Supplementary Note

Underdiagnosis of autistic females and increased male susceptibility to X-linked causes of autism are frequently invoked to explain the male bias of autism. Here, we demonstrate that these factors likely contribute to but do not fully explain autism's male bias.

Underdiagnosis of autistic females

Females often face greater barriers to receiving an autism diagnosis compared to males, due to factors such as clinician bias, gendered behavioral expectations, and sex differences in the extent to which core symptoms of autism are expressed versus "masked"[1]. This has led some to speculate that the male preponderance of autism is driven by underdiagnosis of autistic females[2]. While diagnostic bias likely contributes to the male skew of autism, a male-biased sex ratio persists even when only considering autism cases that are readily clinically ascertainable and thus less susceptible to significant diagnostic bias. For example, the Baby Siblings Research Consortium screened for autism in the younger siblings of over one thousand autistic individuals and still found a 3M:1F sex bias among affected siblings[3,4].

Bias is also unlikely to influence the diagnosis of profoundly affected autistic individuals, such as those with intellectual disability (ID). Consistently, sex ratio estimates in autism with ID range from 2– 3.5M:1F, whereas sex ratio estimates for autism without ID are higher, from 4-9M:1F[1,5–8]. It is tempting to speculate that this increased sex ratio in less profoundly affected individuals reflects diagnostic bias, although there are also significant differences in the genetic architecture of autism with and without ID that could influence a sex bias[9–12]. Regardless, it is clear that even readily ascertainable cases of autism show a significantly male-biased sex ratio.

Finally, the male bias of autism is consistently observed across countries with diverse cultures and gender norms. A recently published study of the global prevalence of autism

reported a male bias in each of over two hundred surveyed countries and regions from the Americas, Europe, Asia, Africa, and Australasia[13]. Taken together, these findings suggest that sociocultural factors or underdiagnosis of autistic females cannot fully account for the male bias in autism.

Enhanced male vulnerability to X-linked causes of autism

Another common explanation for autism's male bias is the increased vulnerability of males to X-linked – and particularly X-linked recessive – causes of autism, as males have just one copy of X-linked genes. Indeed, 33 of 91 X-linked autism genes are reported to follow a strictly recessive mode of inheritance (MOI) in OMIM[14], suggesting a substantial contribution of X-linked recessive mutations to autism (**Fig. S1A**, **Table S1**, and **Supplementary Methods**). However, even X-linked recessive mutations can sometimes affect females due to their variable expressivity, or to skewing of X-chromosome inactivation (XCI). For example, a detailed phenotypic analysis of 19 females heterozygous for mutations in *KDM5C*, an X-linked gene classically thought to cause autism in a recessive manner[15], found that 15 of the 19 exhibited moderate ID[16]. Thus, while XLR mutations certainly contribute to male risk for autism, they also can increase risk for autism and related neurodevelopmental symptoms in females.

Moreover, 13 of the 91 X-linked autism genes (**Fig. S1A**) exhibit an X-linked dominant MOI. For several reasons, these X-linked dominant forms would be expected to bias autism toward females. Firstly, many X-linked dominant causes of autism in females are typically lethal in males. For example, protein-truncating variants in *MECP2* and *DDX3X* cause syndromic autism in females (Rett and *DDX3X* syndrome, respectively) but are nearly always fatal in males[17,18]. Secondly, paternal X chromosomes, which are transmitted only to daughters, tend to carry more *de novo* mutations than maternal X chromosomes, which are transmitted to both sons

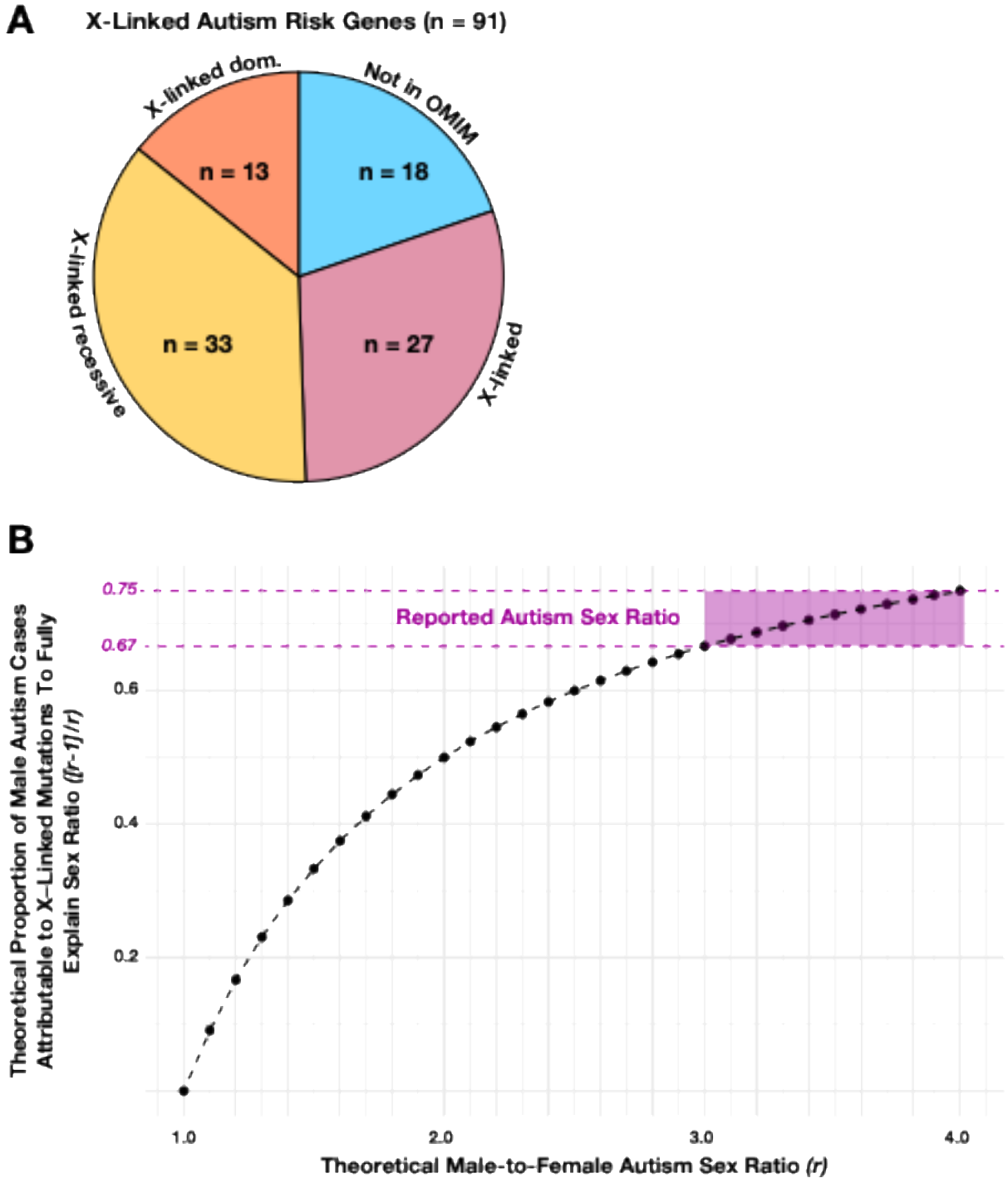
and daughters[19]. Therefore, females are more likely than males to inherit a *de novo* mutation in an X-linked autism gene with a dominant MOI[20]. Consequently, even X-linked dominant causes of autism that are not lethal in males will disproportionately affect females. This female-biased prevalence has been consistently reported in the literature and acts in opposition to the observed male bias in autism. For example, four sex-stratified analyses of *de novo* mutations (DNMs) in X-linked genes associated with autism or developmental disorders (DDs) identified more female-enriched genes (genes significantly enriched for DNMs in affected females only) than male-enriched genes, despite males constituting the majority of the cohort in each of the four studies (**Table S2**)[20–23].

Finally, 27 of the 91 X-linked autism genes follow neither a strictly recessive nor dominant MOI (X-linked MOI; **Fig. S1A**). As a result, mutations in these genes are not expected to uniformly contribute to a male bias in autism[20,24,25]. Considering all of these observations together, it appears unlikely that enhanced male vulnerability to X-linked mutations can fully explain the strong male bias of autism.

This conclusion is further supported by mathematical modeling of autism's male bias. Previous studies of general DDs and ID, both with male-biased sex ratios of 1.4M:1F, have shown that X-linked mutations cannot account for the excess of male cases in these conditions[20,26]. Using the approach described by Ropers and Hamel[26], we find that 67 – 75% of male cases of autism would require X-linked etiologies that do not affect females (e.g., X-linked recessive mutations) to explain a sex ratio of 3-4M:1F (**Fig. S1B** and **Supplementary Methods**). This proportion is much higher than what has been reported in previous studies. For example, the most common X-linked cause of autism in males, Fragile X Syndrome, accounts for only 1 – 6% of all cases of autism[27]. Broader searches for X-linked autism genes have been of great interest

but have largely failed to identify loci that explain a substantial proportion of male autism risk. For example, one of the largest autism exome sequencing studies to date (n = 11,986 autism cases; 17% female) found no X-linked gene with a significant excess of deleterious variants in sex-stratified case-control analyses[28]. In contrast, 102 significant autism risk genes were identified on the autosomes. Even targeted searches for X-linked variants that disproportionately affect males have only explained an additional 2-3% of male cases of autism[29]. Thus, though some male autism cases are attributable to X-linked etiologies, and efforts to identify additional X-linked risk factors are ongoing[30,31], it seems implausible that enough X-linked variation remains undiscovered to fully explain the observed male bias of autism.

Supplementary Figures



Supplementary Figure 1. Enhanced male susceptibility to X-linked, and particularly X-linked recessive, causes of autism cannot fully explain its male bias. (A) Pie chart of modes of inheritance (MOIs) of X-linked autism genes. Most X-linked autism genes do not show a clear X-linked recessive mode of inheritance. X-linked autism genes obtained from *SFARI Gene*[32]. MOIs obtained from *OMIM*[14]. X-linked dom: X-linked dominant. (**B**) Mathematical modeling[26] of theoretical proportion of male autism cases required to be attributable to X-linked mutations that do not affect females to explain the reported 3-4M:F sex ratio of autism. 67 - 75% of male cases of autism would need to be due to X-linked causes to explain the full male bias of autism, a proportion far above what has been seen in sequencing studies of autistic males.

Supplementary Tables

Table S1. OMIM modes of inheritance of X-linked SFARI genes

(an Excel spreadsheet available in Supplementary Material)

Study	**Proportion of females in affected cohort**	**Proportion of X-linked autism/DD genes significantly enriched for DNMs in females only**	**X-linked autism/DD genes significantly enriched for DNMs in females only**	**X-linked autism/DD genes significantly enriched for DNMs in males only**
Turner et al., 2019[22]	11,178/27,603 (0.40)	5/5 (1.0)	*DDX3X*, *HDAC8*, *NAA10*, *USP9X*, *WDR45*	None
Zhang et al., 2020[21]	956/5,748 (0.17)	1/1 (1.0)	*DDX3X*	None
Martin et al., 2021[20]	5,618/13,462 (0.41)	6/8 (0.75)	*CDKL5*, *HDAC8*, *NAA10*, *PDHA1*, *SMC1A*, *STAG2*	*KDM5C*, *UPF3B*
Wang et al., 2022[23]	16,530/46,234 (0.36)	10/10 (1.0)	*DDX3X*, *HDAC8*, *KDM6A*, *MECP2*, *NEXMIF*, *PDHA1*, *SMC1A*, *USP9X*, *WRD45*, *ZC4H2*	None

Table S2. Summary of published sex-stratified analyses of *de novo* mutation (DNM) burden in genes associated with autism or other developmental disorders (DDs).

Supplementary Methods

Analysis of modes of inheritance of X-linked autism genes

A list of 91 X-linked genes associated with autism was obtained from the Q4 2024 release of the *SFARI Gene*[32] database. *OMIM* (omim.org)[14] was used to systematically identify their mode of inheritance (MOI). 18 X-linked genes (*AGTR2*, *CDK16*, *CD99L2*, *DDX53*, *FAM47A*, *IL1RAPL2*, *MAGEC3*, *MAOB*, *PCDH11X*, *PJA1*, *PLXNA3*, *PTCHD1-AS*, *RHOXF1*, *SLC7A3*, *SYAP1*, *TRPC5*, *TSPYL2*, *VSIG4*) had no associated OMIM phenotypes. For all other X-linked genes with at least one associated OMIM phenotype, genes were grouped into the following categories based on OMIM reported MOIs: (1) X-linked recessive only (XLR), (2) X-linked dominant (XLD), (3) multiple modes of inheritance reported (*i.e.*, both XLR and XLD), and (4) X-linked only (*i.e.*, only X-linked reported in OMIM, with no reporting of either XLD or XLR MOIs).

Modeling the contribution of X-linked mutations needed to explain the observed male bias of autism

As demonstrated by Ropers and Hamel[26], the proportion of males that must have an X-linked mutation that does not affect females to fully explain the male case excess in a condition with a male-to-female ratio of r is given by $(r-1)/r$. Under the assumption of a male-to-female ratio of 3-4:1 in autism, this suggests that 67-75% of autistic males would have to have an X-linked cause, respectively, to fully explain the observed male bias.

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