Sex differences in placental markers and later autistic traits

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Abstract

Autism and related neurodevelopmental traits are more prevalent in males. Placental complications are also more frequent in male pregnancies. It is unclear if sex differences in placental function can predict sex differences in autistic traits. To assess this, concentrations of angiogenesis-related markers, placental growth factor (PlGF) and soluble fms-like tyrosine kinase (sFlt-1) were assessed in maternal plasma of expectant women in the late 1st (mean = 13.5[SD = 2.0] weeks gestation) and 2nd trimesters (mean = 20.6[SD = 1.2] weeks gestation), as part of the Generation R Study, Rotterdam, the Netherlands. Subsequent assessment of autistic traits in the offspring at age 6 was performed with the 18-item version of the Social Responsiveness Scale (SRS). Associations of placental protein concentrations with autistic traits were tested in sex-stratified and cohort-wide regression models, controlling for various confounders including placental weight. Mediation analysis was conducted to study if placental proteins mediated the effects of biological sex on autistic traits in childhood. sFlt-1 levels were significantly lower in males in both time-points but showed no association with autistic traits. PlGF was significantly lower in male pregnancies in the 1st trimester, and significantly higher in the 2nd trimester, thus levels increased faster in males during gestation. Higher PlGF levels in the 2nd trimester and the rate of PlGF increase were associated with higher autistic traits in linear regression models (PlGF-2nd : n = 3469,b = 0.24[SE = 0.11], p = 0.03). In sex-stratified analyses, these associations were significant in females but not males. Mediation analyses showed that higher autistic traits in males were partly explained by higher PlGF or a faster rate of PlGF increase in the second trimester (PlGF-2nd : n = 3469, ACME: b = 0.006, [SE = 0.002], p = 0.004). In conclusion, placental sex differences in PlGF levels are linked to sex differences in autistic traits.

Introduction

Sex differences in prenatal physiology may be contributing to sex differences in autism liability (1). Male fetuses are exposed to higher levels of steroid hormones, such as testosterone, following the activation of the testes in the second trimester of gestation. A series of retrospective studies found that several steroid hormones, and estrogens in particular, were higher in maternal serum (2) and in the amniotic fluid of individuals that were later diagnosed as autistic, when compared to neurotypical pregnancies (3, 4).

The aetiology and source of elevated steroidogenesis in autism remains unclear. Prenatally, steroid hormones are derived from various endocrine sources that are mainly regulated by the placenta, which aromatises androgens to estrogens and induces steroidogenesis from the maternal and fetal adrenals. The placenta also affects foetal growth by facilitating nutrient transfer and the production of several growth factors. A “placenta-brain” axis has been proposed, of particular developmental significance, given that many neurotransmitter precursors (e.g. serotonin) are synthesised in the placenta (5–7).

In autism, atypical placental morphology indicating excess proliferation (8, 9), increased placental inflammation and increased placental size (10) have been reported in clinical cohort studies. A large epidemiological study of pregnancy complications (n = 54,000 autism cases) recently reported that “placental pathology” was the most likely explanation for the observed association of preeclampsia and low birth weight with autism, as well as of a more general diagnosis of hypertension during pregnancy with autism (11, 12). Polycystic ovarian syndrome (PCOS), a maternal condition that affects the placenta and is associated with elevated prenatal androgens, has been found to increase the likelihood of autism in their offspring in multiple studies and cohorts around the world (13–15). Male placentas are producing more steroids and are more prone to complications, such as early miscarriage, pregnancy-induced hypertension and spontaneous preterm birth (16–18)

‘Generation R’ is a longitudinal birth cohort of almost 10,000 individuals in Rotterdam, the Netherlands that monitors their participants’ development, from pregnancy to adolescence. In this cohort, sex differences have been reported both in autistic traits (e.g., on the Social Responsiveness Scale - SRS), autism diagnoses, and in markers of placental function during pregnancy. Specifically for the latter, the levels of placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and plasminogen activator inhibitor (PAI-2) were all found to be lower in 1st -trimester maternal plasma of male pregnancies, even after controlling for placental weight differences between the sexes (19). PlGF and sFlt-1 have opposing regulating properties on angiogenesis, via activation and suppression of VEGF-related signalling respectively. These markers have been proposed for prenatal screening for a variety of conditions involving placental vascular health, such as gestational hypertension and preeclampsia (20). However,

it has not yet been examined if sex differences in these placental markers are associated with sex differences in neurodevelopmental outcomes, such as autism spectrum traits (21).

In this study we investigate whether these placental proteins and their sex-related changes throughout pregnancy are associated with the development of autistic traits or with an autism diagnosis in children of the 'Generation R' birth cohort. To this aim, we studied changes in placental markers in the 1st and 2nd trimester of pregnancy in male and female pregnancies. We hypothesize that sex differences in placenta marker may partially mediate sex differences in neurodevelopment, with male-specific hormonal patterns would be associated with higher autistic traits or a diagnosis of autism.

Methods

The cohort

This project utilised data that were collected as part of the 'Generation R Study', a prospective cohort of expectant women and their children, in Rotterdam, NL. The protocol and details of the study have been reported in detail in other publications. Participants have consented to use of their samples and clinical information for the identification of environmental or genetic parameters that contribute to developmental and health-related outcomes. The children of this cohort are followed-up regularly, with both in-person and questionnaire-based measures of their overall development.

For this analysis, only singleton live births were included (Fig. 1). These corresponded to deliveries that took place between April 2002 and January 2006. Other cohort characteristics, with a breakdown for fetal sex, are included in Supplementary Table 1. With regards to ethnicity, participating mothers and fathers reported parental national origin based on classifications recommended by 'Statistics Netherland' and this was further divided into groups for the purposes of statistical analysis (Supplementary Table 4). In addition to the initial written, informed consent by the participating mothers, this study was approved by the review board and the Medical Ethics Committee of the Erasmus Medical Centre.

Placental markers & clinical information

The concentrations of sFlt-1 and PlGF were measured on two separate occasions during pregnancy, at the end of the first and second trimester. Measurements were with immune electrochemoluminence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, The Netherlands) in ng/ml and pg/ml, respectively, were performed. Further details on the protocols of recruitment and sample processing have been previously reported (19). Processing and weighing of placentas after labour was conducted by specialist midwives and has also been described previously (22).

Demographic data on maternal age, ethnicity, educational level and clinical history were obtained through self-administered questionnaire at recruitment, with a response rate of 93%. Clinical information on the pregnancies, including data on birth weight, gestational age at blood draws and at birth, as well as on birth complications were obtained from medical records, completed by community midwives and obstetricians. Specifically, preeclampsia (PE) was defined according to international guidelines on blood pressure elevation (140/90 mmHg or greater), in combination with proteinuria after the 20th gestational week. Pregnancy-induced hypertension ('PIH') were defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy (23). Spontaneous preterm birth (from here on "preterm birth") was defined as non-induced delivery onset before the completion of 37th week of gestation. Designation of 'small for gestational age' (from here on "SGA") was defined as a sex and gestational age-adjusted birthweight below the 10th percentile. The pregnancy complications (preeclampsia, PIH, spontaneous preterm birth and SGA) were classified as placenta-related.

Autistic Traits

The score on the Social Responsiveness Scale (from here on "SRS") is derived from a questionnaire, comprised of items detailing social motivation, interaction, communication, and autism-related behavioural traits that are specific to the population in question. In this study, participating parents were invited to respond to an 18-item abridged version of the questionnaire for children, when their participants' children were 6 years old. Items were scored on a Likert scale; 0 (not true); 1 (sometimes true); 2 (often true); and 3 (almost always true. The abridged 18-item questionnaire has been previously described in published

Generation-R studies, and has correlation of over 0.93 to the full SRS and a Cronbach's α-value of 0.92 (24, 25). Higher scores indicate greater challenges with social communication and more autism-related behavioural traits. Additional details on recruitment and neurodevelopmental follow-ups of the children in 'Generation R' have been previously reported (26).

Statistical Analysis

Baseline characteristics were compared between males and females with Mann-Whitney U-tests or Chi-squared tests where appropriate. Multivariate imputations by chained equations (MICE algorithm) were used to impute missing values for demographic variables of the mothers and children. These included maternal age, BMI, educational attainment, and ethnicity, as well as birth weight and the age of SRS measurement for the children.

The concentrations for sFlt-1 and PlGF were compared between the sexes, via pairwise Mann-Whitney U-tests, as well as via multiple linear regression models. To facilitate statistical comparisons with the latter method, distributions of plasma-derived placental markers were first log-transformed as the dependent variable, with fetal sex, gestational age (at the time of plasma collection) and placental weight (at birth) as independent predictors. In addition, the change of PlGF concentration between the two time-points ("PlGF-e") was computed with the following model:

PlGF-e = ([PlGF]_t2 -[PlGF]_t1) / (gestational age_t2 - gestational age__t1),

with 't1' denoting the first and 't2' the second of time-points of PlGF measurement

With regard to autistic traits, z-scores of SRS scores were computed according to the properties of their distribution in the entire cohort (from here on "autistic traits"). Extreme outliers (n = 95) were then reduced to a maximum value specified by adding three times the interquartile range (IQR) to the upper quartile of the IQR (SRS z-score = 3.1).

For the association of placental proteins with autistic traits, every placental variable (including the rate of PlGF change - 'PlGF-e') was untransformed and studied separately in three multivariable regression models. Model 1 controlled for the sex and age of the child at the time of SRS scoring. Model 2 further controlled for the following cohort covariates: maternal age, maternal BMI in the beginning of the pregnancy, maternal ethnicity and maternal education level. Finally, Model 3 was additionally controlled for potential confounder variables that may also be considered mediators (and thus possibly constitute overadjustment); namely total birth weight (adjusted for gestational age at birth) and placental weight at birth.

Nominally significant results for Model 3 were further scrutinised in sensitivity analyses, that restricted the cohort to the following categories: First, in pregnancies of European maternal ethnicity, given previously reported differences in SRS scores of potentially cultural origin (27). Second, in pregnancies without any reported placental or other complications (PIH, PE, SGA or preterm birth). Third, in pregnancies and children without an autism diagnosis by age 6, in order to check if the observed effects were driven chiefly by diagnosed individuals or could be generalised to the undiagnosed population (Supplementary Table 3).

Results

Neurodevelopmental outcomes

The current analysis was restricted to children in the Generation R cohort (n = 7,293) with, placental marker measurements in maternal plasma at two time-points of pregnancy (Fig. 1). Scores for autistic traits, as measured on the SRS at age 6 (mean age = 74 months, SD = 5.8 months) were available in 3,469 children. Autistic traits differed significantly between the sexes, with males scoring significantly higher than females (Cohen's D = 0.31, $p < 0.0001$)(Fig. 2A). Autistic traits also correlated with the age of the child at the time of assessment, and the age of the mother (Supplementary Table 1).

Placental markers in maternal plasma

Placental markers showed varying degrees of change between the two time-points of measurement 1st and 2nd trimesters. sFlt-1 increased marginally between the 1st and 2nd trimesters (U-test, $p = 0.028$). On the contrary, PlGF increased more sharply between the two time-points of measurement (U-test $p < 0.001$). Levels of both placental-derived proteins correlated significantly

between time-points of measurement (PlGF: Pearson's r = 0.45, p < 0.0001; sFlt-1: Pearson's r = 0.72, p < 0.0001) and with each other in varying degrees (2nd trimester PlGF-sFlt-1: Pearson's r = 0.12, p < 0.0001)(Supplementary Fig. 1), as well as with a variety of maternal characteristics, including maternal age (for sFlt-1) and maternal BMI at the start of pregnancy (for both) (Supplementary Table 2).

Sex differences were assessed via pairwise Mann-Whitney U-tests (Table 1) and with multiple regression models that controlled for gestational age and placental weight (Supplementary Table 3). In the 1st trimester, as previously reported, all three placental markers were significantly lower in pregnancies of males (19). In the 2nd trimester, sFlt-1 levels continued to be significantly lower in the pregnancies of males, compared to females. On the contrary, PlGF levels in the second time-point were significantly higher in males (Fig. 2B). The rate of change in PLGF levels was significantly higher in males and this composite measure was introduced into further association analyses as an independent variable. By comparison, the change in sFlt-1 between time-points did not show any sex differences and was not included in further. analyses.

Table 1
Placental proteins in maternal plasma at two time-points of measurement (1st: mean = 13.5 weeks, 2nd: mean = 20.6 weeks), and a longitudinal variable corresponding to the rate of PlGF change between them. P-values for sex-difference correspond to pair-wise comparisons via U-tests.

1st trimester	N	Mean	Mean Males	Mean Females	p - value for sex difference
PlGF ng/ml	2912	0.054	0.053	0.056	0.025
sFlt-1 ng/ml	2910	5.65	5.35	5.98	< 0.0001
2nd trimester					
PlGF ng/ml	3469	0.231	0.236	0.224	0.0027
sFlt-1 ng/ml	3467	5.88	5.59	6.20	< 0.0001
Longitudinal					
PlGF - change	2627	0.025	0.026	0.024	0.0014
sFlt-1 change	2623	0.015	0.014	0.016	0.913

Association of placental markers with neurodevelopmental outcomes

There was no association of sFlt-1 levels at any single time-point, to the autistic traits of males or females.

PlGF levels, in the 1st and 2nd trimester, correlated to autistic traits in multiple regression models that controlled for sex and age at point of autistic traits ascertainment (Model 1), as well as cohort covariates (e.g. maternal age, BMI, educational attainment: Model 2). When including potential confounders (birth weight and placental weight: Model 3), this effect was significant in the 2nd trimester (β = 0.244 [SE = 0.112], p = 0.03) but not the 1st (Model 3: β = 0.645 [SE = 0.035], p = 0.068).

In sex-stratified analysis, higher PlGF levels were associated with more autistic traits in females in both the 1st and 2nd trimesters (1st trimester - Model 3: β = 0.882 [SE = 0.408], p = 0.031/ 2nd trimester - Model 3: β = 0.393 [SE = 0.143], p = 0.001). This was not significant in male-only comparisons when controlling for cohort covariates and confounders (Models 2 and 3).

The rate of increase in PlGF levels was positively correlated to the autistic traits in both males and females (Model 3: β = 2.19[SE = 0.91], p = 0.017). In sex-stratified analysis, this was more evident in females, with males showing a significant association in Models 1 and 2 (β = 2.37[SE = 1.21], p = 0.049), but not in Model 3, which controlled for the effects of birth weight and placental weight (β = 1.52[SE = 1.49], p = 0.305).

Table 2
Association of z-scores of child SRS Scores to placental protein concentrations in maternal plasma,. Model 1 covariates: age of child at SRS measurement. Model 2 covariates: age of child at SRS measurement, maternal age, maternal BMI in the beginning of the pregnancy, maternal ethnicity and maternal education level. Model 3 covariates: as in Model 2 with the addition of potential confounders; placental weight and birth weight-adjusted for gestational age at birth.

	MALES				**FEMALES**				**BOTH**			
1st trimester	N	β	SE	p	N	β	SE	p	N	β	SE	p
PlGF												
Model 1	1504	0.898	0.525	0.087	1408	**1.732**	**0.357**	**< 0.0001**	2,912	**1.38**	**0.31**	**< 0.0001**
Model 2		0.478	0.511	0.351		**1.081**	**0.352**	**0.0022**		**0.795**	**0.302**	**0.008**
Model 3		0.434	0.610	0.477		**0.882**	**0.408**	**0.031**		0.645	0.035	0.068
sFlt-1												
Model 1	1502	0.005	0.008	0.525	1408	0.006	0.005	0.213	2,910	0.006	0.004	0.198
Model 2		0.006	0.007	0.377		0.004	0.005	0.481		0.005	0.004	0.284
Model 3		0.006	0.009	0.479		0.007	0.006	0.288		0.006	0.005	0.258
2nd trimester												
PlGF												
Model 1	1804	**0.378**	**0.137**	**0.006**	1665	**0.386**	**0.117**	**0.001**	3,469	**0.384**	**0.090**	**< 0.0001**
Model 2		0.181	0.134	0.177		**0.235**	**0.115**	**0.041**		**0.210**	**0.088**	**0.017**
Model 3		0.10	0.18	0.570		**0.393**	**0.143**	**0.006**		**0.244**	**0.112**	**0.030**
sFlt-1												
Model 1	1802	0.004	0.005	0.469	1665	-0.001	0.004	0.751	3,467	0.001	0.003	0.738
Model 2		0.000	0.005	0.99		-0.003	0.004	0.407		-0.0014	0.003	0.629
Model 3		0.001	0.006	0.836		-0.002	0.005	0.731		-0.0001	0.004	0.984
Longitudinal												
PlGF-change												
Model 1	1367	**3.028**	**1.241**	**0.015**	1260	**2.981**	**0.971**	**0.0022**	2,627	**3.000**	**0.786**	**0.0001**
Model 2		**2.374**	**1.210**	**0.049**		**1.961**	**0.947**	**0.0387**		**2.067**	**0.765**	**0.007**
Model 3		1.525	1.485	0.305		**3.050**	**1.107**	**0.006**		**2.187**	**0.912**	**0.017**

Sensitivity analysis

Sensitivity analyses were performed by excluding potential confounders for autistic traits (e.g., specific ethnicities or an autism diagnosis) or for the levels of placental markers levels (e.g., pregnancies with placental complications). These analyses were consistent with the main effect for both PlGF levels in the 2nd trimester and the rate of PlGF increase (Supplementary Table 4).

Mediation analysis

Mediation analysis showed that the sex difference in autistic traits (higher SRS in males, Cohen's D = 0.31, $p < 0.0001$) was significantly mediated by PlGF levels in the 2nd trimester, with higher PlGF levels being linked to higher autistic traits in males

more than females (ACME: 0.0005, p = 0.002). The rate of PlGF-elevation also mediated part of the association of sex with SRS Scores (ACME: 0.004, p = 0.042). This was not found for sFLt-1 at either time-point, despite pronounced sex differences in its concentrations.

Table 3
Sex differences in 2nd trimester prenatal PlGF levels mediate a part of the sex differences in SRS scores at age 6.

Placental markers	***Mediation of the effect of sex on autistic traits***			
1st trimester	ACME (CI)	p-value of mediation	ADE (CI)	p-value of added effect
PlGF	-0.004 (-0.01–0.00)	0.10	0.26 (0.21–0.31)	< 0.0001
sFlt-1	-0.003 (-0.01–0.00)	0.24	0.26 (0.20–0.31)	< 0.0001
2nd trimester				
PlGF	**0.005 (0.001–0.01)**	**0.004**	0.24 (0.19–0.29)	< 0.0001
sFlt-1	-0.001 (-0.01–0.00)	0.86	0.25 (0.19–0.29)	< 0.0001
Longitudinal				
PlGF - change	**0.005 (0.00–0.01)**	**0.026**	0.24 (0.18–0.30)	< 0.0001

Discussion

This study investigates sex differences in placental markers of angiogenesis and their association with neurodevelopmental outcomes in the children.

The analysis focused on the levels of the placenta growth factor (PlGF) and the soluble fms-like tyrosine kinase-1 (sFlt-1), which are produced by the placenta and have opposing functions on angiogenesis, via activation or inhibition of VEGF signalling respectively. In addition, PlGF has been shown to induce proliferation of neuronal Schwann cells, to facilitate cortical expansion in rodents (28), and increase the permeability of the blood brain barrier (29).

These two placental markers were previously studied in the late 1st trimester and found to be significantly lower in the pregnancies of males (19). In this study, we find sex differences in their concentrations continue to be significant, independently of placental weight differences. Specifically, sFlt-1 continues to be significantly lower in males, with little change between time-points. However, the levels of the placental growth factor (PlGF), are significantly higher in males, due to faster increase between semesters. This is the first study to report on this significant sex difference longitudinally in the general population.

The rapid increase of PlGF into the 2nd trimester could be attributed to the effects of fetal androgens, which are rapidly elevated in males during mid-pregnancy, following the activation of the testes (30). Consistently, in cellular and human studies outside of pregnancy, PlGF levels have been found to correlate to the levels of steroid derivatives and DHEAS (31, 32). DHEAS is also significantly higher in the placentas of males, as shown via RNA-Seq in a large clinical cohort (16).

Clinically, very low concentrations of PlGF are considered a biomarker of preeclampsia (20). This is true in both male and female pregnancies and can be found as early as the 1st trimester. However, the significance of *high* PlGF levels ,and particularly during

mid-/late pregnancy, remains unclear. In the same longitudinal birth cohort, it has been shown that PlGF concentrations correlate to infant growth rates of body weight and head circumference, which are also higher in males, compared to females (33, 34).

In this study, we show, for the first time, that sex differences in PlGF levels are also linked to neurodevelopment. Specifically, we find that high levels of PlGF are associated to higher autistic traits in the children. This effect is more apparent in females, following stratification by sex, but also shown to significantly interact with sex and mediate higher autistic traits in males (Table 2). The lack of a significant linear association in the males-only analysis could then be attributed to a 'ceiling effect' in males, given their higher baseline PlGF levels in the 2nd trimester. In sensitivity analyses, this effect persisted in females, when autistic people or complicated pregnancies were excluded from the cohort (Supplementary Table 4).

Large epidemiological studies have previously associated placental dysfunction to a later diagnosis of autism (11). Smaller clinical cohorts have also shown differences in the morphology and shape of placentas in autism, compared to controls (9, 10). This is the first study to link placental markers to the autistic traits in the general population. The pathophysiological process for this effect remains unclear, as PlGF was not shown to affect the developing nervous system directly. The reported effect sizes for PlGF are small and indicate that other regulatory molecules may be more informative for autistic traits. However, it remains possible that high levels of PlGF may affect the fetal blood-brain-barrier, as shown in animal models (29), and potentially prolong exposure of the fetal brain to growth factors and steroids (35). This process would interact with biological sex, given baseline sex differences in steroid levels, placental function, and growth rates. Recent evidence in an animal model is consistent with this hypothesis, showing that placental steroids were sufficient to shape the social behaviour of mice, in sex-specific ways (36). More research would be needed in both humans and experimental models, in order to understand this phenomenon.

In addition, these findings could be interpreted as evidence of a 'male-type' shift in females with high autistic traits. Similar male-type patterns have been noted in autistic females' personality traits, facial structure, and brain structure (37–39). Autistic females also have higher prevalence in steroid-related symptoms and conditions (including PCOS and metabolic dysfunction) (13, 40, 41). In tun, these conditions have been variably linked to the effects of the placenta, which is hypothesised to 'programme' the developing endocrine and nervous system of the fetus (7, 42). Therefore, pregnancies that lead to higher autistic traits, may be linked to other 'male-type' shifts in physiology, which may include higher steroid levels, as previously shown (35), and influence lifelong health outcomes.

In conclusion, this study has shown that sex differences in placental functionality are associated to autistic traits in childhood. Specifically, male-type increases of PlGF in the 2nd trimester positively correlate to autistic traits in females. More research would be needed to understand the mechanisms by which fetal sex interacts with the placenta, as well as environmental and genetic risk factors for autism.

Declarations

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

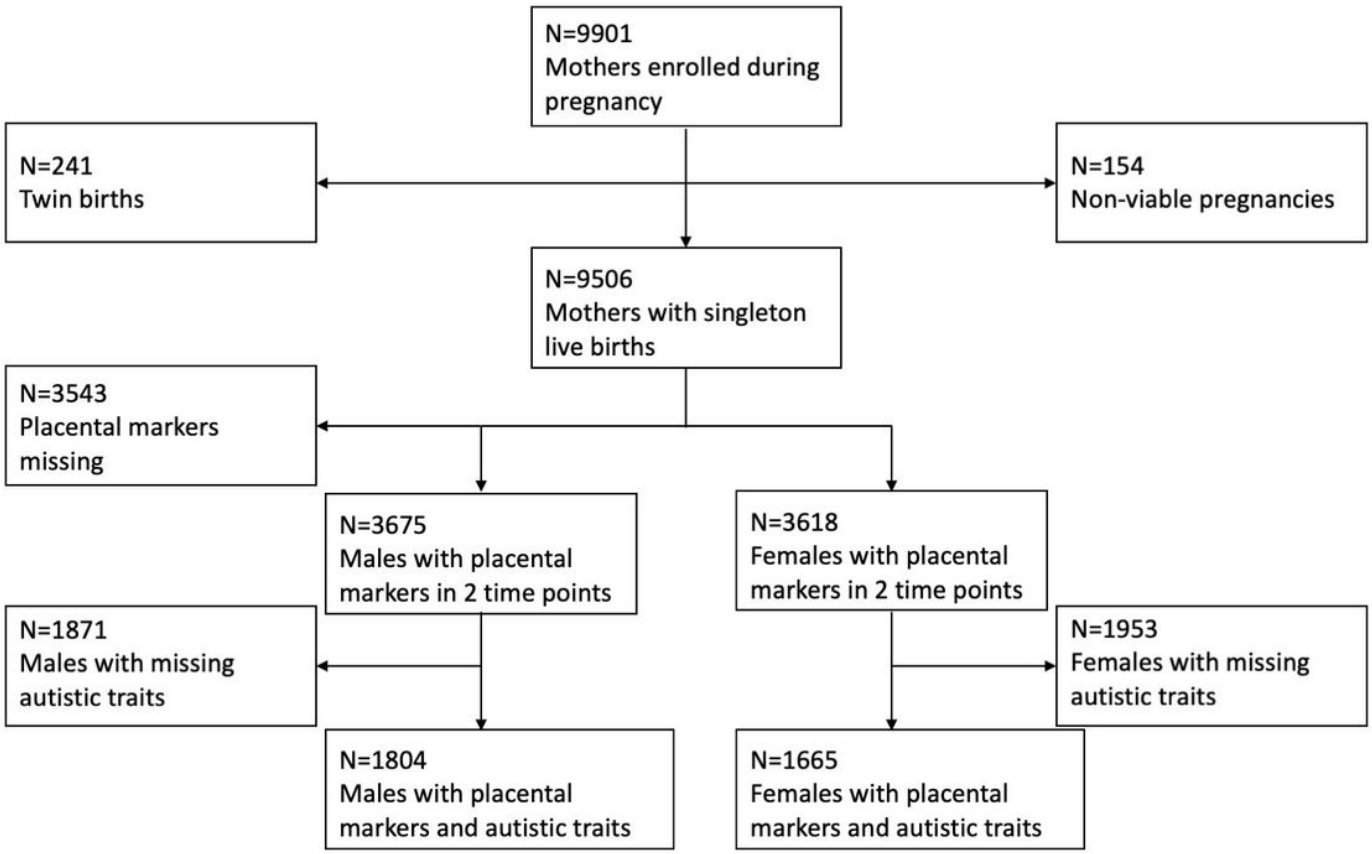

Figure 1

Flowchart of the study with the sample sizes used for comparison of placental markers in association with autistic traits and a diagnosis of autism.

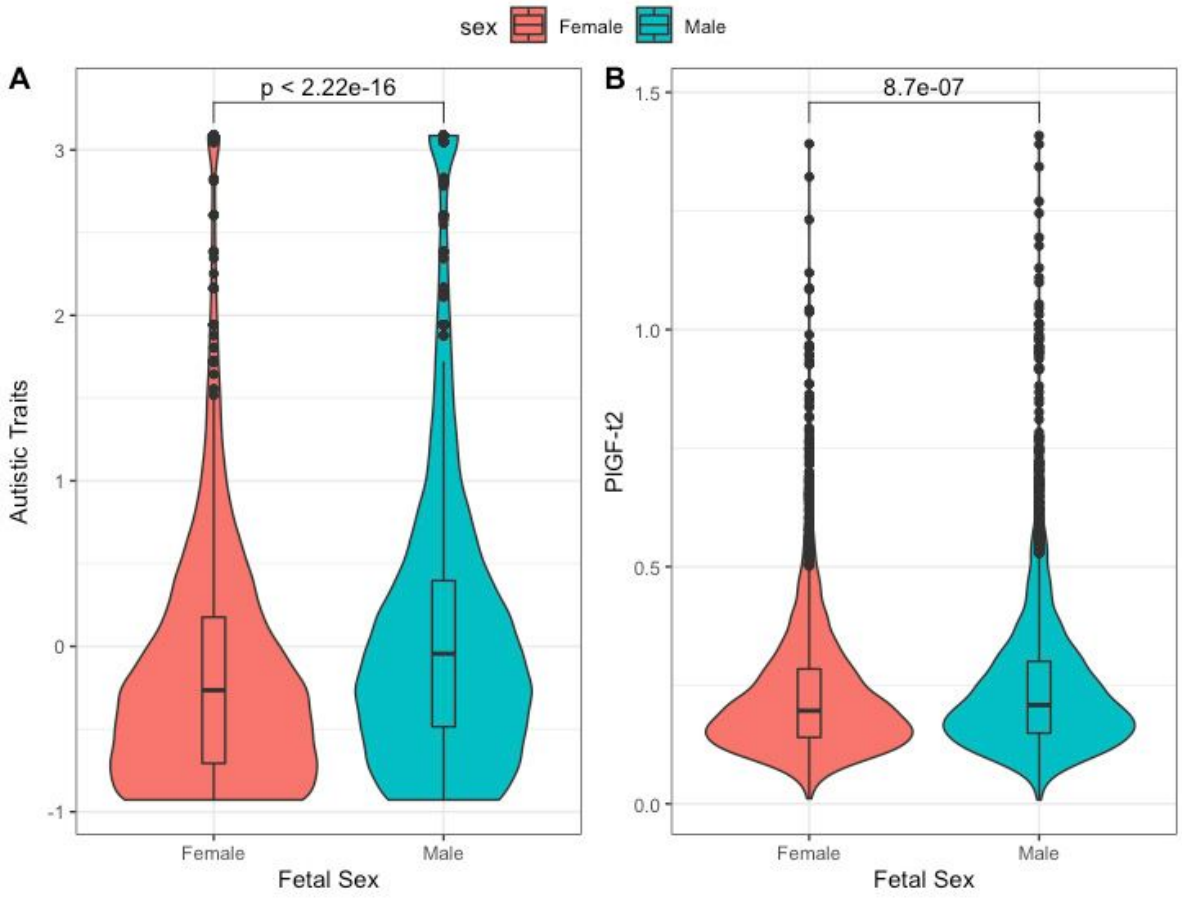

Figure 2

Males have significantly higher (A) autistic traits and (B) PlGF levels in maternal plasma at the 2nd trimester. Values are presented in z-scores. P-values are of U-tests of autistic traits and PlGF concentrations respectively.

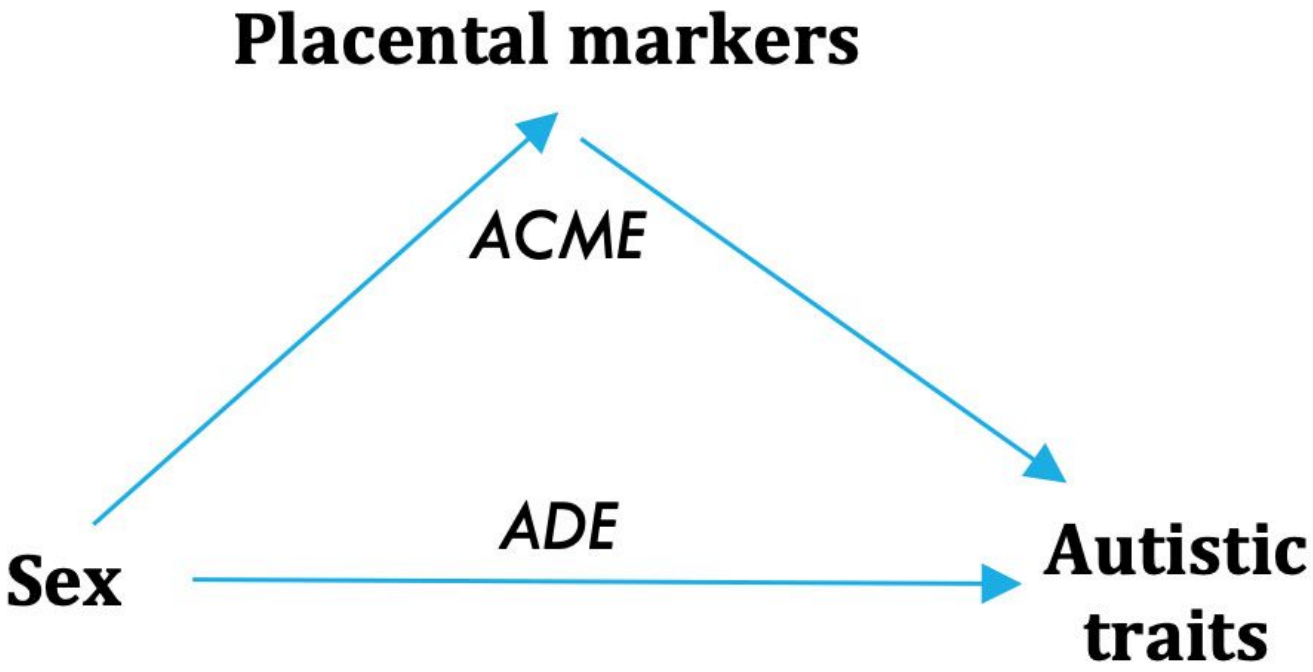

Figure 3

Mediation of placental markers on sex differences in autistic traits ACME: average causal mediated effect, ADE: average direct effect , CI: confidence interval

Supplementary Files

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- SupplementaryMaterial.pdf