effects, and (3) measure the differential expression for each contrast using the eBayes procedure. In other words, we fit a single model but pulled out the appropriate contrasts in two steps.

Next, we transformed platform-specific probe *p*-values to HGNC symbol *p*-values using AnnotationDbi (available from Bioconductor (Huber et al., 2015)). We resolved many-to-one mapping ambiguities by FDR-adjusting the minimum *p*-value of all probes for a given gene symbol (i.e., calculating a within-gene FDR correction). We then used Fisher's method to perform a meta-analysis of the *p*-values obtained from the differential expression analysis. For *K* studies, Fisher's

TABLE 1 The table details all studies included in the meta-analysis, and the number of probes available after establishing a final probe set. We filtered each data set so that all subjects in the ASD group had a diagnosis of autism spectrum disorder. Some typically developing subjects from the Glatt et al. data sets were considered "Type-1 errors"

Study ID	Probes (intersect)	Females (TD)	Males (TD)	Females (ASD)	Males (ASD)
GSE6575	39,561	3	9	8	36
GSE18123	19,532	34	48	24	80
Glatt et al. Wave I	28,424	28	40	11	49
Glatt et al. Wave II	28,424	35	56	28	85
CHARGE	39,561	15	75	15	103
Kong et al., 2013	19,532	7	10	7	46

method scores each gene based on negative two times the sum of the logarithm of the p -values:

$$\chi^2_{2K} = -2 \sum_i^K \log p_i$$

This score follows a χ^2 distribution with $2K$ degrees of freedom (Mosteller & Fisher, 1948). Thus, for each gene, we computed a p -value directly from this score. We corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

2.3 | Differential expression analysis of RNA-Seq data

We used DESeq2 (Version 3.6; Love, Huber, & Anders, 2014) 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 single model was fit using the formula $\sim \text{ASD} + \text{Sex} + \text{ASD}:\text{Sex} + \text{Age}$ (where Age is the age of death). Interaction and sex-independent main effects were then extracted from the model by specifying the relevant contrasts to the DESeq2::results function. Again, we fit a single model but pulled out the appropriate contrasts in two steps. We corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

2.4 | Adjustment of latent batch effects

To ensure that latent batch effects did not inflate the discovery of false positives, we performed all analyses above with adjustment for batch effects using sva (Version 3.26; Leek, 2014; Leek, Johnson, Parker, Jaffe, & Storey, 2012), applying the following steps: (a) estimate the number of surrogate variables while specifying the $\text{ASD} * \text{Sex}$ interaction as the variable of interest (via a 4-level factor that contains "MaleASD", "FemaleASD", "MaleControl", and "FemaleControl") and Age as an adjustment variable, (b) use the sva function (or, in the case of Salmon-generated counts, the svaseq function) to estimate the surrogate variables, and (c) include the surrogate variables in the differential expression model(s) described above. Generally speaking, using sva yielded more conservative results than not using sva. All tables and figures show results generated with sva except where otherwise noted.

2.5 | Pathway analysis and knowledge integration

We performed pathway analysis using GSEA (Version 3.0; Subramanian et al., 2005) in PreRanked mode with classic enrichment and 1,000 permutations. Enrichment scores were calculated for specific MSigDB (Version 6.1; Liberzon et al., 2011; Subramanian et al., 2005) gene sets, including the curated KEGG (c2.cp.kegg; Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017), Gene Ontology Biological Process (c5.bp; The Gene Ontology Consortium, 2017), Reactome (c2.cp.

reactome; Fabregat et al., 2018), and MSigDB Hallmark (hall; Liberzon et al., 2015) sets.

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

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

where positive FC refers to upregulation in ASD for the sex-independent main effects. Then, these transcript-level ranks were converted into gene-level ranks based on the top transcript-level rank. For the microarray meta-analysis, we ranked genes using the χ^2 test statistic (as calculated from Fisher's method). Note that since this latter metric is agnostic to the direction of expression changes (i.e., only large χ^2 test statistics suggest dysregulation), we focused here on pathways enriched with a positive score (effectively making this pathway enrichment test one-tailed). In both cases, we set $\alpha = 0.30$.

3 | RESULTS

3.1 | Evidence for sex-dependent autism biomarkers

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

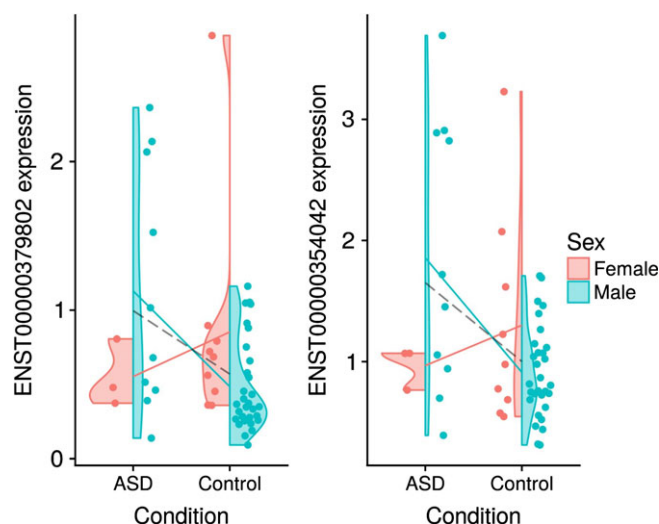


FIGURE 1 The violin plots show the base-2 logarithm-transformed expression for the two transcripts with the largest interaction effect from the RNA-Seq data (i.e., those with the smallest corrected p -value). The solid lines show sex-specific mean expression differences. The dashed line shows the sex-independent (i.e., pooled) mean expression difference. The left and right transcripts are associated with the DSP and SLC13A4 genes, respectively [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 The 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

Transcript ID	Gene symbol	Transcript biotype	Log 2 FC	p -adj (SVA)	p -adj (no SVA)
ENST00000354042	SLC13A4	Protein coding	3.27	.293	.1136846
ENST00000379802	DSP	Protein coding	3.19	.293	.6534814
ENST00000262551	OGN	Protein coding	2.97	.299	.8169099
ENST00000371625	PTGDS	Protein coding	1.74	.299	.0329544
ENST00000223357	AEBP1	Protein coding	1.85	.529	.8713166

From the analysis of the RNA-Seq data derived from postmortem brain tissue, we find no transcripts with significant (FDR-adjusted p -value $<.05$) sex-dependent dysregulation, although one of these transcripts showed a significant interaction prior to batch correction with sva. To illustrate what a sex-autism interaction might look like, Figure 1 shows the per-group expression profiles for the two transcripts with the largest interaction effect (i.e., those with the smallest corrected p -value). Table 2 characterizes those transcripts with the most sex-dependent dysregulation.

From the meta-analysis of the blood-based microarray data, we find four genes with significant (FDR-adjusted) sex-dependent

dysregulation: TTF2, UTY, KCNJ8, and NCS1. Table 3 characterizes those genes with the most sex-dependent dysregulation. For Fisher's method, a very small p -value in only one study could cause the meta-analysis to post a significant result (Tseng, Ghosh, & Feingold, 2012). Therefore, it is useful to inspect visually how each study contributed to the results of the meta-analysis. Figure 2 shows how each study contributed to the meta-analysis findings by plotting the aggregate Fisher score for each gene with large sex-dependent dysregulation, along with the study-wise nominal significance (unadjusted p -value $<.05$). Notably, several of the most significantly dysregulated genes are at least nominally significant in more than one study.

TABLE 3 The table shows genes with the most sex-dependent dysregulation (and their chromosomal position) for the meta-analysis results, 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

	Location	Fisher	Fisher p -adj	Fisher (no SVA)	Fisher p -adj (no SVA)
TTF2	1p13.1	52.80272	.0080909	30.30688	1.0000000
UTY	Yq11.221	49.17813	.0352693	45.32043	.1644908
KCNJ8	12p12.1	48.80700	.0409512	41.60015	.7040022
NCS1	9q34.11	48.69443	.0428454	33.16947	1.0000000
RAP2C	Xq26.2	47.97863	.0571022	25.06737	1.0000000
CHST11	12q23.3	47.29425	.0750785	21.86910	1.0000000
MAP1B	5q13.2	47.18032	.0785676	44.44789	.2320088
CRHR1	17q21.31	47.15296	.0794259	43.43498	.3450932
PAFAH1B1	17p13.3	46.36425	.1087281	17.26173	1.0000000
SH3BGR	21q22.2	46.35975	.1089168	32.82178	1.0000000
PAK3	Xq23	45.67211	.1430652	46.45645	.1048620
FMO1	1q24.3	44.77510	.2038808	32.85009	1.0000000
TROVE2	1q31.2	44.42306	.2341680	21.84958	1.0000000
BNC2	9p22.3-p22.2	44.40285	.2360233	39.13101	1.0000000
DCUN1D1	3q26.33	44.08602	.2672865	40.02468	1.0000000
HECA	6q24.1	44.06996	.2689620	33.92752	1.0000000
RORA	15q22.2	43.52061	.3335278	34.11106	1.0000000
ARHGAP35	19q13.32	43.40139	.3494222	41.54671	.7186470
CAMK1D	10p13	43.34605	.3570412	23.46336	1.0000000
SLC27A6	5q23.3	43.26356	.3687130	42.46235	.5041354
SNX13	7p21.1	43.07811	.3963678	27.35225	1.0000000
ACKR2	3p22.1	43.06703	.3980622	36.05068	1.0000000
KCNN2	5q22.3	42.76385	.4479560	44.03212	.2731511
TEX35	1q25.2	42.72490	.4547740	40.79263	.9608426
TMEM56	1p21.3	42.56794	.4833811	34.90678	1.0000000
SYTL4	Xq22.1	42.49851	.4965789	40.68910	.9997724
AZGP1	7q22.1	42.48820	.4985441	27.40362	1.0000000

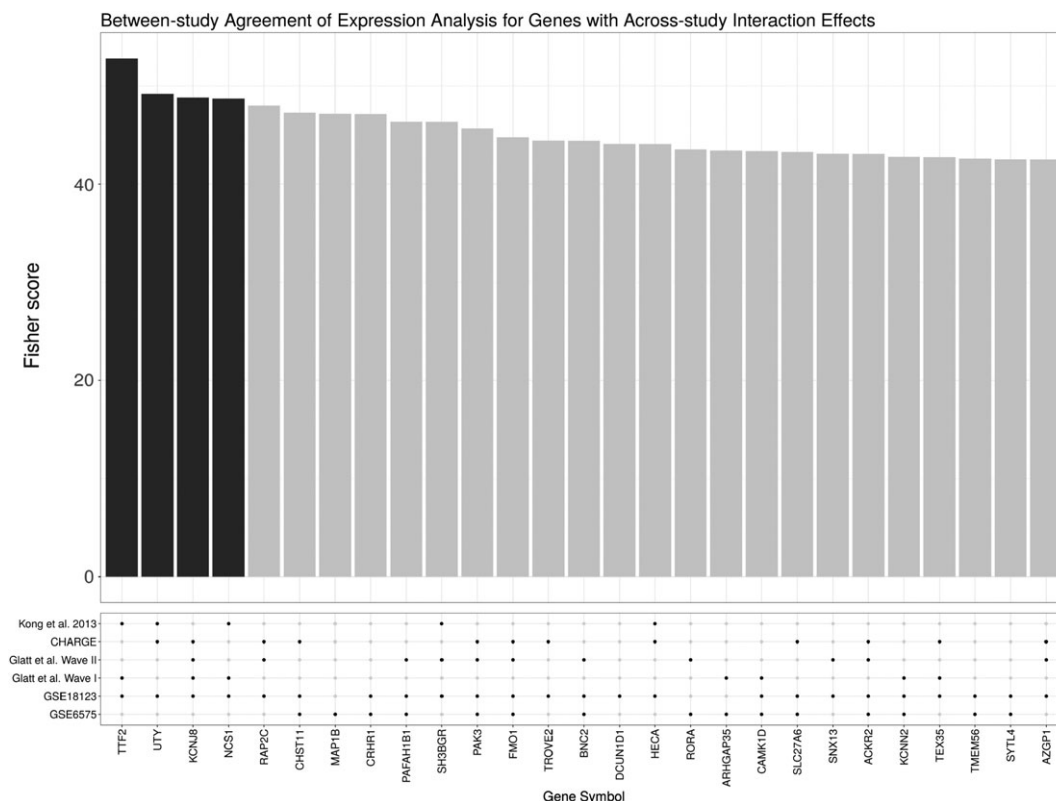


FIGURE 2 The figure shows genes with the most significant sex-dependent dysregulation (i.e., a sex-autism interaction) according to the meta-analysis of the microarray data. Above, the bar plot shows the χ^2 score for each gene as calculated using Fisher's method (where the dark bars indicate that the gene has an FDR-adjusted p -value $< .05$). Below, the dot plot shows whether a gene showed a nominally significant sex-dependent dysregulation at an unadjusted p -value $< .05$ for a given study. Note that most genes selected for by the meta-analysis show at least nominal significance across multiple studies

3.2 | Evidence for sex-independent autism biomarkers

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

From the analysis of the RNA-Seq data derived from postmortem brain tissue, we find seven transcripts with significant (FDR-adjusted p -value $< .05$) sex-independent differential expression. Of these, only one transcript showed significant upregulation in ASD (with all others showing downregulation). Figure 3 shows the expression profile for the two transcripts with the most significant sex-independent main effects. Table 4 characterizes those transcripts with significant sex-independent dysregulation. Interestingly, several of the transcripts called differentially expressed by the analysis are annotated as non-coding RNA species.

From the meta-analysis of blood-based microarray data, we find over 20 genes with significant (FDR-adjusted) sex-independent dysregulation. Table 5 characterizes those genes with the most sex-independent dysregulation. As in figure 2, figure 4, shows how each

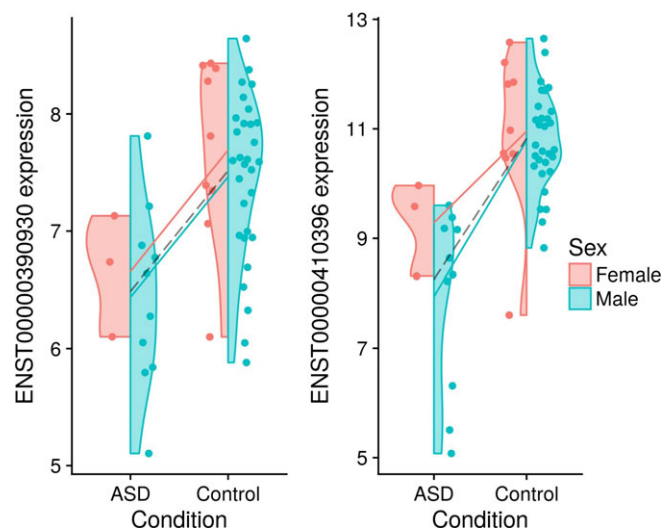


FIGURE 3 The violin plots show base-2 logarithm-transformed expression for the two most significant main effects (i.e., of the ASD condition) from the RNA-Seq data. The solid lines show sex-specific mean expression differences. The dashed line shows the sex-independent (i.e., pooled) mean expression difference. The left and right transcripts are associated with the SNORD17 and RNU2-2P genes, respectively [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 4 The 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

Transcript ID	Gene symbol	Transcript biotype	Log 2 FC	<i>p</i> -adj (SVA)	<i>p</i> -adj (no SVA)
ENST00000390930	SNORD17	snoRNA	−2.98	1.54e−05	.0000102
ENST00000410396	RNU2-2P	snRNA	−4.76	4.04e−05	.0000000
ENST00000613119		snRNA	−3.23	9.18e−05	.0000000
ENST00000258526	PLXNC1	Protein coding	0.48	.00468	.4273372
ENST00000393775	IGSF11	Protein coding	−1.18	.00468	1.0000000
ENST00000459255	SCARNA10	snoRNA	−1.71	.00468	.0014803
ENST00000618786	RN7SL1	Misc RNA	−1.35	.0124	.0026454

study contributed to the meta-analysis findings by plotting the aggregate Fisher score for each gene with large sex-independent dysregulation, along with the study-wise nominal significance (unadjusted *p*-value <.05). Again, most genes selected as statistically significant by the meta-analysis are at least nominally significant in more than one study.

3.3 | Pathway enrichment of ASD biomarkers

In an effort to summarize the biological relevance of the biomarker profiles generated above, we used the complete ranked lists of the

differentially expressed transcripts and genes in four separate gene set enrichment analyses to identify common differentially regulated pathways. Four enrichment profiles were generated using the sex-dependent RNA-Seq brain biomarkers, sex-independent RNA-Seq brain biomarkers, sex-dependent microarray blood biomarkers, and sex-independent microarray blood biomarkers.

Figure 5 shows the KEGG pathways enriched by the biomarkers as ranked by the analysis of the RNA-Seq data. Interestingly, all significant enrichment occurred in the same direction. Figure 6 shows the KEGG pathways enriched by the biomarkers as ranked by the analysis

TABLE 5 The table shows genes with the most sex-independent dysregulation (and their chromosomal position) for the meta-analysis results, 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

	Location	Fisher	Fisher <i>p</i> -adj	Fisher (no SVA)	Fisher <i>p</i> -adj (no SVA)
ARHGAP35	19q13.32	69.93173	.0000060	59.58783	.0004856
GIMAP8	7q36.1	59.63083	.0004774	52.83287	.0079551
UCHL3	13q22.2	55.73392	.0024208	26.61452	1.0000000
SPART	13q13.3	55.39389	.0027862	42.18348	.5444711
MAGED2	Xp11.21	54.65897	.0037735	31.43801	1.0000000
ZNF721	4p16.3	54.38812	.0042188	46.99662	.0833787
TCEAL8	Xq22.1	54.30254	.0043699	31.39363	1.0000000
KIF13B	8p12	53.86902	.0052226	44.83078	.1953472
COX19	7p22.3	51.12904	.0160066	51.00574	.0167250
POLR1A	2p11.2	50.82535	.0181073	32.52993	1.0000000
NUCB2	11p15.1	50.20003	.0233305	49.91295	.0260057
EIF3A	10q26.11	49.84351	.0269484	47.30791	.0736972
GNG5	1p22.3	49.73328	.0281743	23.65365	1.0000000
HNRNPA3P1	10q11.21	49.54482	.0304006	51.23881	.0152201
KLF1	19p13.13	49.43419	.0317868	35.96265	1.0000000
GNPDA1	5q31.3	48.90133	.0393978	41.09008	.8257563
SART3	12q23.3	48.88131	.0397143	52.43134	.0093729
CCNC	6q16.2	48.87932	.0397440	16.14519	1.0000000
UBE2A	Xq24	48.87429	.0398222	23.54974	1.0000000
ESF1	20p12.1	48.70005	.0427109	39.78234	1.0000000
ZNF740	12q13.13	48.63177	.0438971	46.72013	.0930420
MTERF4	2q37.3	48.49211	.0464282	37.44203	1.0000000
CCP110	16p12.3	47.96099	.0574500	28.55662	1.0000000
JUND	19p13.11	47.85553	.0599253	37.46297	1.0000000
MTERF1	7q21.2	47.82145	.0607453	29.44710	1.0000000
ZNF569	19q13.12	47.57334	.0670814	36.67860	1.0000000
PGM1	1p31.3	46.90219	.0876872	37.26441	1.0000000
ECI2	6p25.2	46.79028	.0916801	51.52112	.0135729
ARHGAP8	22q13.31	46.67208	.0960926	46.17090	.1155403

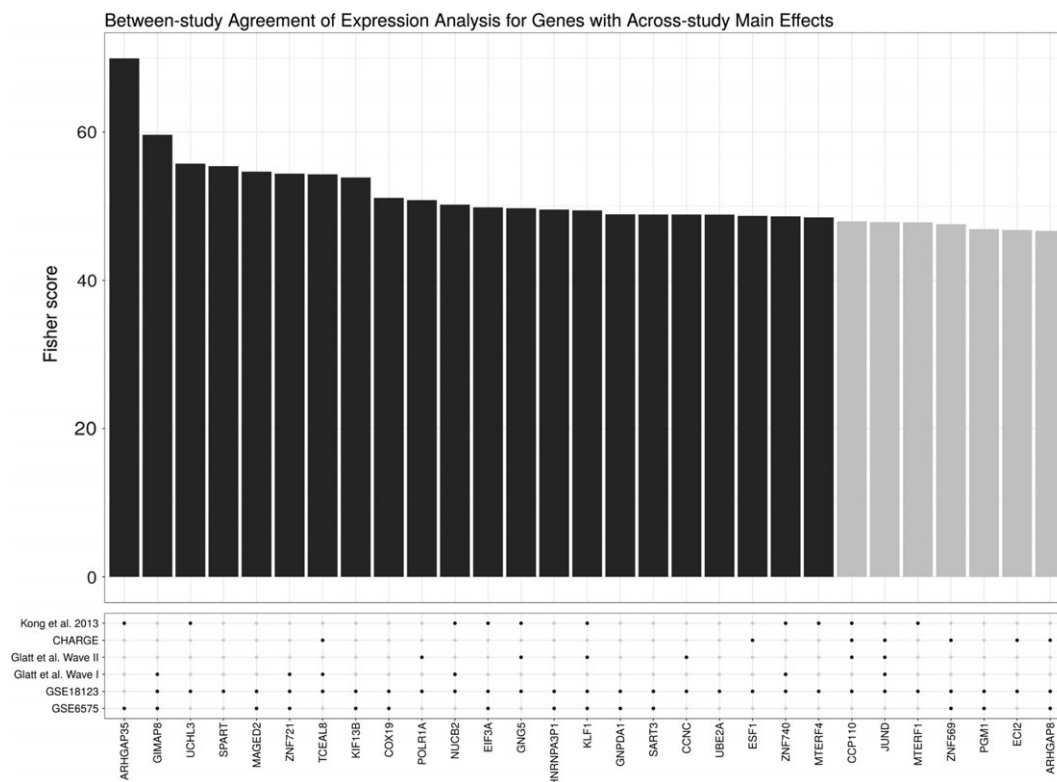


FIGURE 4 The figure shows genes with the most significant sex-independent main effects (i.e., of the ASD condition) according to the meta-analysis of the microarray data. Above, the bar plot shows the χ^2 score for each gene as calculated using Fisher's method (where the dark bars indicate that the gene has an FDR-adjusted p -value $< .05$). Below, the dot plot shows whether a gene showed a nominally significant sex-independent main effect at an unadjusted p -value $< .05$ for a given study. Note that most genes selected for by the meta-analysis show at least nominal significance across multiple studies

of the microarray data. Unlike the RNA-Seq enrichment analysis, the meta-analysis enrichment analysis is agnostic to direction. Figure 7 compares the overlap between these significant pathways. For the sex-dependent analyses, no pathways are enriched in both the RNA-Seq and microarray data. However, for the sex-independent analyses, two pathways are enriched in both data (i.e., Ribosome and Proteasome), though this observation might have occurred by chance. Note that we also tested for enrichment among the Gene Ontology Biological Process, Reactome, and MSigDB Hallmarks gene sets, all of which show more examples of overlap between the separate sex-independent analyses (see the Supplementary Information for more details). We make the complete pathway enrichment results for the interaction and main effects found in the RNA-Seq and microarray data sets all available at <https://doi.org/10.5281/zenodo.1421429>.

4 | DISCUSSION

In this report, we present an analysis of several ASD transcriptomic studies, including a meta-analysis of six blood-based microarray data sets and an exploratory analysis of RNA-Seq data derived from post-mortem brain. In both analyses, we focus on identifying sex-dependent and sex-independent biomarker profiles for ASD by modeling the sex-autism interaction directly. In addition to identifying transcript and gene biomarkers, we use gene set enrichment analysis to summarize the observed dysregulation at the pathway level,

contrasting sex-dependent pathway enrichment with sex-independent pathway enrichment. In doing so, we find that some pathways are across both tissues (i.e., Ribosome and Proteasome), though this observation might have occurred by chance.

Despite small sample sizes in all studies, we found evidence for the existence of some sex-dependent biomarkers in human tissue. The meta-analysis identified four genes with significant (FDR-adjusted) sex-dependent dysregulation in the blood: TTF2, UTY, KCNJ8, and NCS1. One of these, TTF2, plays an important role in normal thyroid development (De Felice & Di Lauro, 2004). Interestingly, a loss of thyroid hormone homeostasis has been linked to ASD (Berbel, Navarro, & Román, 2014; Khan, Harney, Zavacki, & Sajdel-Sulkowska, 2014). As it is well-known that thyroid diseases have a sex-specific presentation (Bauer, Glenn, Pilhatsch, Pfennig, & Whybrow, 2014), it seems plausible that thyroid abnormalities could contribute to a sexually dimorphic ASD signature. Some thyroid-disrupting environmental chemicals have also been linked to an altered risk for autism (Braun et al., 2014; Lyall, Croen, Sjödin, et al., 2017), including one study showing sexually dimorphic associations (Lyall, Croen, Weiss, et al., 2017). The other, UTY, is a Y-chromosome gene (with considerable homology to an X-chromosome homolog), making any interpretation of its differential dysregulation difficult. Two other genes, KCNJ8 and NCS1, are involved in potassium and calcium channel activity, respectively, the latter of which has been implicated in autism (Handley, Lian, Haynes, & Burgoyne, 2010). Although the RNA-Seq analysis did not yield any significant

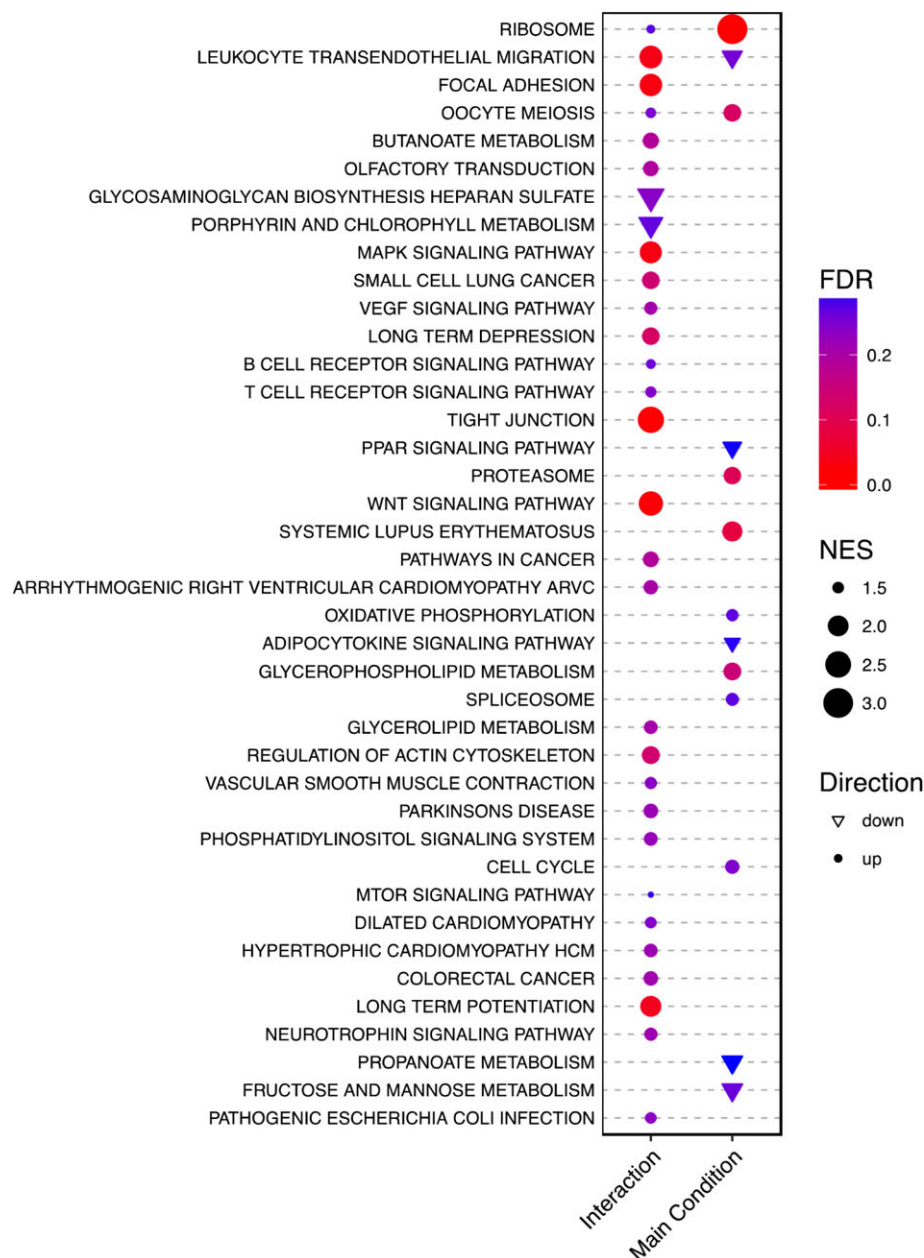


FIGURE 5 The dot plot shows results from a GSEA of the RNA-Seq data against the MSigDB KEGG pathways. For the sex-autism interaction and the main effect results, a KEGG pathway (y-axis) has a circle (or triangle) if it is enriched (or depleted). The size of the points indicates the absolute normalized enrichment score. The color indicates the FDR. Note that only points with an FDR < 0.3 are plotted (see Methods). The enrichment score is defined as the degree to which a gene set is overrepresented at the top or bottom of a ranked gene list as calculated by GSEA. The ratio of this to the expected enrichment score of all permutations is the normalized enrichment score (NES) [Color figure can be viewed at wileyonlinelibrary.com]

interactions, it is not surprising considering this data set contained only three female ASD probands. Nevertheless, the large (albeit non-significant) effect sizes warrant repeat studies with bigger cohorts and more female ASD probands.

By modeling the sex-autism interaction directly, we are able to follow-up the sex-dependent analysis with a secondary sex-independent analysis for any transcript or gene whose expression did not significantly interact with sex. Using the same regression model, we contrast the pooled male ASD probands and female ASD probands against the pooled male controls and female controls to calculate the main effects (which we can interpret as sex-independent biomarkers).

Here, over 20 transcripts and genes exceeded the threshold for FDR-adjusted significance. Interestingly, for the RNA-Seq data, several of the significant biomarkers are not protein-coding genes, highlighting the value of using nonpoly-A-selected libraries to quantify both coding and noncoding transcripts. For the microarray meta-analysis, several of the sex-independent biomarkers are associated with key neurodevelopmental processes, including some X-chromosome genes. For example, *MAGED2*, differentially expressed in ASD probands, is located on an X-linked intellectual disability hotspot (i.e., Xp11.2; Langnaese, Kloos, Wehnert, Seidel, & Wieacker, 2001; Moey et al., 2016) which, if causally relevant, could contribute to the male risk bias.

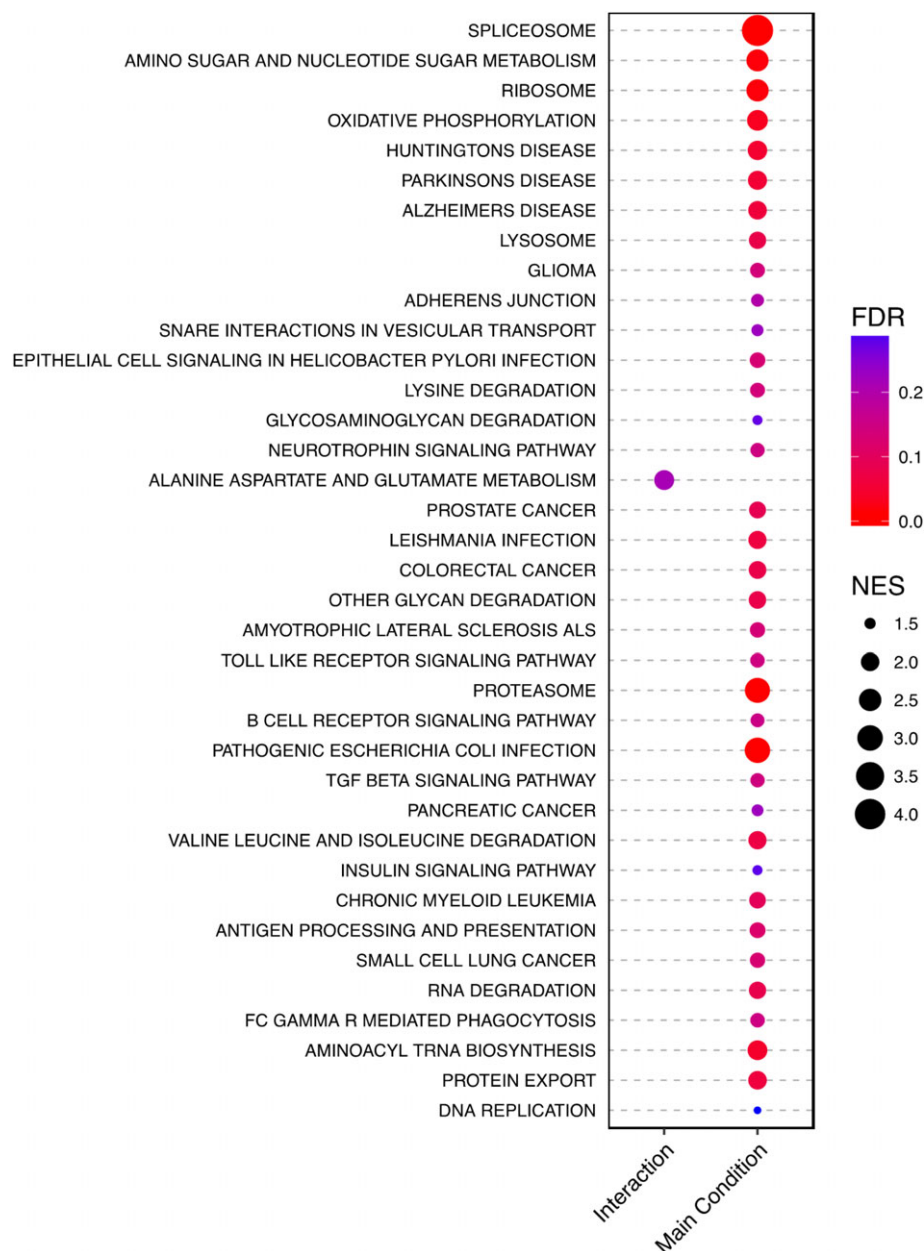


FIGURE 6 The dot plot shows results from a GSEA of the meta-analysis data against the MSigDB KEGG pathways. For the sex-autism interaction and the main effect results, a KEGG pathway (y-axis) has a circle if it is enriched. The size of the points indicates the absolute normalized enrichment score. The color indicates the FDR. Note that only points with an FDR < 0.3 are plotted (see Methods). The enrichment score is defined as the degree to which a gene set is overrepresented at the top or bottom of a ranked gene list as calculated by GSEA. The ratio of this to the expected enrichment score of all permutations is the normalized enrichment score (NES) [Color figure can be viewed at wileyonlinelibrary.com]

For both the microarray meta-analysis and the RNA-Seq analysis, we tested the ranked sex-dependent and sex-independent biomarker profiles separately for pathway-level enrichment. We found some pathway enrichment for the sex-dependent profiles, and even more for the sex-independent profiles. Importantly, very few of the enriched pathways were the same for both the interaction and main effects. This suggests that males and females exhibit unique pathway-level signatures that, if causally relevant, might further suggest the existence of both sex-specific and common ASD pathophysiologies. Although few KEGG pathways are enriched among the sex-dependent results, there are dozens of significantly enriched sex-dependent pathways across other tested gene sets (see Supplementary Information

for more details). Among the pathways enriched in the sex-independent meta-analysis results, there are a number of pathways for known neurodevelopmental and neurodegenerative diseases, including Huntingtons, Parkinsons, Alzheimers, and amyotrophic lateral sclerosis (ALS), suggesting that at least some of these ASD biomarkers may have functions important to general brain health. Considering that the sex-dependent and sex-independent biomarkers and pathways differ, it seems plausible that molecular diagnostics could benefit from modeling sex-specific processes directly.

In addition to finding that pathway enrichment differs considerably between the sex-dependent and sex-independent biomarker profiles, we found that some sex-independent pathways (e.g., Ribosome

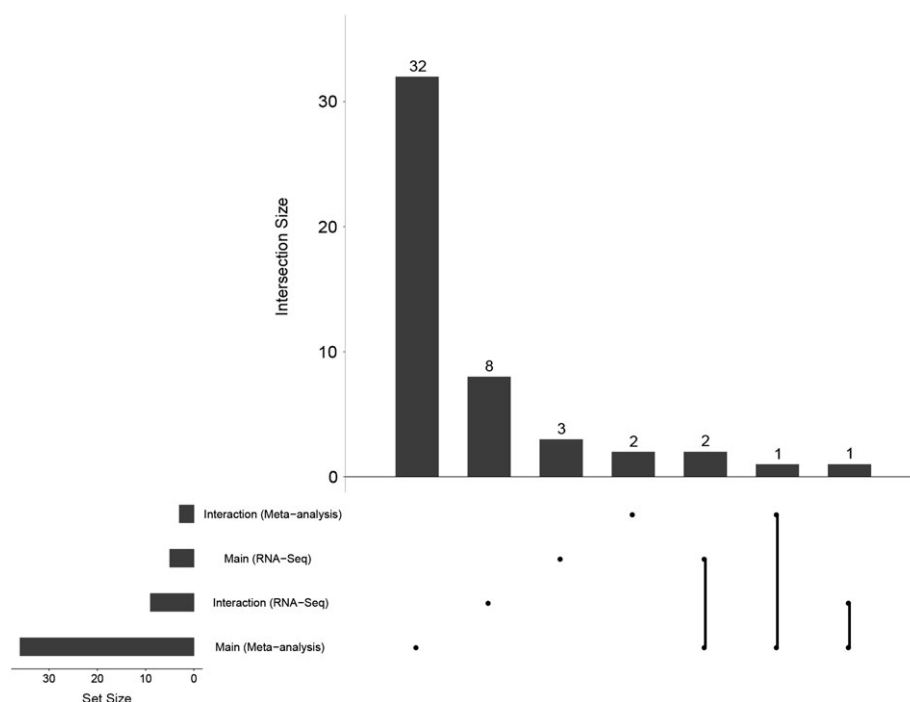


FIGURE 7 The UpSet plot (Lex, Gehlenborg, Strobel, Vuilleumot, & Pfister, 2014) shows set intersections (and their sizes) from a GSEA of four results against the MSigDB KEGG pathways. Set identity is indicated by the joined lines. Set size is indicated by the top bar chart. The bar chart on the left 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)

and Proteasome) were enriched across both the RNA-Seq and microarray data. Interestingly, this overlap exists despite the fact that analyses were performed on different human tissues, and with different transcript quantification assays. In fact, more than 50 Gene Ontology pathways were enriched among both sets of ranked sex-independent biomarkers, even though no gene products showed significant differential expression in both data. This overlap is consistent with a broad literature supporting common pathway-level signatures across the widely heterogeneous population of ASD probands. If true, it may be advantageous to model pathway-level dysregulation directly, for example in machine learning applications Quinn, Lee, Venkatesh, and Nguyen (2018).

When we compare our pathway enrichments to the previous ASD mega-analysis pathway enrichments (Tylee, Hess, et al., 2017), we observe several complementary results. First, we found positive enrichment of the MAPK pathway in our sex-dependent RNA-Seq results, agreeing with the male-specific enrichment of Mek targets found in the Tylee et al. study (Tylee, Hess, et al., 2017). Second, we found an enrichment of the ribosome-related pathway in both of our sex-independent analyses, agreeing with the ribosome-related pathway enrichment identified by the sex-independent mega-analysis (Tylee, Hess, et al., 2017). Third, we found an enrichment of the Toll-like receptor (TLR) signaling pathway in our sex-independent meta-analysis results, agreeing with the TLR 3 and 4 signaling pathway enrichment identified by the sex-independent mega-analysis (Tylee, Hess, et al., 2017). Importantly, these complementary results exist despite considerable differences in statistical methodology and data set inclusion. The Wright et al. study which generated the RNA-Seq

study did not test for KEGG enrichment. However, we both found SNORD17 to be differentially expressed independent of sex (Wright et al., 2017).

Our analysis is not without limitations. First, although we used *sva* to adjust for latent batch effects, it is still possible that some residual batch effects remain because they coincide with the diagnostic label (e.g., undocumented comorbidities or medication use). This would confound the discovered biomarker profile, causing spurious results. Second, as with any observational study, it is impossible to conclude whether the gene expression signatures, and their associated pathways, are causally related to ASD. Third, this analysis is likely underpowered to detect all sex-autism interactions, owing to the small sample sizes and disproportionately smaller female cohorts. Yet, based on the extant literature, which clearly highlights sex as an ASD risk factor, and the results published here, we believe that modeling the sex-autism interaction should become a mainstay of ASD transcriptomic research. Advantageously, interaction modeling is compatible with the most commonly used softwares for batch-effect correction (Leek et al., 2012), RNA-Seq analysis (Love et al., 2014), and microarray analysis (Smyth, 2004). Yet, this analytical technique cannot offer any benefit if transcriptomic studies continue to systematically exclude female subjects (e.g., Alter et al., 2011; Hu et al., 2009; Sara-chana et al., 2010). Although there seems to exist a strong skew in the prevalence of male ASD, this very fact underlies the importance of studying female ASD: a complete understanding of the molecular basis of ASD will require the intentional study of both sex-dependent and sex-independent mechanisms, as well as their differences and commonalities.

ACKNOWLEDGMENTS

SCL and TPQ designed the experiments, performed the analyses, and drafted the manuscript. JL provided statistical expertise and helped revise the manuscript. SWK, IHP, and SJG contributed data and helped revise the manuscript. TMC, SV, and TN supervised the project and helped revise the manuscript. This research was partially supported by the Australian Government through the Australian Research Council's Linkage Projects funding scheme (LP140100240). SWK is supported by a grant from the National Institute of Health (R01MH107205). IHP is supported by grants from the National Institute of Environmental Health Sciences (1R01ES015359; 2R01ES015359; 1P30ES023513; 3P01ES011269), the National Institute of Health (UG3OD023365), and the United States Environmental Protection Agency (RD83543201).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Lee SC, Quinn TP, Lai J, et al. Solving for X: Evidence for sex-specific autism biomarkers across multiple transcriptomic studies. *Am J Med Genet Part B*. 2018; 1–13. <https://doi.org/10.1002/ajmg.b.32701>