Sex differences in human perinatal development and autism

Alexandros Tsompanidis

Department of Psychiatry
University of Cambridge

This dissertation is submitted for the degree of
Doctor of Philosophy

Peterhouse November 2021

Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Acknowledgements, the Preface and specified in the text. It is not substantially the same as any work that has already been submitted before for any degree or other qualification except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit of 60,000 words as set by the Degree Committee for Clinical Medicine and Clinical Veterinary Medicine.

Thesis Title: Sex differences in human perinatal development and autism

Alexandros Tsompanidis

Thesis Summary:

Autism is a neurodevelopmental condition that is more frequently diagnosed in males than females. To explain this, in 2014, the prenatal sex steroid theory was proposed. This extended the fetal testosterone theory, published in 2004. The prenatal sex steroid theory proposes that exposure to higher levels of prenatal sex steroids (e.g., prenatal androgens and estrogens) that are on average higher in male fetuses are associated with higher likelihood for autism and elevated autistic traits. This background literature is reported in Chapter 1. In this thesis, eight novel studies are reported that test and extend the prenatal sex steroid theory by investigating perinatal factors related to sex differences in physiology. **Study 1** (described in Chapter 2) reports a case-control analysis of steroid levels in the amniotic fluid of males who were later diagnosed as autistic, linked with the Danish Biobank (n = 98 cases, n = 177 controls). This included univariate analyses of both prenatal androgens and estrogens, as well as the aromatisation ratio. All estrogens, but not testosterone, on average were elevated in autistic males. **Study 2** (described in Chapter 3) reports a prospective cohort study (the Cambridge Ultrasound and Pregnancy [CUSP] study) of pregnant women and their infants in Cambridge (n=219), who were assessed for their autistic traits during pregnancy and late infancy. Steroid hormone levels were assessed in maternal serum. Estradiol levels correlated with both maternal autistic traits and the male infants' autistic traits, but there was no correlation with female infants' autistic traits. **Study 3** (described in Chapter 4) reports a large prospective cohort study in Rotterdam (Generation-R) that studied the levels of placental function markers in maternal serum (n=3469), their sex differences in the general population, their association with both autistic traits in childhood (assessed using the Social Responsiveness Scale - SRS), and with likelihood for autism in males. Male-like patterns in placental angiogenic markers, high placental growth factor (PlGF) and low soluble fms-like tyrosine kinase-1 (sFlt-1) levels, respectively correlated with higher autistic traits in females and an autism diagnosis in males. Chapter 5 describes Studies 4, 5, and 6, all based on a longitudinal cohort, the Cambridge Human Infant Longitudinal Development [CHILD] Study. This included prenatal (n=41) and postnatal (n=27) brain MRI imaging

and salivary testosterone measurements during mini-puberty. **Study 4** found that both male and female infants experienced transient increases in testosterone postnatally (2 to 6 months), but this did not correlate to their autistic traits at 18 months. **Study 5** focused on total brain volume and surface area in infancy, as well as rate of brain growth perinatally, all of which correlated negatively with the infant's autistic traits. **Study 6** found that this was driven by low volume in regions that show sex differences and are involved in face recognition. Chapter 6 describes two genetic studies, which found that autism-related genetic variance (rare and common variance respectively) overlaps with X-linked genes that show sex differences in the placenta (**Study 7**) and correlates with the genetics for early age of menarche (**Study 8**). Chapter 7 brings all of the findings from Studies 1 to 8 together to draw conclusions and consider limitations and future directions. Based on these analyses, I then propose a new theory on the role of the placenta in mediating sex differences in human perinatal development and autism.

Acknowledgements

I have been fortunate enough to have many people around me, who supported me in my doctoral research and during the writing of this thesis. I am deeply grateful to all.

First and foremost, I would like to thank my supervisors, Simon Baron-Cohen and Rosie Holt for their unwavering trust, patience, and respect they afforded me, even when faced with my Mediterranean character and occasional exasperation. Second, my advisors Topun Austin and Laura Blanken, who overcame so many bureaucratic hurdles, so I could explore my ideas.

In addition, I would like to thank my fellow doctoral scientists at the Autism Research Centre, Elizabeth Weir, Sarah Hampton, Tanya Procyshyn and Aicha Massrali, as well as the administrators Becky Kenny, Emma Baker, and Jo Davis, who helped make our work environment enjoyable and relaxing. In particular, I am grateful of the assistance of all of our interns and visiting students, Katie Maxwell, Alanna Shand, Leona Strauss, Eglė Padaigaitė, who helped enormously with the organising of our studies. A big thank-you to Ezra Aydin, who first welcomed me to the ARC and offered me hospitality.

My time in Cambridge wouldn't have been the same without Peterhouse and our wonderful MCR. My tutor Chris Lester, the Fellows and Master of Peterhouse, all ensured I had everything I needed during my time as a student and a new place to call home. There are so many friends I made at Peterhouse, and I hope we will continue meeting and reminiscing for years to come. I will also never forget Jenny's generosity, Catherine's festive company, Daniel's cooking and all the lessons in British culture from Olly.

I always wanted to become a researcher. But my journey from Greece to Cambridge has not been easy. I would be remiss if I did not thank the people who made this aspiration possible. First, my research supervisor and mentor in Athens, Despina Sanoudou, who entrusted a young, naïve undergraduate student with his own research project and instilled a sense of endless possibility and academic inspiration. Second, my dear friend Lorenzo Martini, who first accompanied me to Cambridge, listened to my many theories and taught me so much I didn't know. Third, all my Greek friends who accepted me as I am and still share stories over wine, every time I return.

Last but not least, I would like to thank all my family in Greece. Without their constant support, I wouldn't have made it far. Μαμά, μπαμπά και αδερφέ, ευχαριστώ για όλα.

Preface

The first two studies presented in this thesis have been peer-reviewed and published in scientific journals. Each of these two publications have been authored by several researchers, who worked collaboratively to set up and complete the studies, as well as address the reviewers' comments. As the first author in both journal articles, I conducted all statistical analyses, as well the writing of the text and interpretation of the results. The last authors of these two studies, Prof Simon Baron-Cohen, Dr Rosemary Holt, Dr Alexa Pohl, supervised my analysis and reviewed the text, which formed the basis of two chapters in this thesis:

Study 1 (presented in Chapter 2): published in 'Molecular Psychiatry', July 2019, with the title "Foetal oestrogens and autism".

Study 2 (presented in Chapter 3): published in 'Molecular Autism', July 2021, with the title "Maternal steroid levels and the autistic traits of the mother and infant".

The other Studies in this thesis have not yet been published, but specific findings have been presented in conferences (Study 3 in Chapter 4) and reviewed by my supervisors, Prof Simon Baron-Cohen and Dr Rosemary Holt, as well as by my thesis advisors: Dr Laura Blanken, Prof Henning Tiemeier, Prof Topun Austin, and Dr Varun Warrier.

For Studies 5 and 6, Luca Villa and Roger Tait conducted the pre-processing, clearing and segmentation of the structural brain imaging data (prenatal and postnatal), which I then analysed in association with clinical and neurodevelopmental variables (presented in Chapter 5).

Table of Contents

List of Figures

List of Tables

Chapter 1
Introduction

1.1 Autism in the population

Autism is a condition that is characterised by behavioural patterns that include challenges in social interactions and communication, as well as unusually restricted interests, and repetitive behaviours (RRBs). These two domains (social and non-social) represent the 'core' features of autism, as described in the Diagnostic and Statistical Manual of Mental Disorders - Version 5 (American Psychiatric Association 2013). In addition, the condition often presents with a combination of 'associated' features, such as resistance to change, social attention deficits, language delays, sensory hypersensitivity and co-occurring anxiety and depression (Croen et al. 2015).

Since its first description as "childhood schizophrenia", autism, as a behavioural construct, has shifted and changed definitions, reflecting societal attitudes, the need for disability advocacy and the availability of mental health provisions. In 1978, 75% of autistic people had co-occurring intellectual disability (ID) (Rutter 1978) whereas by 2021 only 25% of autistic people have co-occurring ID. This reflects growing awareness of autism without ID, or what used to be called Asperger Syndrome, which was only introduced in DSM in 1994. Autistic traits have also been defined with a variety of psychometric tools, forming a wider autism spectrum of behaviour and cognition in the general population (Baron-Cohen et al. 2001). Whilst diagnosis of autism remains categorical, this spectrum had been proposed since 1988, in order to account for the heterogeneity in severity, behaviour and the ranging comorbidities that were observed in autistic people. After publication of the fifth edition of the DSM in 2013, all related diagnostic labels ('Asperger's syndrome', 'Pervasive Developmental Disorder' etc.) were dropped in favour of the single umbrella term 'autism spectrum disorder'. A clinical diagnosis of the condition is generally given under a liability-threshold approach, in cases where these traits accumulate and interfere with daily functioning and thus necessitate the provision of services that can support learning needs, disabilities and improve quality of life.

These changes have led to more people receiving a diagnosis, with prevalence increasing from 4 per 10,000 in the 1970s to 1 to 2% in most countries today (Stoltenberg et al. 2010). Increased awareness of the condition and complex sociological factors may also be contributing to this increase (Madigan et al. 2019). The social advocacy movement for neurodiversity has also gained momentum worldwide, rightly arguing for the representation and inclusion of autistic people in every part of society including in the workplace (Baron-Cohen 2017). In addition, increased awareness of gender differences in autistic people's behaviour, as well as a possible implicit gender bias in some of the of instruments used to detect and diagnose autism, has resulted in more diagnoses in girls and women (Hull et al. 2020). Addressing the latter has also led to a reduction in the sex ratio of clinical diagnoses, which dropped from 4:1 males to females in 1978, two decades ago, to an estimate of 2:1 (males to females) more recently (Loomes et al. 2017).

The unifying of the previous diagnostic categories has also increased the phenotypic heterogeneity of the condition (Mottron and Bzdok 2020). While a diagnosis still requires a patient to exhibit both social challenges and RRBs, these may vary from person to person, but also depend on age, gender, socioeconomic background, and the co-occurrence of intellectual disability. With regard to gender differences in particular, these appear to affect non-social behaviours and psychiatric comorbidities, rather than the severity of the social challenges (Kaat et al. 2021). For example, young autistic girls may not show restricted interests in vehicles or spinning objects, but rather fixate on specific colours and animals (e.g. horses). In adulthood, autistic women also appear to 'mask' many aspects of their condition in social scenarios, more often than autistic males (Hull et al. 2020). This appears to lead to greater severity of comorbid depression and anxiety in women compared to men (Lai et al. 2019). In addition to these behavioural variations, diagnostic criteria may also differ in each country. In the UK, the NICE guidelines specify that autistic traits on their own are not sufficient for a diagnosis, unless these infringe significantly on a person's quality of life and learning potential (National Institute for Health and Care Excellence 2012). However, this link between an autism diagnosis and disability is now being challenged, with many advocating the inclusion of people without concurrent disabilities or learning difficulties, but who still feel that their 'neurodiverse' personality and behaviour set them apart to their peers (Baron-Cohen 2017). For this reason, the evaluation of autistic traits in the general population may be a better way to

study the condition. Several questionnaires have been validated to this extent and designed to be specific to developmental stages (e.g. infancy or adolescence), as well as to be as gender neutral as possible (Appendix 1).

During the last two decades, there has also been more research into the physiological origins and developmental nature of the autism. There is now growing recognition that autism extends beyond brain and behaviour and includes a series of physiological cooccurring conditions, such as food intolerances or gastrointestinal pain in as many as 70% of autistic people; much higher than epilepsy (at 15%) or attention deficit and hyperactivity (at 40%) (Holingue et al. 2018; Croen et al. 2015). In genetics, twin-heritability studies, exome sequencing studies and recent genome-wide-association studies have all confirmed that most of the liability in autism can be attributed to genetic factors (Grove et al. 2019; Gaugler et al. 2014). This is also true for the wider spectrum of autistic traits in the undiagnosed population (Warrier et al. 2019). Larger genome-wide association studies (GWAS) are needed to better capture common genetic variance, but the findings on heritability are consistent with the earliest studies that reported both an overabundance of *de novo* copy-number variants (Sebat et al. 2007) and high monozygotic twin-pair concordance rates relative to dizygotic pairs (Le Couteur et al. 1995). In short, autism is a genetic and neurodevelopmental condition that affects many aspects of development from conception onwards.

Despite this large genetic component, it is also evident that autism affects males and females differently and with different frequencies. The sex ratio remains consistently skewed towards males, even after diagnosing more girls and women with autism and recognition of compensatory mechanisms, such as 'camouflaging' (Lai et al. 2020). Research is ongoing to understand this sex difference in liability. It remains unclear how a condition with a largely genetic aetiology, that is largely independent of the X-chromosome, can affect more males than females (Werling and Geschwind 2015). The main framework for understanding this suggests that typical sex differences increase autism likelihood* in males more than females (Baron-Cohen et al. 2011). Simon Baron-Cohen first reported that two dimensions of traits that are distributed differently in males and females, are also shifted towards male profiles in autistic individuals (Baron-Cohen 2001 ; 2002). These dimensions are 'empathising' (E) and 'systemising' (S), referring respectively to the cognitive drives to identify and respond to people's mental states (i.e.,

'theory of mind'), or to analyse and understand the workings or mechanics of rules-based systems (Baron-Cohen et al. 2003).

Since then, the resulting "extreme male brain" (EMB) theory has received both praise and criticism. Critics argue that the EMB theory contributed to the stereotype that autism is exclusively a condition found in males. However, this is not in line with the focus that the theory placed in understanding autism in both sexes and particularly in autistic females. The theory in fact proposed that both autistic males and females are similar in showing a trend towards S>E (systemizing more than empathizing). Based on sex differences on average in undiagnosed individuals, this can be interpreted as a 'male-like' shift specifically in the distributions of E-S traits. Critics also erroneously claim that the EMB theory held back diagnosis of autism in females. Yet, in the two decades following the theory's publication, diagnostic rates in females have increased steadily and more rapidly than in males, leading to a reduction in the sex ratio. The E-S/EMB theory has now been tested (Baron-Cohen et al. 2014) and replicated in a large population of both diagnosed (n = 36,000) and undiagnosed males and females (n = 600,000), with findings in line with all 10 of the theory's predictions (Greenberg et al. 2018).

It remains unclear if the S>E profile can largely be attributed to physiological "masculinisation" processes (such as the effects of prenatal sex steroid hormones) or to postnatal social variables and how both of these variables may interact with other established autism likelihood factors (e.g., genetic predisposition, pregnancy factors).

1.2 The fetal testosterone theory

To shed light on these questions, the fetal testosterone theory was first proposed, which argued that factors that determine sex differentiation in early development (i.e. fetal testosterone) may also increase the likelihood for autism and related traits (Baron-Cohen et al. 2004). This hypothesis was formulated based on studies in both humans and non-human animals.

---

* *In this thesis the term 'likelihood' is used, instead of 'risk', to avoid the implication that autism is a disorder without positive attributes or that it ought to be mitigated or prevented.*

First, testosterone shapes brain development *in utero*, and Geschwind and Galaburda had previously proposed that prenatal testosterone may drive sex differences in neurodevelopmental disorders such as language delay, in brain lateralisation and handedness, and in spatial ability (Geschwind and Galaburda 1985). Second, use of amniocentesis in human pregnancies (to screen for chromosomal aneuploidies) allowed clinical researchers to confirm that male fetuses undergo a brief surge in amniotic testosterone levels, between the 1st and 2nd trimester of gestation (Welsh et al. 2014). This was attributed to the activation of the fetal testes, following their descent from the abdomen, and thought to facilitate the differentiation of the male urogenital tract. This time-point of gestation in which males on average are exposed to elevated androgens is called the 'prenatal masculinisation window' (PMW) and its disruption has been linked to medical conditions like hypospadias and cryptorchidism (Aaronson et al. 1997; Rodprasert et al. 2020).

However, linking the effects of the PMW to neurodevelopment and behaviour in humans has proven to be less straightforward. In rodent models, paradigms involving removal of gonads and exogenous administrations of hormones uncovered a similar sex differentiation window, occurring immediately after birth. McCarthy et al have tied early steroid exposure to male-like traits, including mating behaviour, aggression and sociability (Margaret M McCarthy 2008). Interestingly, the main 'masculinising' agent in rodents is not testosterone, but rather estradiol that is aromatised from testosterone in the brain (Margaret M. McCarthy 2009). McCarthy's group have also uncovered the paracrine and cellular properties of the sex differentiation system in rodents, showing that estradiol can regulate the rates of neuronal proliferation/apoptosis, in region-specific ways, utilising the brain's immune system, in the form of prostaglandins and glial cells (Hoffman et al. 2016; McCarthy and Wright 2017)

Primates and humans rely on testosterone for 'masculinising' the brain and body, rather than its aromatised derivative, estradiol. This is made clear in humans born with rare genetic forms of complete androgen insensitivity, due to deleterious mutations in the androgen receptor (Hughes et al. 2012). Individuals with this condition are chromosomally male (XY), have typical or slightly elevated estradiol levels, feminised external genitalia, and undescended gonads, which are often only discovered in puberty, because of amenorrhoea. On the other hand, in even more rare cases of genetic aromatase

deficiency in XY-individuals, the external genitalia, sexual orientation and behaviour during childhood all appear to be typically male (Rochira and Carani 2009; Chen et al. 2015).

The first study to test the fetal testosterone theory was organised in Cambridge by Baron-Cohen and his team. This was an ambitious longitudinal project, based on a prospective cohort of pregnant women who had underwent amniocentesis and agreed to follow-ups of their children for two decades or more. The participants were recruited when they consented to amniocentesis as part of their clinical care and gave permission for the remaining amniotic fluid to be stored and analysed for prenatal testosterone levels. All children were followed up at multiple time-points and their parent was asked to fill in a series of questionnaires about their child's cognitive development and invited to attend appointments for in-person and cognitive performance assessments. Individuals later diagnosed as autistic were excluded from the analysis, as the sample size (approximately n = 600) did not have the statistical power for a case-control analysis.

Instead, the project focused on linear models that regressed prenatal testosterone levels in amniotic fluid, in relation to the continuous, behavioural traits or psychometric scores of the children, as rated by their parents. These studies largely confirmed the initial hypothesis. Amniotic testosterone was positively associated with systemising, attention to detail and restricted interests in childhood and was negatively associated with empathising, eye-contact, vocabulary development and theory of mind (Knickmeyer et al. 2005; 2006; Auyeung et al. 2006; Chapman et al. 2006). Amniotic testosterone levels were also associated positively with autistic traits, in both infancy and childhood, as measured by two separate psychometric tools that corresponded to each developmental time-point (Auyeung et al. 2009; 2012). Although participants did not overlap completely between the different studies, the cohorts were not completely independent either, so these studies cannot be considered independent replications of the same hypothesis.

To address potential ascertainment bias stemming from amniocentesis, an independent cohort of children and adolescents with congenital adrenal hyperplasia (CAH) was recruited and assessed for their autistic traits. CAH is genetic condition that predisposes individuals to high levels of adrenal hormones that also include testosterone. Females with the condition often show virilisation and ambiguous genitalia, because of a fetal increase in their testosterone. The first study in this population reported a significant

result, showing that females with CAH had significantly higher autistic traits during adolescence via a one-tailed t-test analysis (Knickmeyer et al. 2006).

Independent research groups have also attempted to replicate these findings on autistic traits. However, an independent study that included amniotic testosterone, as well as a population with CAH, utilised a different outcome measure of autistic traits, called the CAST (Childhood Autism Spectrum Test). No significant differences in autistic traits were found in children with CAH and no association of amniotic testosterone levels with CAST scores was found in multiple regression models, that controlled or stratified for sex (Kung, Spencer, et al. 2016).

The same research group revisited the theory and attempted to approximate androgen exposure in a larger paediatric cohort in Cambridge, rather than assay for fetal testosterone directly. This approximation was based on measuring of the anogenital distance (AGD) by specialist paediatric nurses during early childhood (Kung et al. 2021). The AGD roughly corresponds to the length of the pelvic floor, between the anus and genitals, and is one of the most consistently sexually dimorphic biometric measurements immediately after birth, as well as during prenatal development (Aydin et al. 2019). As yet no evidence in humans has confirmed that AGD correlates to prenatal testosterone levels in both/either males or females, although some evidence for this exists in postnatal life and in animal models (Zhou et al. 2016; Hotchkiss et al. 2007; Sanchez-Ferrer et al. 2017). In the study regarding autistic traits, the authors used the children's version of the Autism Spectrum Quotient (AQ) as an outcome and reported no association with the children's AGD. However, when the AQ was graded on a Likert scale (rather than a binary grade for each item), a significant association was noted in the combined cohort of both males and females, but this was not significant after controlling for sex. Once again no interaction term with sex was accounted for in their models (Kung et al. 2021).

With amniocentesis being gradually phased out, in favour of non-invasive prenatal screening procedures, the potential to replicate the original studies is disappearing. Different kinds of clinical samples have been used but these may not capture the fetal circulation as closely as amniotic fluid. For example, a series of studies using cord blood have also reported mixed findings, in terms of a link between steroid hormones and later autistic traits. Simple linear regression models did not find a link in longitudinal cohorts sampled from the general population (Whitehouse et al. 2012; Jamnadass et al. 2015), or

in enriched cohorts of siblings of autistic children. However, a significant interaction was noted in the latter group, in cases where the autistic sibling was female (Park et al. 2017). Cord blood is collected after birth though and may be confounded by both the process of labour, as well as include a mixture of fetal and maternal serum. In addition, the period of sampling does not correspond to the PMW, so these studies may not be considered a direct test of the prenatal testosterone theory, which focuses on the prenatal masculinisation process.

In sum, the evidence for the prenatal testosterone theory remains mixed. However, most studies and the formulation of the theory itself relied on a few particular assumptions:

1. That testosterone alone is responsible for the sex differentiation of the brain prenatally. Testosterone is only one of the androgens that may act via the androgen receptor and change gene expression. Others include androstenedione and to a certain extent, DHEAS. Interestingly, the former has been found to be elevated postnatally in autistic adults (Ruta et al. 2011). In addition, sex differentiation need not rely solely on androgens or the downstream effects of steroid hormones, as sex chromosomes and growth rates may also contribute to these developmental differences.
2. That a single measurement of the concertation of testosterone can convey information on cumulative exposure. However, the endocrine system during pregnancy is complex and multi-factorial (Makieva et al. 2014). Steroid concentrations can change rapidly within a few days, as evidenced by the PMW. Diurnal rhythms also add considerable variance within each day.
3. That the hypothesised association of prenatal testosterone levels with autistic traits will be linear in nature and affect both males and females in the same way. Most of the studies above featured statistical models that controlled for any sex differences in linear models (with autistic traits as the outcome). But this statistical measure may miss significant differences in the way hormones affect the brains of males and females. In addition, particularly in males, testosterone may not lead to "masculinised" physiology in a linear way. In fact, the effects of testosterone are often characterised by 'ceiling effects', whereby a minimum concertation that is present in most males, is sufficient to create an effect, with further increases in concentration reaching a plateau in terms of outcomes (Breedlove 2010).

4. That any differences in steroid hormone exposure can be attributed to the PMW and are fetal in origin. Yet almost all steroid hormones increase during gestation, driven simultaneously by maternal, fetal and placental synthesis of steroids and their precursors, in both males and females (Makieva et al. 2014).
5. That the genetic liability for autism and the fetal hormonal environment act additively to increase autism likelihood, rather than interact. The target genes of testosterone in developing neurons *in vitro* show a statistically significant enrichment for autism-related genes (Quartier et al. 2018). But other studies have failed to find an enrichment for autism genes in the regulatory networks of steroid levels (Mitra et al. 2016), indicating that genetics and steroid hormones may not be additive, but rather interact in more complex ways, in males and females, in their role on autism likelihood.
6. That investigating the wide distribution of autistic traits in undiagnosed individuals would be informative for clinical autism itself.

In order to address many of the assumptions above, Baron-Cohen's group, decided to test the theory, for the first time, in amniotic fluid samples of autistic individuals (Baron-Cohen et al. 2015). This was made possible in Denmark, where a national psychiatric registry and a national biobank are linked via unique patient identifiers. Thus, mothers consented to have their amniotic fluid samples prospectively linked to their child's later autism diagnosis. These were matched to undiagnosed controls, corresponding to the same time period and matched for all relevant variables (birth order, gestational age, sex of fetus, maternal age, birth weight, APGAR score, etc). Furthermore, the assays were not restricted to testosterone alone, but included a series of steroid hormones that were adjacent on the Δ4 synthesis pathway (progesterone, 17 alpha hydroxyprogesterone, androstenedione, testosterone), as well as cortisol. As expected, steroid hormones correlated significantly with each other, for each individual. Unsupervised principal component analysis confirmed that a single 'steroidogenic' factor, derived from all assayed hormones, was elevated in males diagnosed with autism. A low number of females with autism prevented a sex-stratified analysis or the study of sex-by-diagnosis interactions.

Since then, a series of other clinical studies of pregnancies that led to autism have confirmed that a series of prenatal steroids, rather than testosterone alone, are

associated with autism likelihood. A large study that collected the prenatal test results of n=2586 cases in California, found that low levels of estriol, as well as atypical levels of the human choriogonadotropin (both too low and too high) were associated with autism in the children (Windham et al. 2016). Human choriogonadotropin (hCG) is a placental-derived hormone that induces steroid synthesis, similarly to GnRH. A subsequent case-control comparison of estrogens in maternal serum reported higher levels of estradiol (Bilder et al. 2019) in pregnancies of autistic people. Finally, a novel study of steroid levels in the fetal meconium reported that a series of steroid hormones (including androgens like androstenedione) were elevated in the siblings of autistic children and that this correlated with their autistic traits in infancy (Terloyeva et al. 2020). This source tissue was considered to be more informative than cord blood, as meconium reflects cumulative exposure throughout gestation, rather than a brief moment in time.

The findings from Danish Biobank and the ones that followed it, reinforce the original hypothesis of a prenatal steroid component for autism likelihood. However, they did not provide evidence for an interaction with fetal sex or conduct a univariate case-control comparison for testosterone in amniotic fluid. Moreover, the findings added complexity to the original hypothesis by implicating many different kinds of prenatal steroids, necessitating a wider clinical assessment of the endocrine system in autism.

1.3 Autism and brain development

Autism is neurodevelopmental condition that is diagnosed based on behavioural observations. Years of research have uncovered relative consistency in its behavioural and genetic architectures (Meng Chuan Lai et al. 2020a; Pinto et al. 2014). However, the condition is still lacking a common neurological phenotype or a biomarker that can easily be ascertained in a clinical setting and particularly during prenatal development.

Enlarged total brain volume has been consistently observed in infants later diagnosed with autism (Courchesne, Carper, and Akshoomoff 2003; Courchesne et al. 2007). A landmark series of longitudinal studies by Hazlett and colleagues identified that this enlargement begins as early as 12 months of age (Heather Cody Hazlett et al. 2011; H C Hazlett et al. 2017). Prenatally, other studies have reported atypical prenatal head growth

trajectories after 22 weeks gestational age (GA) in children later diagnosed with autism (Abel et al. 2013; Bonnet-Brilhault et al. 2018). Early brain overgrowth is also consistent with post-mortem findings in autistic children and adolescents showing deficits in synaptic pruning (Tang et al. 2014).

In terms of brain regions, increased amygdala volume (Schumann et al. 2010), prefrontal cortex volume (Courchesne et al. 2011) and a larger cerebellum (E. B. E. Becker and Stoodley 2013) have all been separately implicated in autism. The functional significance of these structural differences are unclear but many studies have attempted to study correlations to specific autistic traits, such as restricted interested and behaviours in 3-year-old children (Pote et al. 2019). In brief, differences in cortical thickness between cases and controls are largely heritable and thought to reflect differences in synaptic efficiency, long-range connectivity and firing patterns (Panizzon et al. 2009). Indeed, there is genetic overlap between autism and the determinants of cortical thickness across the entire brain (Romero-Garcia et al. 2019). Similarly, differences in the volume of the amygdala could also account for associated features in autism, including social anxiety and sensory overload (S Baron-Cohen et al. 2000).

Studies of brain structure have also attempted to link specific regions to autistic traits and the effects of sex steroid hormones. The same longitudinal cohort which was used to study the 'fetal testosterone' theory in Cambridge, was also profiled with MRI in childhood and a correlation was found between amniotic testosterone levels and the volume of brain regions relating to theory of mind, such as the superior temporal sulcus.

More recently, testosterone was shown to affect the development of human stem cell-derived brain organoids, leading to overproliferation of excitatory neurons, over inhibitory neurons (Kelava et al. 2022).

On the other hand, estradiol in animal models was reported to regulate GAD (the rate-limiting enzyme in the formation of GABA), increasing its levels in the arcuate nucleus and inducing the stellation of neighbouring astrocytes (M. M. McCarthy et al. 1995; Blutstein et al. 2009; Mong, Nuñez, and McCarthy 2002). Similar observations have been made in rhesus macaque (Noriega et al. 2010) and in neurons of the human neonatal hypothalamus (Perrot-Sinal, Auger, and McCarthy 2003).

In brief, these lines of evidence would be consistent with various reports over the years of an imbalance between excitation and inhibition in autism, favouring the latter over the former, and potentially leading to symptoms such as epilepsy or sensory overload in autism (E. Lee, Lee, and Kim 2017). Other factors, such as cortical brain overgrowth and dysfunction of the amygdala have also been proposed as potential biomarkers for the condition. However, given the condition's considerable behavioural and genetic heterogeneity, it is unlikely that a single, neurobiological cause will ever be identified. Yet the condition's consistent sex ratio and likely prenatal onset both suggest that sex differences in the womb may be significant determinants of liability.

1.4 Autism as a 'steroidopathy'

Individual hormones and neurotransmitters have often been targeted as potential treatments of autism symptoms (e.g., melatonin for insomnia)(Rossignol and Frye 2014) or parsimonious ways to understand the underlying function of the autistic brain (e.g. serotonin, excitation/inhibition imbalance etc)(Muller et al. 2016; Lee et al. 2017). Bringing all these findings together has been challenging. Finding a common denominator of pathophysiology may be improbable, given the considerable phenotypic variance in the condition itself. Yet, the steroid hormone system and its regulation via the hypothalamus, may provide a framework to understand at least a subset of the symptoms associated with autism. For example, both melatonin and serotonin synthesis may be linked to steroids such as estradiol, via their interaction on the hypothalamus (Chowdhury et al. 2010) or genetic effects on enzymes such as monoamine oxidase and aromatase (Epperson et al. 2014; González et al. 2007). In addition, estradiol interacts with the GABAergic system to regulate the rates of neuronal inhibition at the synapse (Mukherjee et al. 2017).

Consistently, studies show that steroid-related comorbidities symptoms are more common in autistic populations and particularly in autistic women. This has been found in at least two independent populations (the UK and Israel), via several self-report questionnaires (Pohl et al. 2014; Simantov et al. 2021). Autistic women reported a series of symptoms at higher frequencies than the general population, including irregular menstrual cycles, dysmenorrhea, atypical puberty onset and acne. Hyperandrogenic

symptoms also correlated to the autistic traits of the wider population, independently of age or BMI (Simantov et al. 2021).

Besides self-report data, epidemiological studies have also revealed higher rates of polycystic ovary syndrome (PCOS) in women with autism (Cherskov et al. 2018). Interestingly, history of hirsutism and PCOS are also more frequent in mothers of children with autism. The latter finding has been replicated in very large studies and in different populations (Sweden, the UK, Finland and Israel), as well as in a meta-analysis (Cherskov et al. 2018; Kosidou et al. 2016; Berni et al. 2018; Chen et al. 2020; Katsigianni et al. 2019). This link was independent of other metabolic symptoms associated with PCOS or familial likelihood of autism, but in one of the studies an interaction with the sex of the child was noted, with girls showing higher ORs than boys, in the presence of equivalent symptoms in the mother (Cesta et al. 2016; 2020).

Steroid regulation is not the only endocrine system to be affected in autistic individuals. Most epidemiological studies report an overabundance of metabolic comorbidities, often secondary to obesity and a sedentary lifestyle (Croen et al. 2015). Less is known for adult long-term health and for the causes of elevated mortality, that are sadly seen in this population (Hirvikoski et al. 2016). Social and psychogenic causes (e.g., elevated anxiety or reduced access to medical assistance) are difficult to disentangle from an underlying endocrine pathology. But these clinical findings are consistent enough to warrant further attention.

Several studies have implicated genetic variance relating to steroid regulation. For example, aromatase and the estradiol receptor (*ER-β)* are both down-regulated in the post-mortem brains of autistic individuals (Crider et al. 2014). Polymorphisms in the *ER-β* were significantly associated with AQ (Autism-Spectrum Quotient) scores in a candidate gene study (Chakrabarti et al. 2009). In addition, *RORa*, a transcriptional activator of aromatase, is under-expressed in both lymphoblastoid lines, as well as post-mortem samples of autistic people (Sarachana and Hu 2013). As mentioned before, the target genes of testosterone in developing neurons *in vitro* also shows a statistically significant enrichment for autism-related genes (Quartier et al. 2018). Moreover, a study that created polygenic risk scores (PRS) for markers of physical sex differentiation (e.g., facial masculinity), as well as PRS for hormone levels, found that these correlated to the PRS for reduced social functioning in a large cohort of autistic and non-autistic

individuals (the SPARK, n>9400)(McKenna et al. 2021). Finally, a high-throughput functional analysis of all implicated molecules in autism revealed that both aromatase and the estrogen receptor (but not androgens) represented two important nodes in the molecular networks identified by the analysis (Diaz-Beltran et al. 2017).

In accordance with findings implicating steroid hormones, exposure to endocrine-disrupting chemicals has also been implicated to adverse neurodevelopmental outcomes. These include environmental pollutants, such as very fine pesticide-related particles (R. Raz et al. 2015; Pagalan et al. 2019), as well as more complex compounds that disrupt steroid pathways (Long et al. 2019). However, more recent meta-analysis for the latter remains inconclusive, when assaying the levels of these chemicals in maternal serum (Hamra et al. 2019).

Although only one study has directly implicated fetal cortisol levels in autism (Baron-Cohen et al. 2015), many other studies of the prenatal environment also indicate that stress and/or inflammation may play an important part. A couple of studies have reported elevated rates of infection in pregnancies of children that developed autism (Zerbo et al. 2015; Al-Haddad et al. 2019). Autism rates were also found to be higher in cases of significant maternal stressors (Beversdorf et al. 2005), including recent experience of hurricanes (Kinney et al. 2008), a history of childhood trauma or domestic abuse, even after controlling for other perinatal complications (Roberts et al. 2013; 2016). An interaction effect between reported maternal stress and the genotype of the serotonin transporter gene in the mothers has also been indicated by a recent candidate-gene study (Hecht et al. 2016). Interestingly the clinical severity of maternal nausea and vomiting during pregnancy also correlate to the child's autistic traits (Whitehouse et al. 2018).

The role of the prenatal environment and associated hormonal factors is further strengthened by the interesting finding that autism occurrence is seasonal, with higher rates in pregnancies conceived in the winter, rather than in the summer, in a large epidemiological study in Scotland (Mackay et al. 2016). The authors speculated that vitamin D could be mediating these seasonal patterns. Consistently with this, low vitamin D levels, in cord blood and neonatal blood spots, have also been implicated in separate studies in the Netherlands and in Sweden (Vinkhuyzen et al. 2018; Lee et al. 2018). Vitamin D is a steroid hormone with placental as well as estrogenic effects, as it has been

shown to be partly synthesised by the placenta and act via the estrogen receptor in various tissues (Shin et al. 2010; Lisse et al. 2011).

Prenatally, the regulation of steroidogenesis is a more complex phenomenon compared to postnatal life, as it involves several steroidogenic sources that act together through a series of barriers-links to remodel endocrine homeostasis during pregnancy (Figure 1.1).

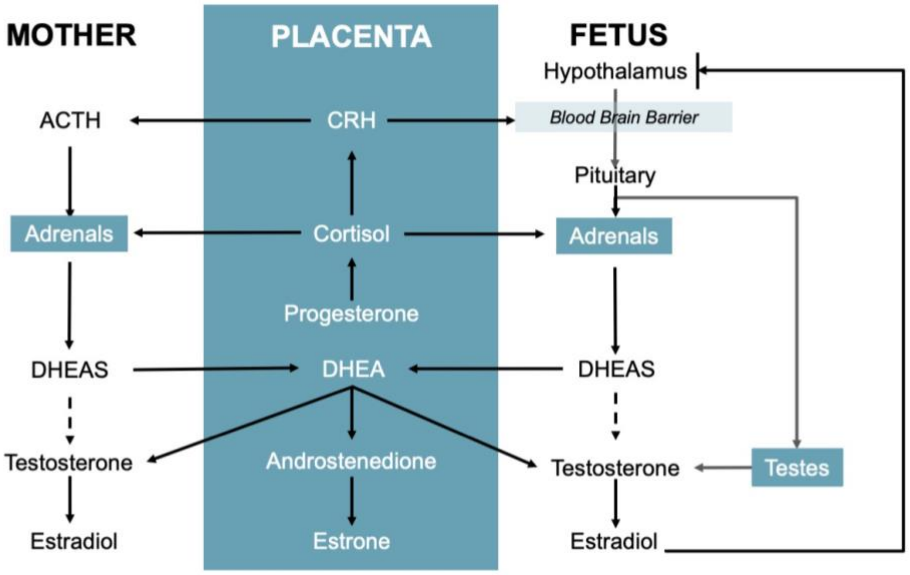


Figure 1.1: The sex-steroid system during pregnancy

These include the maternal and fetal hypothalamus, adrenals and gonads, their respective blood-brain-barriers and the interaction of mother and fetus within the placenta (Murphy et al. 2006). During pregnancy, this maternal-fetal unit acts in concert to increase steroidogenesis from all sources, under a positive feedback loop mediated by placental production of human choriogonadotropin (hCG) and corticotropin releasing hormone (CRH). These steroidogenic agents are released into the maternal and fetal circulations and are able to 'recruit' both HPA/G axes for steroid production. Typically, these are further rapidly modified via a series of enzymes, maintaining relative homeostasis during pregnancy, and regulating the hormones' effects and feedback loops. Examples of these rate-limiting regulatory steps in the placenta include the oxidisation of cortisol to cortisone (via the 11β dehydrogenase), the sulfation of DHEAS to DHEA. (via the SULT2A1 sulfotransferase) and the aromatisation of androgens to estrogens (via CYP19A1 aromatase) (Murphy et al. 2006; Makieva, Saunders, and Norman 2014). These modifications ensure that these hormones target specific tissues, rather than affect fetal development continuously throughout gestation. In addition, they interact with

developmental time-points, as the rate of increase an enzyme may be different to the rate of increase of the hormonal substrate, allowing for differential effects at different time-points of gestation. For example, the ratio of cortisol to corticosterone increases in the last trimester, with the former strengthening the newly formed fetal lungs before delivery (Watterberg and Scott 1995).

However, pregnancy complications and fetal sex may affect these homeostatic mechanisms. Males have significant differences in placental gene expression, compared to females (Gonzalez et al. 2018; S. Gong, Johnson, et al. 2018), as well as increased androgenic burden via testicular steroidogenesis and different growth trajectories during pregnancy (Broere-Brown et al. 2016). In addition, pregnancy complications and conditions such as PCOS or maternal stress may imbalance prenatal steroidogenesis by their respective effects on aromatisation of androgens (Manuel Maliqueo et al. 2013) or the inactivation of glucocorticoids (Peña, Monk, and Champagne 2012; Welberg, Thrivikraman, and Plotsky 2005).

Prenatal steroids exert organisational effects on the developing brain. Yet they also exert long-term 'conditioning' effects on the HPA/G axis of the fetus, potentially leading to life-long effects relating to steroid exposure. For example, high levels of androgens in utero can affect the differentiation of the ovaries and lead to PCOS in later life, which in turn is also associated with higher androgens in adulthood (Risal et al. 2019). As mentioned before, maternal PCOS is predictive of an autism diagnosis in their children, in at least three separate populations (Cherskov et al. 2018; Kosidou et al. 2016; Rotem et al. 2021). But autism itself is also associated with more steroid-related symptoms and PCOS in autistic women (Pohl et al. 2014; Simantov et al. 2021). This is why a potential 'steroidopathy' in autism may be transgenerationally transmitted and exert continuous and cumulative effects in autistic individuals. In addition, higher circulating steroids during postnatal life (Ruta et al. 2011) may also be linked to prenatal hormone levels. Evidence for such 'conditioning' effects can also be found in complex, steroid-related postnatal outcomes (e.g. age of menarche or the pubertal growth spurt), which are also regulated by the maturation of the HPA/G axis and prenatal factors that act on it (Dela Cruz and Pereira 2012; Matagne et al. 2004; Zambrano et al. 2014). Obesity and cardiovascular risk may also be linked to the prenatal endocrine environment(Calkins

and Devaskar 2011) and consistent epidemiological trends have been reported in autistic individuals (Lyall et al. 2011; 2022).

For this reason, research of a 'steroidopathy' in autism ought to start prenatally, consider multiple hormonal pathways, life-long health outcomes and employ a 'systems' approach that considers all components of the maternal-placental-fetal interface.

In brief, converging clinical evidence from independent cohorts and genetic analyses, indicate that hormonal homeostasis is affected in autism prenatally and in some cases throughout one's life. Steroid hormones other than testosterone are likely also significant in understanding of this 'steroidopathy'. However, it remains unclear from where these steroid hormones originate, why they are elevated prenatally, how they may affect males and females differently and specifically how male physiology interacts with steroid synthesis pathways to affect neurodevelopment. The research in this thesis contributes to answering these questions.

1.5 The placenta as a mediator of autism likelihood factors

The placenta is an organ formed predominantly from the trophoblast cells of the developing fetus. As a selective barrier between the mother and fetus it directly regulates the transfer of nutrients, prevents the transfer of pathogens, and produces a series of factors that impact fetal neurodevelopment (Figure 1.1). It is heavily involved in the hormonal regulation of the pregnancy, producing growth factors (e.g., IGF1), metabolic signals (e.g., leptin), immune system cytokines and neurotransmitter precursors (e.g., serotonin). The placenta is also responsible for aromatising testosterone into estradiol, producing a very high quantity of estrogens that increases gradually throughout pregnancy (Tal et al. 2015).

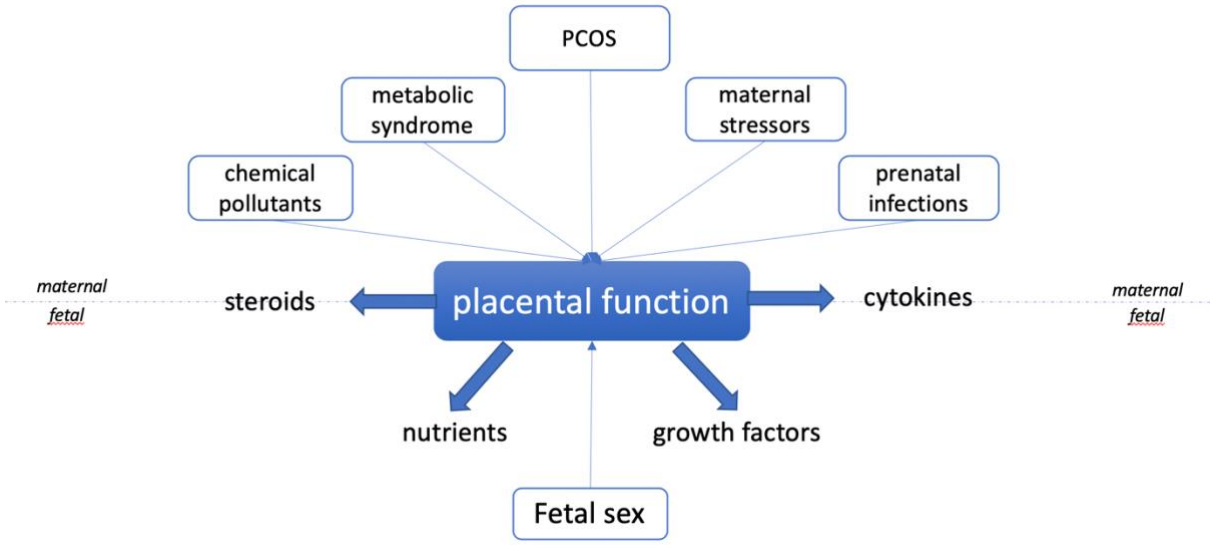


Figure 1.2: Likelihood Factors for autism converge on the placenta. PCOS: polycystic ovary syndrome

Several of the environmental factors and pregnancy complications that have been associated with autism also link to the function of the placenta. These include preeclampsia (Dachew et al. 2018), hypertensive disorders of pregnancy (Curran et al. 2018), low birthweight for gestational age and gestational diabetes (Xiang et al. 2015; Wan et al. 2018). The largest epidemiological study of pregnancy complications in autism (n=54,000 cases) reported that "placental pathology" was the most likely explanation of their observed pattern of associated complications (preeclampsia and low birth weight)(Maher et al. 2020).

Maternal comorbidities that have been linked to autism, namely PCOS, high BMI, concurrent infection or stress, all affect placental function as well, leading respectively to reduced placental aromatisation, insulin resistance and vascular dysfunction respectively (Maliqueo et al. 2013; 2017).

Crucially, placental function is sexually dimorphic, with male placentas producing more steroids (Gong et al. 2018) and fewer vascular protective factors than females (Brown et al. 2014). Male placentas are consistently more likely to malfunction, cause hypertension, preeclampsia and even spontaneous miscarriage (Murji et al. 2012). Placentas also respond to pregnancy complications in sex-specific manners, with males often being less

able to adapt to maternal stressors (Davis and Pfaff 2014) and maternal obesity (Maliqueo et al. 2017).

This 'male disadvantage' in placental physiology and adaptability may be also mediating male susceptibility to autism. In cases of autism, atypical placental morphology (i.e. more trophoblast inclusion bodies) (Straughen et al. 2017), increased placental inflammation and increased placental size (Park et al. 2018) have been reported in small, clinical cohorts. Similar reports have been published regarding the placentas of neonates with an autistic sibling (Walker et al. 2013).

Genomic studies of the placenta in autism have also revealed significant overlap between molecular function and neurodevelopment. Whole-methylome comparison between autism cases and controls reveal that the placentas of autistic individuals had significant epigenetic differences, compared to neurotypical pregnancies (Zhu et al. 2019). The 400 associated genes overlapped significantly with genes associated with autism that were identified by exome sequencing (SFARI list), as well as with the genes expressed in autistic individuals' brains post-mortem. Methylome profiles in the placenta have also been associated with neurodevelopmental outcomes (e.g. social challenges), as well as with later cognitive traits, such as IQ, in preterm infants (Santos et al. 2020). More recently, a study that used a mouse model to study the placenta-brain axis discovered that placenta-derived steroids were shaping behavioural outcomes relating to social behaviour. Interestingly, disrupting steroid synthesis in these mice selectively with a gene-knockout led to social deficits in males but not females, further indicating that placental effects on neurodevelopmental interact with sex (Vacher et al. 2021).

Therefore, the placenta and its steroid synthesis capacity may represent the mediators of a series of maternal, fetal, genetic and environmental likelihood factors that affect neurodevelopment and lead to autism (Figure 1.1).

1.6 Excess masculinisation or gender incoherence?

Since the fetal testosterone theory was proposed and the findings of the longitudinal Cambridge cohort were reported, different kinds of studies have come forward with evidence for a wider prenatal steroid imbalance in autism that extends beyond testosterone. More research is needed to confirm this and create a clinical framework for

understanding the origins and effects of this phenomenon in the long-term health of autistic people. However, the role of sex is still unclear, as is the relevance of these hormonal findings for understanding the skewed sex ratio in autism diagnoses. In brief, it is still unclear if there is still merit to the idea of autism as a condition of "excess masculinisation", as the findings on testosterone remain mixed (Baron-Cohen et al. 2011).

In the past few years, new methods have been developed and more evidence has accumulated that further supports the idea of an overlap between autism pathophysiology and sex differentiation mechanisms. For example, a new method was utilised by Tan and colleagues for scanning and comparing facial features via three-dimensional photogrammetry. This has been employed to scan for sex differences in the faces of populations of adults and children and then to train software to apply a "masculinity" score, based on those differences. Tan and colleagues used this method to scan the faces of autistic adults, autistic children, as well as their siblings, and found that all three groups showed "hypermasculinised" facial features, compared to their typical peers (Tan et al. 2015; 2017; 2020). In addition, traits such as social difficulties also correlated with facial masculinity in both males and females. This has been replicated in an independent cohort and shown to correlate to autism genetics as well (McKenna et al. 2021).

A similar phenomenon was reported in the brain structure of autistic adults, measured via MRI. Specifically, brain regions that are consistently larger in males were found to be even larger in autistic males, with a similar trend in autistic females (Lai et al. 2013). In addition, a high-throughput proteomic study that compared the biochemical profiles of n=78 autistic brains to n= 96 neurotypical controls, in circulating plasma, found that autistic women had "masculinised" levels for all of the measured metabolites (O'Neill et al. 2019).

However, this 'sex-by-diagnosis' interaction is not always found to be unidirectional towards the male end of the distribution. For example, a study of functional neural connectivity patterns (via fMRI), analysed this interaction in autistic adults and found that the direction of effect depended on the network in question. While the default network showed a pronounced shift towards 'male-like' patterns in both autistic males and autistic females, other networks (e.g. sensorimotor connectivity) showed 'female-

like' shifts in autism (Floris et al. 2018). A significant lack of "masculinisation" in autistic males, has also been reported in a few post-pubertal secondary sex characteristics, such as voice pitch, grip strength and bone density (Neumeyer et al. 2013; Kern et al. 2013). In one of the most comprehensive studies on sex differences in autistic biometry, pre-pubertal features were indeed "masculinised", (e.g., head circumference in females), but secondary characteristics in adulthood were consistently "androgynous". The authors termed this phenomenon as "gender incoherence" and autism as a "gender defiant" condition (Bejerot et al. 2012).

Behaviourally, as mentioned previously, autistic people show masculinised profiles, in terms of systemising and empathising (Greenberg et al. 2018). However, this is not mirrored in other personality traits that characterise males more than females, such as aggression or sociopathy. It is also worth noting that autistic people also show higher rates of gender identity incongruence, as well as atypical sexual orientation, compared to their peers (Bejerot and Eriksson 2014; Warrier et al. 2020). In humans, the development of gender identity and sexual orientation depend on many different factors, including social learning, which may be altered in autistic people. Nevertheless, a role for hormonal processes that contribute to the sex differentiation of the human brain cannot be excluded.

Excess masculinisation and gender incoherence may appear as contradictory hypotheses that cannot be attributed to the same steroid imbalance. However, the effects of steroids are dependent on the timing (e.g., pre-, or post-puberty) and the specific outcome in question. Prenatally, sex steroids are thought to act in a unidirectional, masculinising "programme", on top of a female default. By contrast, pubertal secondary sex characteristics induce both a masculinising (largely androgen-dependent) and a feminising "programme" (largely estrogen-dependent), based on the different gonadal substrates they acquired prenatally (i.e., testes in males, ovaries in females and so on). The interplay between androgens and estrogens, via aromatisation, regulates complex phenotypes such as the growth spurt in males, menarche in females and a negative feedback loop on the hypothalamus via inhibitory signalling (N Pitteloud et al. 2008). However, much less is known about the way that sex steroids affect the development of the human brain and particularly if their organisational/activational effects extend beyond reproductive behaviour. In animal models these effects are clearer but there are

significant differences compared to humans (e.g. estrogens are masculinising rodents but not primates)(Konkle and McCarthy 2011). Fortunately, new cutting-edge experimental methods have started to enable discoveries in humans as well. A recent study of stem-cell-derived brain organoids have now shown that androgens have significant effects on neuronal proliferation, cortical differentiation, and the balance between excitation and inhibition (Kelava et al. 2020).

In conclusion, since the first proposal of the "extreme male brain" and "prenatal testosterone" theories, considerable evidence has accumulated that supports the notion of an overlap between sex differentiation processes and autism, which could in turn partly explain male vulnerability to the condition. Steroids other than testosterone may be at play, as well as complex interactions with genetics and sex-related biometry (e.g., growth rates). This thesis will attempt to further explore these links and expand upon the fetal testosterone theory by investigating additional physiological sex differences in human perinatal development and their link to autism.

1.7 Outline of the studies in this thesis

In this thesis, I investigate factors relating to perinatal sex differentiation, in association with autistic traits, as well as in clinically diagnosed cases of autism. These perinatal factors include steroid hormone levels, placental function, genetics, and the growth rate of the brain. In terms of the timing for steroid exposure, both prenatal (during the PMW) and postnatal measurements (during 'mini-puberty') are included. In terms of neurodevelopmental outcomes, various developmental stages are covered, from early infancy to late childhood. Finally, in the analysis of their effects, a particular focus is given to statistical interactions with sex, in order to study the ways males and females may respond differently to the same hormonal, neurological or genetic factors.

Overall, the thesis includes six different studies, corresponding to four independent clinical cohorts. To avoid repetition in terms of methodologies, studies that are based in the same cohort of participants, are presented in the same chapter. An additional Genetics chapter reports on two separate analyses that are utilise publicly available data on rare and common genetic variance respectively.

In brief the chapters in this thesis correspond to different cohorts and are further organised in studies as follows:

Chapter 2 - **Study 1**: Sex steroids and autism

- Cohorts: Danish Biobank (n= 98 male cases, n= 177 male controls)
- Predictors: Steroid hormones in amniotic fluid
- Timing: First to second trimester (mean=14.9 weeks)
- Outcome: Clinical diagnosis of autism

Chapter 3 - **Study 2**: Sex steroids and traits

- Cohorts: the Cambridge Ultrasound Siblings and Parents study (n=122)
- Predictors: Steroid hormones in maternal serum
- Timing: First trimester (mean=12.7 weeks)
- Outcome: Autistic traits of the mother and the infant

Chapter 4 – **Study 3**: Sex and the placenta

- Cohorts: Generation R, the Netherlands (n=3469 cohort, n=65 with autism)

- Predictors: Placental proteins that show sex differences (PlGF, sFlt-1, PAI-2)
- Timing: First (mean= 13.5 weeks) and second trimester (mean=20.6 weeks)
- Outcome: Autistic traits of the infants and a clinical diagnosis of autism.

Chapter 5 – **Studies 4, 5 and 6**: Sex and Infant Development

- Cohorts: the Cambridge Imaging and Longitudinal Development (CHILD) cohort, Cambridge, (n=41 prenatal & n=27 postnatal)
- Predictors: Mini-puberty testosterone (Study 4), perinatal brain growth on MRI (Study 5) and regional brain volume in infancy (Study 6)
- Timing: Third trimester (Study 5) and second month postnatally (Studies 4-6)
- Outcome: Autistic traits of the infant (Q-CHAT) & family history of autism

Chapter 6 **- Study 7**: Autism genes and gene expression differences in the placenta

- Cohorts: Placentas from pregnancies at the Cedars-Sinai Medical Centre of UCLA (n=17 females, n=22 males)(Gonzalez et al. 2018)
- Predictors: Lists of genes that show sex differences in autosomes & the X chromosome.
- Outcome: High-confidence autism genes (categories 1 & 2), aggregated by the Simons Foundation (SFARI) (Banerjee-Basu and Packer 2010) .

Chapter 6 - **Study 8**: Genetic correlations between autism & endocrine outcomes

- Cohorts: Various, including UK Biobank, 23andMe, ReproGen Consortium
- Predictors: GWAS summary statistics for postnatal hormone levels & steroid-related traits
- Outcome: Common variance of autism (Grove et al. 2019) & of general psychiatric liability (iPSYCH) (Schork et al. 2019)

Chapter 7 and Chapter 8 do not correspond to specific cohorts but aim to analyse the findings of Studies 1 - 8, in a clinical and evolutionary context respectively.

Chapter 2

Study 1 - Sex steroids and autism

The chapter is based on a study published in 'Molecular Psychiatry', July 2019, with A. Tsompanidis as a joint first author and the title "Foetal oestrogens and autism"

https://doi.org/10.1038/s41380-019-0454-9.

Summary of Study 1

Elevated latent prenatal steroidogenic activity has been found in the amniotic fluid of autistic boys, based on measuring prenatal androgens and other steroid hormones. To date, it is unclear if other prenatal steroids also contribute to autism likelihood. Prenatal estrogens need to be investigated, as they play a key role in synaptogenesis and corticogenesis during prenatal development, in both males and females. In this study, prenatal levels of estriol, estradiol, estrone, and estrone sulfate are tested in amniotic fluid are associated with autism, in the same Danish Historic Birth Cohort, in which prenatal androgens were previously measured, using univariate logistic regression (n = 98 cases, n = 177 controls). Estradiol, estrone, estriol, and progesterone each related to autism in univariate analyses after correction with false discovery rate. A comparison of standardized odds ratios showed that estradiol, estrone and progesterone had the largest effects on autism likelihood. These results for the first time show that prenatal estrogens contribute to autism likelihood, extending the finding of elevated prenatal steroidogenic activity in autism. This likely affects sexual differentiation, brain development and function.

2.1 Introduction

As discussed in the previous chapter, the male-biased prevalence of autism (Fombonne 2009; Baron-Cohen et al. 2011), together with the finding that autistic girls have a higher mutational load than autistic boys (Robinson et al. 2013; Jacquemont et al. 2014; Werling and Geschwind 2015), suggests that males have a higher likelihood of developing autism.

Although autism is strongly heritable and sex-associated genetic mechanisms could contribute to this implication of sexual differentiation in autism (Baron-Cohen et al. 2011; Werling and Geschwind 2015) prenatal hormone exposure and a brief surge in fetal testosterone are critical for sexual differentiation and masculinization in humans (Hines, Constantinescu, and Spencer 2015; Welsh, Suzuki, and Yamada 2014). In line with this, our research group previously found elevated steroidogenic activity during this prenatal masculinization window (PMW) in the amniotic fluid of autistic boys (Baron-Cohen et al. 2015).

Whilst prenatal androgens are responsible for masculinization in humans, prenatal estrogens also contribute to fetal and neonatal brain development (Margaret M McCarthy 2008), and yet these have not been thoroughly investigated for their potential role in autism likelihood. Estrogens and their receptors are widespread in the developing brain in both males and females and regulate many neurodevelopmental processes, including synaptogenesis, apoptosis, and neuronal differentiation (Konkle and McCarthy 2011; MacLusky and Naftolin 1981; González et al. 2007). Estradiol in particular supports synapse formation in the cortex by enhancing excitatory GABA activity (Nunez et al. 2008). In autism, synapse formation (Durand et al. 2012), neuronal differentiation (Li et al. 2014), as well as the GABAergic system (Puts et al. 2017), are all atypical. These provide clues that prenatal estrogens may be involved in autism.

With regard to clinical studies in humans, low estriol in maternal serum during the 2nd trimester of pregnancy significantly increases the likelihood of autism in the fetus, as demonstrated in a large study of n = 2,586 pregnancies that resulted in autism diagnosis in the offspring (Windham et al. 2016). This study may have been confounded by a variety of pregnancy complications, such as pre-eclampsia (Tache et al. 2014) and being small for gestational age (Talge et al. 2011), since these are also

more frequent in autism (Walker et al. 2015; Moore et al. 2012; Kristen Lyall et al. 2012). Thus, further study of prenatal estrogenic activity, particularly in fetal circulation, is warranted. In addition, there is a need to compare different prenatal estrogens to each other, in relation to autism likelihood.

In the study presented below, prenatal levels of estriol, estradiol, estrone, and estrone sulfate were measured in amniotic fluid of boys with and without autism (n = 98 and n = 177 respectively) from the Danish Historic Birth Cohort (HBC) and in the same samples in which an elevated steroidogenic factor was found, following principal component analysis of prenatal androgens and other steroid hormones (Baron-Cohen et al. 2015). Each steroid hormone was correlated to autism likelihood via univariate logistic regression. Differences in the aromatising capacity in autism were also investigated, by comparing the ratio between androgens and estrogens.

2.2 Methods

2.2.1 Participants and Laboratory Methods

The study was approved by the Danish Data Protection Agency and The Danish Ethical Committee of Midtjylland Region. The Danish Historic Birth Cohort was established at Statens Serum Institute, Copenhagen with a grant from The Danish Medical Research Foundation and The Danish Ministry of the Interior and Health (Project no 271-05-0523/09-060179). A full description of the cohort selection procedure is available elsewhere (Baron-Cohen et al. 2015). Briefly, cases and controls were drawn from singleton births between the years 1993-1999 inclusive, whose amniotic fluid samples were stored in the HBC. These corresponded to amniocentesis procedures performed between 14-16 weeks of gestational age. Cases were identified from the Danish Psychiatric Central Register using ICD-10 autism spectrum codes F84.0 (childhood autism), F84.5 (Asperger syndrome), F84.1 (atypical autism), F84.8 (other pervasive developmental disorder), and F84.9 (unspecified pervasive developmental disorder). Any additional amniotic fluid was assayed for estradiol, estriol, estrone, and estrone sulfate, using liquid chromatography-tandem mass spectroscopy (Appendix Table 2.1). As some individuals did not have sufficient remaining sample for analysis, the sample size was slightly decreased. The same data quality screening criteria that were used in

the initial analysis were applied: i.e. removal of outliers > 99%, removal of records where duplicate assay values were > 3 SD (Standard Deviation) apart (Baron-Cohen et al. 2015). After this step, the final sample with high-quality data for all steroids assayed to date consisted of 98 males with autism and 177 control males. This sample was used for all analyses in this paper, unless otherwise specified (Appendix Figure 2.1).

2.2.2 Statistical analyses

A correlation matrix was calculated for all assayed steroid hormones using Pearson's correlation coefficient. The univariate distribution of each of the estrogens was then studied. All hormones showed a substantial rightward skew. These were then transformed using the Box-Cox procedure to reduce their skew, as the distribution of the predictor variable affects the statistical power of logistic regression (Faul et al. 2009). Univariate logistic regression models to determine whether each hormone separately increased autism likelihood in this cohort. Correction of statistical significance thresholds for multiple comparisons was conducted using the Benjamini-Hochberg false discovery rate (FDR). To estimate and compare the aromatising capacity between cases and controls, the aromatisation ratios were calculated, according to previously published recommendations (Sollberger and Ehlert 2016), by log-transforming the raw concentration values and subsequently subtracting them according to the following formula:

Ratio 1= log(testosterone concentration in nmol/l) – log(estradiol concentration in nmol/l)

Ratio 2= log(androstenedione concentration in nmol/l) – log(estrone concentration in nmol/l)

Nonparametric tests were then used to compare these ratios to each other (Spearman's rank correlation coefficient) and between cases and controls (Wilcoxon rank-sum test).

2.3 Results

There were no significant differences between groups in maternal age at birth, paternal age at birth, birth weight, gestational week at amniocentesis, or storage time between groups (Table 2.1).

Table 2.1: Description of Danish Historic Birth Cohort sample

	Control	***Autism***	
	n =177	n =98	
	Mean ± SD	**Mean ± SD**	**p-value**
Maternal age at birth	33.33 ± 5.15	33.53 ± 5.65	0.775
Paternal age at birth	35.30 ± 6.72	35.85 ± 7.44	0.552
Birth weight (g)	3516.81 ± 659.45	3524.55 ± 679.15	0.928
Gestational week at amniocentesis	14.89 ± 1.91	14.90 ± 1.48	0.953
Storage time (years)	14.90 ± 1.58	14.96 ± 1.69	0.770
APGAR score > 6	97%	96%	

Raw data for each of the estrogens are presented in Figure 2.1. Values for the median and interquartile range, as well as raw data categorised by autism diagnosis are available in the Appendix (Appendix Table 2.2).

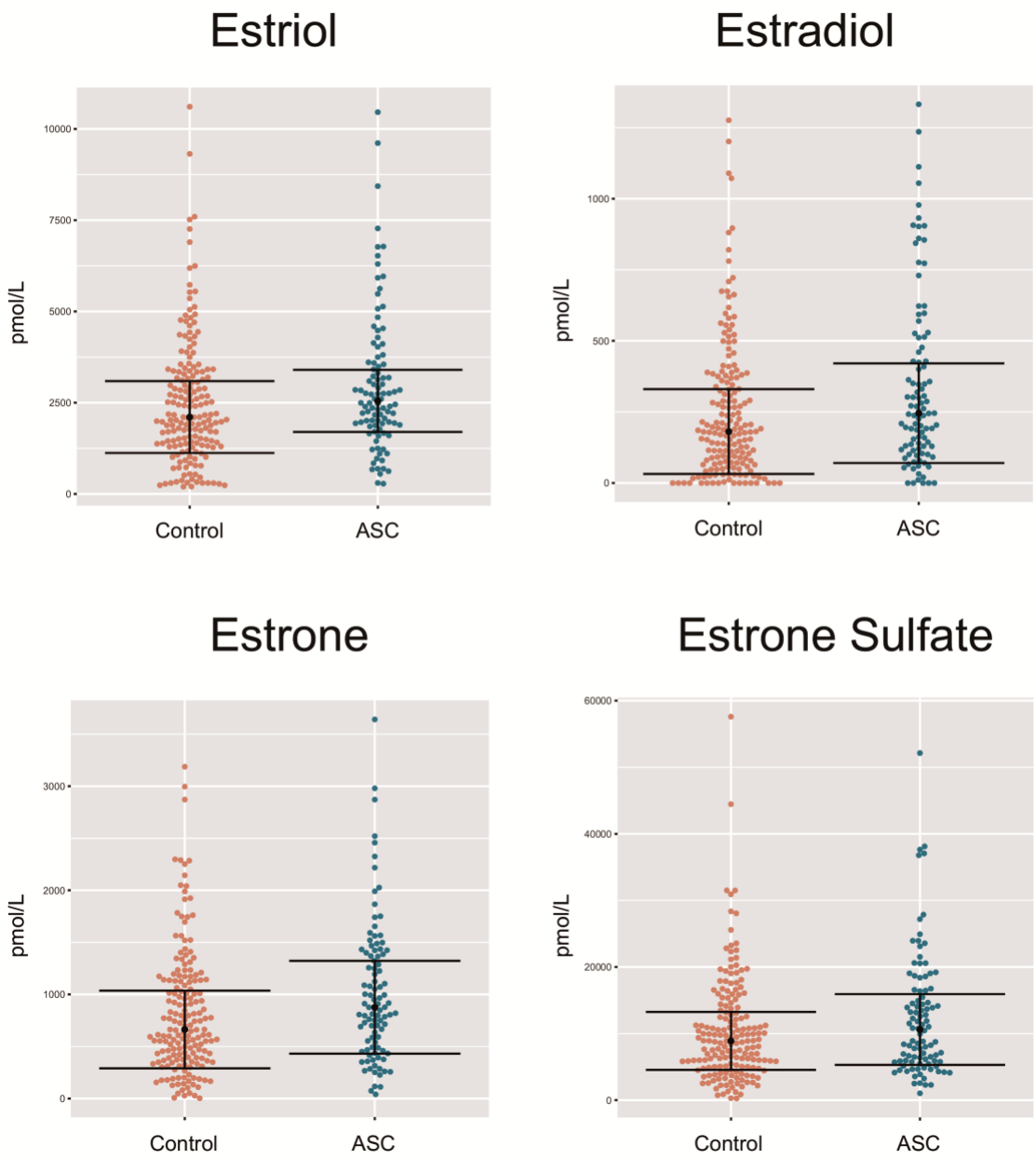


Figure 2.1: Beeswarm plots illustrating the distribution of estriol, estradiol, estrone, and estrone sulfate concentrations. Error bars represent the interquartile range, and the black dot represents the median; (n CTR = 177, n ASC = 98).

Estriol levels were the most predictive of an autism diagnosis, as revealed by univariate logistic regression (β=0.025, FDR-adjusted q=0.034). The same was found for the levels of estradiol and estrone, with both hormones being significantly associated with an autism diagnosis using logistic regression (Estradiol: β=0.029, q=0.031; Estrone: β=0.029, SE=0.011, FDR-adjusted q=0.031) (Table 2.2). Estrone sulfate was also a nominally significant predictor of autism likelihood in the logistic regression model but did not maintain statistical significance following false-discovery rate correction (β=0.033, SE=0.016, z=2.095, p=0.036, q=0.065) (Table 2.2).

2.3.2 Androgens and other steroids

The previously assayed concentrations of androgens and cortisol (Baron-Cohen et al. 2015) in the same subset of samples, were revisited and analysed with the same statistical methods as the estrogens, in order to understand whether the relationship between estrogens and autism likelihood was similar to the relationship between androgens and autism likelihood (Table 2.2, Beeswarm plots of distribution in Appendix Figure 2.2). Of the previously analysed steroid hormones, only progesterone was a significant predictor of autism diagnosis, following univariate logistic regression and correction via FDR in this subset of the cohort (β=0.053, FDR-adjusted q=0.031) (Table 2.2).

Table 2.2: Results of univariate logistic regression for amniotic steroid hormones. Asterisk denotes statistical significance (q<0.05), following correction via FDR.

	Regression Coefficient	***Standard Error***	***z-value***	***p-value***	***FDR-adjusted q value***
Estriol	0.025 *	0.010	2.426	0.015	0.034
Estradiol	0.029 *	0.010	2.757	0.006	0.031
Estrone	0.029 *	0.011	2.603	0.009	0.031
Estrone sulfate	0.033	0.016	2.095	0.036	0.065
Testosterone	0.352	0.304	1.157	0.247	0.247
Androstenedione	0.444	0.270	1.648	0.100	0.128
17-OH Progesterone	0.547	0.271	2.022	0.043	0.065
Progesterone	0.053 *	0.021	2.562	0.010	0.031
Cortisol	0.147	0.111	1.332	0.183	0.206

2.3.3 Comparison

Pairwise correlation analysis (Pearson's) revealed varying degrees of similarity between the steroid hormones. The concentrations of estrogens were significantly correlated with one another at $q<0.05$, adjusted for multiple comparisons via FDR (Figure 2.2, Appendix Table 2.3). Estrone and estriol showed higher correlations to the other steroids than did estrone sulfate and estradiol. In comparison, the previously analysed steroid hormones formed a distinct group, with weaker correlations to estrogens and stronger correlations to each other. Androstenedione and progesterone (Pearson's $\beta=0.59317$, FDR-adjusted $q<0.001$), estrone and estrone sulfate (Pearson's $\beta=0.589$, FDR-adjusted $q<0.001$) were the pairs that were more closely related. Estradiol did not correlate with either testosterone or estriol.

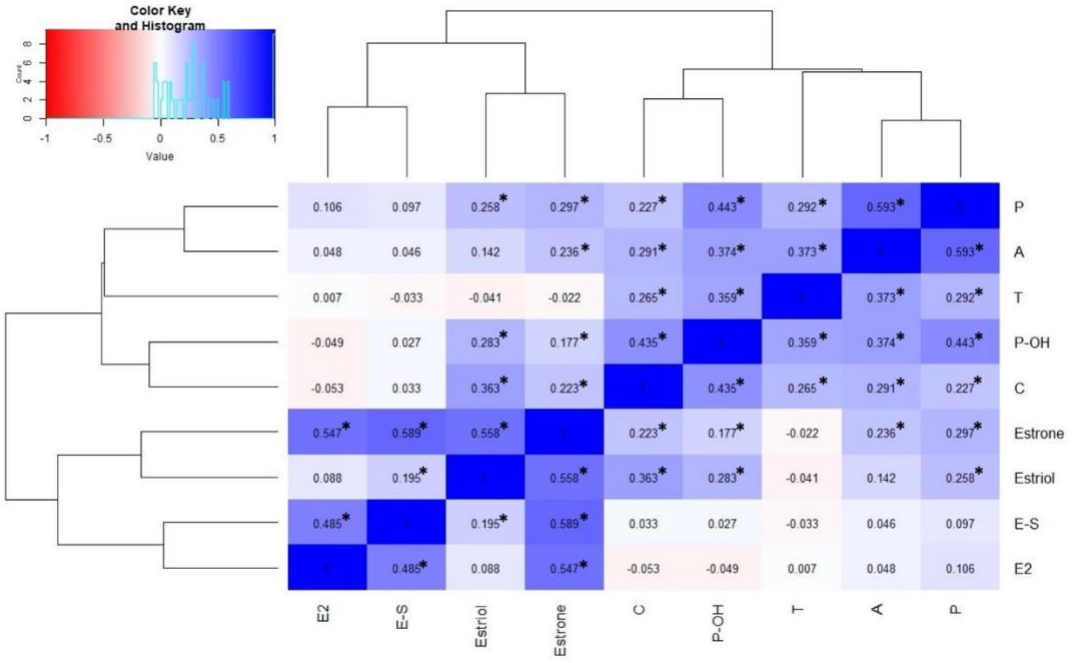


Figure 2.2: Heatmap and dendrogram of the pairwise correlations between the steroid hormones assayed in amniotic fluid. Asterisk denotes statistical significance (q<0.05), following correction via FDR. P: Progesterone; A: Androstenedione; T: Testosterone; P-OH: 17

Standardized odds ratios (ORs) for all analysed hormones were then calculated, to determine which hormones had the largest effect on autism likelihood (Figure 2.3). Each hormone was standardised by its median and interquartile range, so that a one-unit increase in a hormone corresponded to the movement from the 25th to the 75th percentile of its range. Progesterone and estradiol had the highest standardised ORs, with a movement from the 25th to 75th percentile of these hormones incurring nearly a 50% increase in autism likelihood. Estrone and estrone sulfate also had ORs over 1. In contrast, increases in testosterone or androstenedione levels were not associated with increases in likelihood of diagnosis.

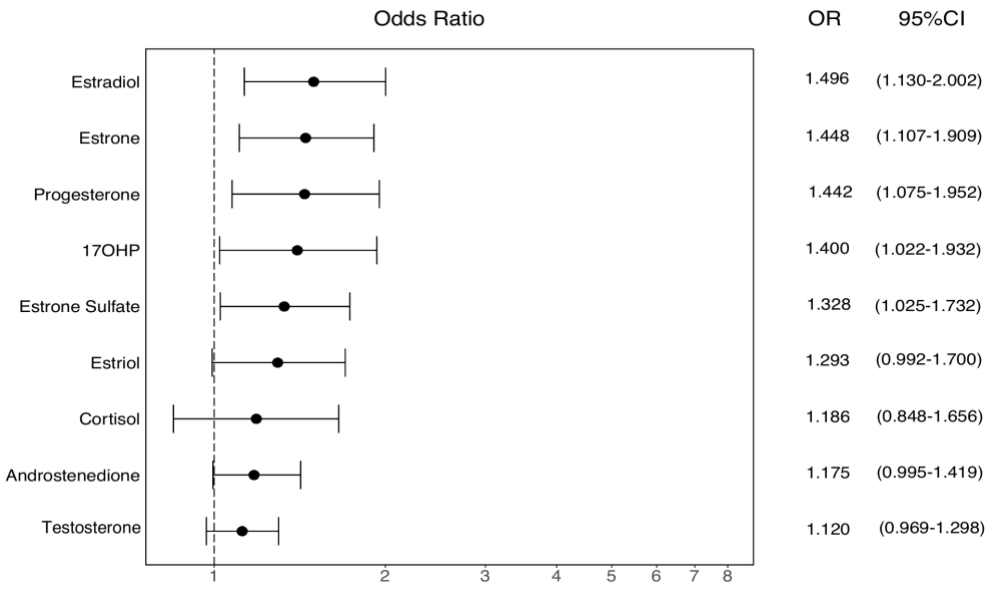


Figure 2.3: Standardized odds ratios (ORs) for autism diagnosis for all amniotic steroids assayed to date.

The aromatising of testosterone (T) to estradiol (E2) and of androstenedione (A) to estrone (E1) was indirectly assessed by calculating their ratios, following log-transformation and subtraction of their raw values. The two ratios were highly correlated with each other (Spearman's *rho*=0.6614, $p<0.001$) but were not significantly different between autism cases and neurotypical controls (T/E2: Wilcoxon rank-sum $w=7551$, p=0.076) (A/E1: Wilcoxon rank-sum $w=7610$ $p=0.093$).

2.4 Discussion

Study 1 reports the first evidence that elevated levels of prenatal amniotic estradiol, estriol and estrone are each associated with autism, with estradiol levels being the most significant predictor of autism likelihood in univariate logistic regression models. These findings complement earlier observations that elevated steroidogenic activity is associated with autism in the same samples derived from the Danish Historic Birth cohort (Baron-Cohen et al. 2015). Estradiol had the strongest positive effect size on autism likelihood, followed by estrone, estriol and progesterone (Figure 2.3). This finding appears to contradict an earlier report. (Windham et al. 2016) that showed that *lower* levels of estriol in 2nd trimester were modestly associated with a later diagnosis of autism in the offspring. However, the samples in Study 1 correspond to a slightly earlier time point in pregnancy compared to Windham *et al.* (mean gestational week=14.9, vs. gestational weeks=15-20) (see Table 2.1)(Windham et al. 2016), which could potentially better capture the steroid surge during the PMW (Welsh, Suzuki, and Yamada 2014). Furthermore, the samples in this study are of different origin, as Windham *et al.* assayed maternal serum, rather than prenatal amniotic fluid**.** Steroid hormone levels in maternal serum do not differ relative to the baby's sex and do not correlate to amniotic levels during the PMW (van de Beek et al. 2004). Therefore, amniotic estrogens are arguably more relevant to the current research question than are maternal serum estrogens.

A discrepancy in estrogen levels between the mother and child could potentially be attributed to the placenta, which acts as an endocrine regulator of the maternal-fetal interface and the main source of estrogen production for the fetus via the aromatisation of fetal androgens (Kaludjerovic and Ward 2012). Several lines of evidence suggest a contributory role for the placenta in the etiology of autism. First, there is increased placental inflammation in autism (Straughen et al. 2017). Second, there is atypical placental morphology (Anderson et al. 2007) and increased placental size (Park et al. 2018) in cases of autism and at high familial risk respectively. Third, complications related to the placenta (pre-eclampsia: (Dachew et al. 2018), hypertensive disorders: (Curran et al. 2018)) are also more frequent in pregnancies that lead to autism. As with autism, placental dysfunction also disproportionately affects males more than females (Murji et al. 2012).

Given the high pairwise correlations between many of the steroid hormones (Figure 2.2 and Appendix Table 2.3), as well as the lack of difference in aromatisation between cases and controls, both suggest that an increase in fetal estrogens is secondary to increased activity along the entirety of the steroidogenic axis in pregnancies that later result in autism (Baron-Cohen et al. 2015). Interestingly, estradiol was not significantly correlated to testosterone (Pearson's β=0.007, p=0.9103) despite their proximity in steroidogenesis. This discrepancy may be because estrogens are also *de novo* produced by the placenta, in addition to being aromatised from fetal and maternal androgens (Escobar et al. 2011; Kaludjerovic and Ward 2012). Thus, a multi-systems approach is needed, in order to clarify the causes of elevated fetal estrogens in autism.

In the brain, estrogen-mediated signaling on GABA-ergic neurons in the hypothalamus is required, in order to suppress the steroidogenic axis (N Pitteloud et al. 2008). Inefficient suppression of this axis in autism could be due to inefficient aromatisation of androgens in the hypothalamus, resistance to estrogen signaling and/or dysfunction of the GABA-ergic system. Prenatally, fetal genetics (e.g. due to mutations in aromatase (Bhismadev Chakrabarti et al. 2009) or aromatase activators (Sarachana and Hu 2013)), pregnancy complications (e.g. placental size (Park et al. 2018)), as well as maternal risk factors (e.g. PCOS (Cherskov et al. 2018)) could all affect various points in this pathophysiological pathway. These speculations would require further testing. Specifically for aromatisation, ratios based on circulating hormone levels may not be sufficient to capture tissue-specific activity, since aromatase is differentially regulated by separate promoters in the placenta, the adrenals and the brain (Simpson et al. 1993).

High levels of prenatal estrogens could dysregulate many aspects of prenatal endocrinology and affect prenatal brain development in areas not restricted to sexual differentiation. Several lines of evidence support a wider role of estradiol as a 'neurosteroid' with many regulatory properties (Srivastava et al. 2010). For example, disruption of estrogen signaling in the developing cerebellum of mice reduces Purkinje cell growth in both males and females, but only reduces social behaviour in male mice, suggesting that the cerebellum may react to estrogenic disruption in a sexually dimorphic way (Hoffman, Wright, and McCarthy 2016a). In early development, estradiol decreases GABA-ergic signaling (Mukherjee et al. 2017) and mediates its

post-natal shift from excitation to inhibition (Ganguly et al. 2001). Estrogens both increase the number of spines on embryonic primary cortical neurons (Srivastava et al. 2010) and induce the recruitment of proteins necessary for excitatory synapse formation, such as neuroligin-1, NMDA subunit GluN1, and post-synaptic density protein 95 (PSD-95) to the spines (Sellers et al. 2015). Higher levels of prenatal estrogens might therefore increase the number of excitatory synapses in the cortex, increasing the likelihood for autism, as suggested by the excitatory/inhibitory (E-I) theory of autism (Rubenstein and Merzenich 2003). The perceptual phenotype in autism is characterized by reduced GABA-ergic inhibition, as shown using paradigms such as binocular rivalry (Robertson et al. 2013a) and attention to detail (Robertson et al. 2013b). Estrogen signaling could thus be a significant modulator of neuronal inhibition, particularly during early brain development and the 'critical period' of cortical plasticity, which is heavily reliant on the GABA-ergic system (Hensch 2005).

Although estradiol (aromatised from testosterone) is the main prenatal masculinising agent in most mammals (Margaret M McCarthy 2008), its role in human sexual differentiation remains unclear. Men with aromatase deficiency have typical development of their urogenital tract (Rochira and Carani 2009), but have cognitive disabilities, lack a growth spurt, and have atypical secondary sexual characteristics such as feminised body proportions (Chen et al. 2015). Estrogens may therefore both feminise and masculinise in humans, varying by target tissue and developmental milieu. In autism, cognitive styles and sexually dimorphic neuroanatomy show some masculinised phenotypes (Lai et al. 2013; Baron-Cohen et al. 2014; Greenberg et al. 2018), but functional connectivity and physical growth show a mixed pattern of masculine and feminine shifts (Floris et al. 2018; Bejerot et al. 2012). Prenatally though, and particularly during the PMW, the process of sexual differentiation is understood to be directionally masculine over an anatomically and physiologically female default. The observed high levels of fetal estrogens could thus be contributing to developmental cognitive differences (Greenberg et al. 2018), according to the "extreme male brain" theory of autism.

There was no statistically significant univariate, logistic association between autism and testosterone or androstenedione, which act via the androgen receptor. Mechanisms through which androgenic signaling could increase autism likelihood, that

may have been missed in this analysis, include additional androgens or other agonists of the androgen receptor (e.g. neurosteroids like dehydroepiandrosterone (Lu et al. 2003)), interaction effects between androgens and estrogens (e.g. coactivation of the androgen receptor by estradiol (Yeh et al. 1998)), as well as non-linear associations of androgens with autism likelihood. Consequently, androgenic activity may still be an important feature in the development of autism, as suggested by related clinical comorbidities (Pohl et al. 2014; Cherskov et al. 2018) and shown in associations with prenatal testosterone to autistic traits in a separate cohort (Auyeung et al. 2010).

This analysis could not be conducted in females, as there were too few diagnosed women in the HBC. Thus, at present, the findings only generalise to males. Furthermore, comparison of the concentrations of androgens and cortisol to estrogens is potentially confounded by the fact that the latter were analysed at a later time-point and underwent an additional freeze-thaw cycle. However, the same the same assay methodology was used, as with the initial analysis for androgens (LC-MS/MS). There were no differences in total storage time between androgens and estrogens (Table 2.1). In addition, clinical ascertainment bias may be a factor, as the samples corresponded to pregnancies in which karyotype screening via amniocentesis was recommended. Controls were matched for and showed no significant differences in clinical confounders such as maternal age. Autism prevalence in the wider Danish biobank amniocentesis cohort (0.8%) was also consistent with prevalence estimates of autism in the Danish population during the same period (Parner, Schendel, and Thorsen 2008). Yet, it is important to note that the findings may not generalise to a younger population, which would not have been recommended amniocentesis at the time.

Another limitation of this study is its reliance on clinical diagnoses from the Danish Central Psychiatric Register, which could not be independently validated. However, a previous validation study of childhood autism diagnoses in the Danish Central Psychiatric Register found that 94% of diagnoses between 1990 and 1999 in the register were valid using a standardised coding scheme (Lauritsen et al. 2010). Similarly, the source of amniotic steroids could not be ascertained, as these could be of fetal, maternal, or placental in origin. Fetal plasma and amniotic fluid are in osmotic equilibrium until the fetal skin is keratinised (typically by 25 weeks of gestation)

(Underwood, Gilbert, and Sherman 2005). Therefore, steroid concentrations in amniotic fluid reflect those in fetal circulation.

In conclusion, this study found that prenatal estradiol, estriol, and estrone are elevated in in boys who went on to develop autism. This extends previous finding of elevated prenatal steroidogenesis in the same cohort (Baron-Cohen et al. 2015). High levels of prenatal estradiol contribute to a greater degree to autism likelihood than other prenatal sex steroids, including testosterone. In the next chapter, prenatal estrogens are further analysed, in association with the autistic traits of both infants and of their mothers.

Chapter 3
Study 2 - Sex steroids and autistic traits

This chapter is based on a study that was published in 'Molecular Autism', July 2021, with A. Tsompanidis as the first author and the title "Maternal steroid levels and the autistic traits of the mother and infant"

https://doi.org/10.1186/s13229-021-00453-7

Summary of Study 2

Prenatal sex steroids have been associated with autism in several clinical and epidemiological studies. It is unclear how this relates to the autistic traits of the mother and how early this can be detected during pregnancy and postnatal development. In Study 2, maternal serum was collected from pregnant women (n=122) during their first ultrasound appointment (mean=12.7 [SD=0.8] weeks). Concentrations of the following were measured via immunoassays: testosterone (T), estradiol (E2), dehydroepiandrosterone sulphate (DHEAS), progesterone (P); and sex hormone-binding globulin (SHBG) which was used to compute the free fractions of estradiol (FEI) and testosterone (FTI). Standardised human choriogonadotropin (hCG) and pregnancy-associated plasma protein A (PAPP-A) values were obtained from clinical records corresponding to the same serum samples. Mothers completed the Autism Spectrum Quotient (AQ) and for their infants, the Quantitative Checklist for Autism in Toddlers (Q-CHAT) when the infants were between 18-20 months old. FEI was positively associated with maternal autistic traits in univariate (n=108, Pearson's r=0.22, p=0.019) and multiple regression models (semipartial r=0.24, p=0.022) controlling for maternal age and a diagnosis of PCOS. Maternal estradiol levels significantly interacted with fetal sex in predicting infant Q-CHAT scores, with a positive relationship in males but not females (n=100, interaction term: semipartial r=0.23, p=0.036) after controlling for maternal AQ and other covariates. The opposite was found for standardised hCG values and Q-CHAT scores, with a positive association in females but not in males (n=151, interaction term: r=-0.26, p=0.005). In conclusion, maternal steroid factors during pregnancy are associated with autistic traits in mothers and their infants.

3.1 Introduction

In the previous chapter (Study 1), prenatal sex steroid levels in amniotic fluid were retrospectively linked to an autism diagnosis in later life. Diagnosis of autism is possible as early as 18 months of age (Howlin and Asgharian 1999). In addition, autistic traits exist along a spectrum in the wider population (Baron-Cohen et al. 2001). These can also be measured in infancy, as demonstrated by the Quantitative Checklist for Autism in Toddlers (Q-CHAT), a novel, dimensional measure of autistic traits shown to predict later autism diagnosis in several validation studies (Allison et al. 2008; Liliana Ruta et al. 2019; Allison et al. 2021). This can have screening potential, especially if linked to additional prenatal measures.

As it was shown in Chapter 2, autistic males have higher levels of estrogens in univariate analyses of amniotic fluid samples (Baron-Cohen et al. 2015; 2019). Additionally, estradiol levels have been found elevated in the maternal serum of pregnancies linked to a later autism diagnosis in the child (Bilder et al. 2019). But maternal estrogens have not yet been tested with regard to autistic traits in infancy.

On the other hand, testosterone levels measured during amniocentesis are negatively correlated with the frequency of eye-contact at 12 months (Lutchmaya, Baron-Cohen, and Raggatt 2002) and, vocabulary size at 18 and 24 months (Lutchmaya, Baron-Cohen, and Raggatt 2001), and are positively correlated with restricted interests, attention to detail (Knickmeyer et al. 2005) and with autistic traits at 18 and 48 months (Auyeung et al. 2010;Auyeung et al. 2009). Steroid-related conditions such as PCOS and placental complications are also more common in autistic people (Cherskov et al. 2018; Pohl et al. 2014; Maher et al. 2018), and autistic adults demonstrate signs of steroid imbalance in various tissues (Ruta et al. 2011; Hu et al. 2009; Crider et al. 2014). Further research is needed to understand the timing of this endocrine imbalance in autism, as well as to study whether it extends to estrogens and autistic traits in the general population.

The prenatal environment shows baseline sex differences in steroid production via the placenta (Gong et al. 2018), as well as in markers of placental formation and function, such as human choriogonadotropin (hCG) (Adibi et al. 2015; Murji et al. 2012; Davis and Pfaff 2014). Atypical levels in both have been found in maternal serum of pregnancies that resulted in an autistic child, with both higher (for estradiol) and lower levels (for

estriol) reported compared to controls (Windham et al. 2016; Bilder et al. 2019). However, these prenatal factors have not been studied together with fetal sex and in relation to both maternal and infant autistic traits.

To evaluate all these prenatal factors, in Study 2 both the mothers and their infants were assessed for autistic traits (via the Autism Spectrum Quotient (AQ) and Q-CHAT respectively) in a new longitudinal cohort. Steroid hormone levels (estradiol, testosterone, DHEAS, progesterone) and placental markers (e.g., hCG) were tested in maternal serum collected during the mother's first ultrasound appointment were predicted by autistic traits measured in the mothers during pregnancy and infants at the 18-month follow-up.

3.2 Methods

3.2.1 Cohort recruitment

Mothers were recruited during their pregnancy, during or immediately before their routine 20-week ultrasound scan (mean gestational age of 20.3 [SD=0.4] weeks), between 2016 and 2018 at the Rosie Hospital, Cambridge University Hospitals NHS Foundation Trust (Aydin et al. 2019b). This study had been approved by the East of England Cambridge Central Research Ethics Committee (REC Ref 16/EE/0004) and the Research and Development Department of Cambridge University Hospitals. Eligibility inclusion criteria for the study were as follows: (1) little/no consumption of alcohol during pregnancy, (2) no smoking or recreational drug use during pregnancy (3) a singleton pregnancy of a fetus (4) whose measurements indicated their size to be appropriate for gestational age (no intrauterine growth restriction (IUGR) or large-for-gestational age (LGA). For the postnatal part of the study, the parents of all live singleton births were asked to take part in a series of developmental follow-ups during the first two years of life of their infant. Participating mothers also gave informed consent for access to all their pregnancy-related clinical records, test results and the biological samples that were obtained during their routine clinical care of their pregnancy, before and after the point of recruitment.

3.2.2 Clinical data collection

Serum samples had been collected at the end of the 1st trimester (mean gestational age of 12.7 [SD=0.8] weeks since conception) by a specialist phlebotomist at the Rosie Hospital, Cambridge. These were initially assayed for the levels of human choriogonadotropin (hCG) and pregnancy-associated peptide alpha (PAPP-A) as part of a national screening programme for biomarkers of Down's Syndrome and other conditions, and any remaining serum was stored at -80C. These samples were retrospectively linked to the participating mothers, following their recruitment at approximately 20-weeks gestation. A total of n=122 of these samples was subsequently thawed and transferred to separate vials (1ml aliquots per sample), which were further anonymised and sent for additional analysis at the Core Biochemical Assays Laboratory (CBAL) at Addenbrookes Hospital, Cambridge. These corresponded to a subset of the study cohort (n=219) (Figure 3.1), since, in many instances, serum had been depleted or discarded after routine prenatal testing prior to recruitment.

In addition, mothers were asked to fill in a Pregnancy History Questionnaire (PHQ), at the point of recruitment (mean gestational age of 20.3 [SD=0.4] weeks). The PHQ is a self-report inventory designed to collect information on metabolic, reproductive, and clinically diagnosed conditions of the mothers, pertaining to their current, as well as previous pregnancies. Maternal hirsutism was ascertained by the question 'During your adult years, have you found coarse, dark hair, growing in any of the following areas?', followed by drawings of multiple body areas that are prone to secondary hair growth (e.g. chest, lower face, upper or lower limbs), as described in previous studies (Barrett et al. 2018). Clinical history of sub-fertility, IVF and related treatments was also assessed in the PHQ.

Following labour, birth records containing information on neonate weight and gestational age at birth were also collected.

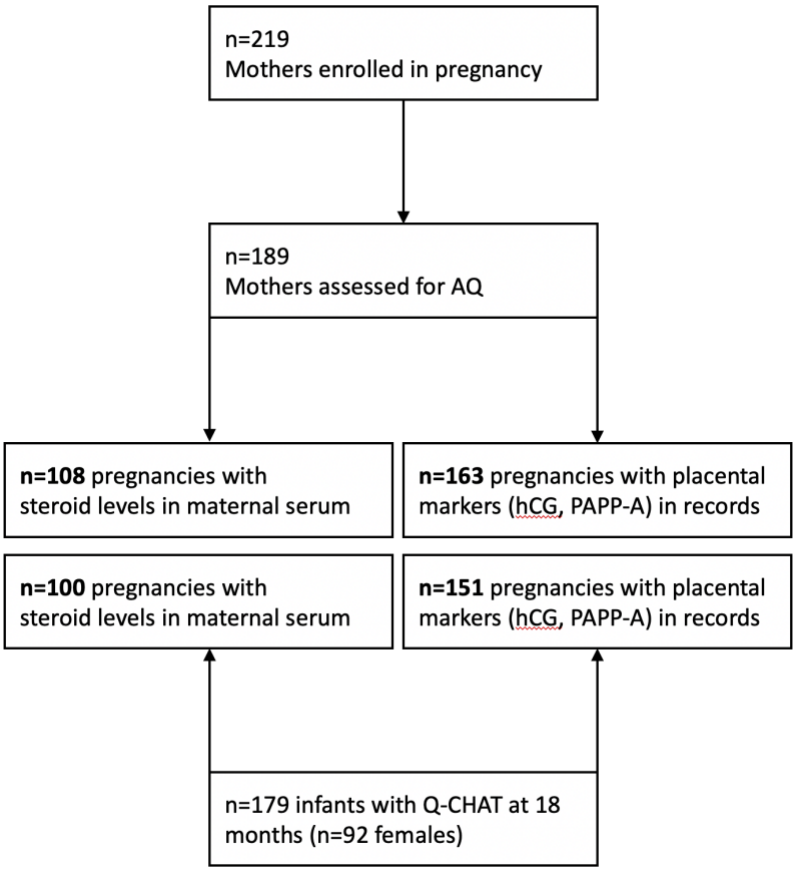


Figure 3.1: Flowchart of the study, showing different cohort sizes for each comparison of autistic traits (AQ or Q-CHAT) to prenatal measurements (steroid hormones or placental markers).

3.2.3 Additional Assays

The following steroids and peptides were assessed in terms of concentration: Testosterone (T), Estradiol (E2), Dehydroepiandrosterone sulphate (DHEAS), Progesterone (P), sex hormone-binding globulin (SHBG). Samples were analysed on a DiaSorin Liaison® XL automated immunoassay analyser using a one-step competitive chemiluminescence immunoassay for each hormone and two monoclonal antibodies for each peptide. All reagents, standards and consumables are those supplied by DiaSorin (DiaSorin S.p.A, 13040 Saluggia (VC), Italy). Batch quality precision data and concentration thresholds of detection for each assay are reported in the Appendix (Appendix Table 3.1).

3.2.4 Autistic Traits

Mothers were asked to complete the Autism Spectrum Quotient (AQ) – Adult version (Baron-Cohen et al. 2001) during or immediately after their 20-week routine ultrasound scan at the Rosie Hospital (mean gestational age of 20.3 [SD=0.4] weeks) . In addition, parents were invited via email to complete an online version of the Quantitative Checklist for Autism in Toddlers (Q-CHAT) (Allison et al. 2008) after their infant reached 18 months of age (mean=570 days, SD=21.3 days). All study data were collected and managed using REDCap electronic data capture tools hosted at the University of Cambridge (Harris et al. 2019; 2009).

For ethical reasons, the parents of children that scored highly on the Q-CHAT at 18 months (score of >39), as well as those with a diagnosed sibling or parent, were offered the opportunity to be profiled by trained staff with the 3di via an extensive, guided phone interview (Skuse et al. 2004; Slappendel et al. 2016). A report with the results of the Q-CHAT and 3di was then sent to the parents, which they could then show their GP or healthcare visitor and start a referral process for diagnosis. No diagnosis was suggested by the research staff, as the participants had not consented to this when they agreed to take part in the study.

3.2.5 Statistical Analysis

Autistic trait distributions (maternal AQ and infant Q-CHAT) were assessed for extreme outliers, as defined by an interval of three times the interquartile range. If present, these were reduced to the highest possible value within the interval to facilitate statistical testing.

All steroid hormone concentration values exhibited varying degrees of positive skew and were log-transformed to reduce this and facilitate subsequent statistical testing via linear regression. Following this, outliers were preserved in the analysis to retain clinical heterogeneity. Human choriogonadotropin (hCG) and pregnancy-associated peptide alpha (PAPP-A) values were retrieved from participants' clinical records. These had been standardised according to multiple of the median (MoM) by the Prenatal Screening

Department of the Trust according to maternal age, gestational age and the national means (Spencer et al. 1992; Wright et al. 2010).

Multivariate analysis was conducted by calculating composite scores for the free fractions of estradiol and testosterone, and by estimating overall steroidogenesis. The free testosterone index (FTI) and free estradiol index (FEI) were calculated via the following formulas (all concentrations in nmol/L) and then log-transformed for further statistical analysis, as previously suggested (Sollberger and Ehlert 2016):

FEI: (100*[E2])/([SHBG])

FTI: (100*[T])/([SHBG])

Latent factor analysis ('nFactors' package) was used to identify the optimal number of common steroidogenic factors based on their correlation matrix. Values for the predicted steroidogenic factor were calculated for each individual via the "Bartlett" method, based on the predicted loadings.

A series of clinical characteristics and group covariates were assessed for pairwise association with autistic traits. In cases of binary traits, differences were tested via Student's *t*-tests. These included comorbidity with PCOS or family history of autism, the latter being defined as present if the participating mothers reported having a first-degree relative (including previous child) that had been diagnosed with autism. A score of clinical severity of maternal hirsutism was devised based on responses in the Pregnancy History Questionnaire (PHQ), which the participating mothers completed following labour. A score of 1 denoted selection of one area of excess hair growth and 2 denoted more than one area. These were further treated as group variables (i.e., "no hirsutism", "one area", "more than one area") and were used in cohort comparisons in terms of hormone levels and autistic traits.

Circulating hormones were log-transformed and assessed for association with autistic traits. For AQ, pairwise Pearson's correlations were first used and then followed-up with a linear regression model with AQ as the outcome variable and the following predictor variables: hormonal concentration, maternal age, comorbidity with PCOS. To account for potential underlying associations between infant sex and Q-CHAT scores and preserve power, only multiple regression models were used in which an interaction term between

infant sex and hormonal concentration was added (for each hormone separately), with Q-CHAT scores as the outcome and addition of the following covariates: maternal age, maternal PCOS, maternal AQ scores, infant age at assessment, adjusted for gestational age at birth. This approach was preferred to stratifying for sex, given the low sample size compared to previous studies (Bilder et al. 2019). Family history of autism was not included as a covariate in these models, given the very small number of mothers with a diagnosed first-degree relative who also had available serum for hormone assays (n=1).

To ensure their validity, multiple regression models with significant findings for AQ or Q-CHAT were further tested for heteroscedasticity via the studentized Breusch-Pagan test and for the non-normality of their residuals via the Shapiro-Wilk test (Appendix Table 3.8).

3.3 Results

3.3.1 Cohort characteristics and autistic traits

Of the n=219 pregnant women who consented to take part in the study, n=17 had a first-degree relative with autism, n=26 had been diagnosed with PCOS, and n=89 responded positively to having excess body hair growth in the past. Overall mean age of the mothers was 32.4 years (SD=4.54). Of this cohort, n=189 completed the Autism Spectrum Quotient (AQ), with scores ranging from 1 to 47 (mean=14.63, SD=8.11) (Figure 3.1), while n=4 reported conceiving via IVF. None of the other women in the cohort declared a diagnosis of subfertility or receiving hormonal medications to assist with conception prior to their current pregnancy.

Women with a family history of autism had a significantly higher AQ score (n=13, mean=28[SD=14.36]) compared to women without any first-degree relatives with autism (n=176, mean=13.64[SD=6.51]) (Cohen's D=1.98, p=0.004) (Table 3.1). Of the women who replied to both questionnaires, those who reported excess body hair in more than one area of their body (n=51) also had significantly higher AQ scores (mean=16.8[SD=6.7]), than those without any sign of excess body hair growth (n=109, mean=13.8[SD=8.9])(Cohen's D=0.36, p=0.021)(Figure 3.2A). This effect persisted after controlling for family history of autism, maternal age, and a diagnosis of PCOS via a linear regression model (r=4.3[SE=1.3], semipartial r=0.21 , p=0.005) (Appendix Table 3.2).

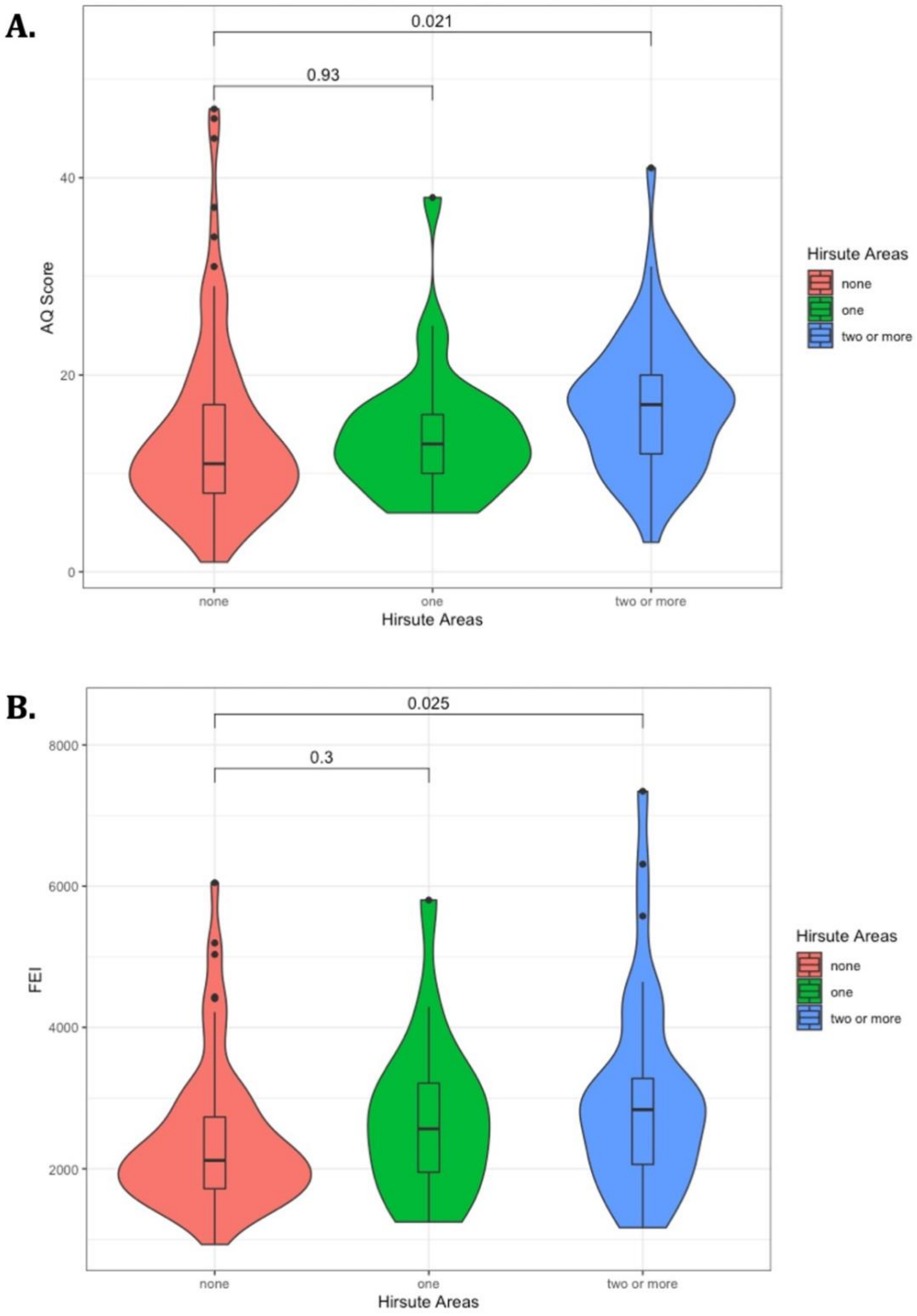


Figure 3.2: Violin-boxplots showing the distribution of (A) maternal autistic traits (AQ) and (B) free estradiol levels (FEI), according to clinical history of hirsutism. Women that reported two or more body areas affected had significantly higher levels of both A

Of these pregnancies, n=178 of the infants were followed-up with the Q-CHAT when they were older than 18 months of age (range: 541 to 671 days after birth), with most being assessed between 18 and 20 months (mean=570 days, SD=21.3 days) (Figure 3.1). Prior to statistical analysis, infant age was adjusted according to the gestational age at birth for each infant (mean=39.56, SD=1.5 weeks post-conception).

Regarding Q-CHAT scores, one extreme outlier was noted in the distribution (Q-CHAT=71), which was reduced to the highest value within an interval of three times the interquartile range (Q-CHAT=53), in order to reduce skewness and facilitate statistical comparisons.

There was no significant difference between Q-CHAT scores of males (mean=30.35[SD=8.13]) and female (mean=29.63 [SD=7.58]) infants at this time-point of assessment (Cohen's D=0.09, p=0.54).

Q-CHAT scores were significantly correlated with maternal AQ scores (Pearson's r=0.21, p=0.008) (Appendix Figure 3.1).

Table 3.1: Cohort characteristics and associations between clinical/demographic factors and autistic traits. Test coefficients are Cohen's D and Pearson's r correlation coefficient.

		AQ				***Q-CHAT***		
Categorical	***N***	***Mean traits [SD]***	***Cohen's D***	***p***	***N***	***Mean traits [SD]***	***Cohen's D***	***p***
History of autism	13 with	28.00 [14.4]	**D=1.98**	**0.0036**	n=14	31.00 [11.0]	D=0.14	0.720
	176 without	13.64 [6.5]			n=165	29.90 [7.5]		
Maternal PCOS	24 with	14.96 [6.7]	D=0.05	0.8043	n=22	27.86 [6.3]	D=0.31	0.115
	165 without	14.58 [8.3]			n=157	30.28 [8.0]		
Fetal sex	87 males	14.15 [8.1]	D=0.11	0.453	87 males	31.00 [8.1]	D=0.09	0.54
	102 females	15.04 [8.2]			92 females	29.63 [7.6]		
History of Hirsutism	109 none	13.8 [8.0]			102 none	29.27 [7.8]		
	29 in one area	13.9 [6.9]	D=0.01	0.93	28 in one area	31.5 [7.5]	D=0.28	0.181
	51 two+ areas	16.8 [6.9]	**D=0.36**	**0.021**	49 two+ areas	30.63 [8.0]	D=0.17	0.326
Continuous	***N***	***Mean factor***	***Pearson r***	***p***	***N***	***Mean factor***	***Pearson r***	***p***
Maternal age	n=154	32.50 [4.5]years	r=-0.05	0.462	n=135	32.60 [4.5]years	r=-0.14	0.065
Birth Weight	n=164	3399.5 [516.6]g	r=-0.04	0.596	n=157	3402.8 [509.8] g	r=-0.03	0.727
Infant age-adjusted					n=148	572,9 [23.8] days	r=-0.09	0.250

3.3.2 Hormone covariates and factor analysis

Hormone concentrations were only available in subsets of the cohort of women that consented to the study. This differed slightly for placental markers that were part of routine prenatal screening (hCG and PAPP-A) and steroid level measurements, which were analysed for research purposes on the remaining serum sample for each participant (Figure 3.1).

The analysed maternal serum samples corresponded to a narrow period of gestation between the late 1st and early 2nd trimester (mean=12.7 weeks, SD=0.8 weeks). Circulating hormones showed varying degrees of correlation with each other (Figure 3.3) and with other demographic and clinical variables. Testosterone, DHEAS and progesterone were all positively correlated with maternal age (Appendix Table 3.3). Unsupervised factor analysis showed that a common latent factor could be derived from estradiol, testosterone and DHEAS that accounts for 32% of the total variance in hormone levels. The values for this 'steroidogenic factor' were predicted for each participant based on the factor loadings and the concentrations of steroids (Appendix Figure 3.2).

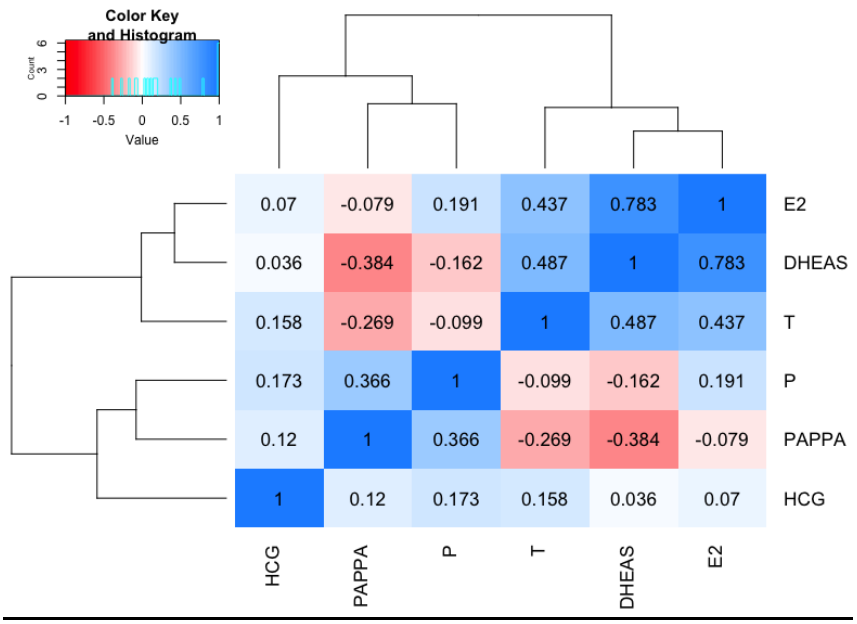


Figure 3.3: Heatmap and dendrogram of the pairwise correlations (Pearson's) of the log-transformed hormone concentrations.

Women with PCOS also had significantly higher levels of estradiol and progesterone, but lower levels of SHBG, compared to women without the condition (Appendix Table 3.4). Maternal age correlated positively with progesterone, but negatively with testosterone, FTI and DHEAS (Appendix Table 3.4). Women with a history of hirsutism in more than one area of their body, also had significantly higher levels of both estradiol (Cohen's D=0.47, p=0.034), FEI (Cohen's D=0.51, p=0.025) and the predicted steroid factor (D=0.50, p=0.028), but not androgens specifically (Figure 3.2B).

3.3.3 Association of hormones with maternal AQ score

The association between hormones in maternal serum and maternal autistic traits was investigated via univariate Pearson's correlation coefficient and with multiple regression (MR) controlled for maternal age and a diagnosis of PCOS (Table 3.2). FEI was significantly correlated with maternal AQ score using both methods (MR: semipartial r=0.24, p=0.022) (Figure 3.4A). Log-transformed estradiol levels were associated with AQ in Pearson's correlation (r=0.20, p=0.036) but this was not statistically significant in the multiple regression model that controlled for other covariates (MR: semipartial r=0.11, p=0.25).

Table 3.2: Pearson's correlation (hormone and AQ) and multiple regression models (MR) for predicting AQ scores, controlled for maternal age and a diagnosis of PCOS.

	Standardised Coefficient	***Standard Error***	***Semipartial Correlation***	***p - value***
Estradiol				
Pearson's	**r=0.20**	**0.09**		**p=0.036**
MR [1]: intercept	r=4.52	12.119		p=0.714
: predictor	r=1.43	1.23	r=0.11	p=0.25
Testosterone				
Pearson's	r=0.09	0.10		p=0.377
MR [1]: intercept	r=17.34	4.50		p<0.001
: predictor	r=0.34	1.11	r=0.03	p=0.761
DHEAS				
Pearson's	r=-0.09	0.10		p=0.379
MR [1]: intercept	r=15.29	6.47		p=0.02
: predictor	r=0.44	0.85	r=0.06	p=0.569
Progesterone				
Pearson's	r=0.09	0.09		p=0.365
MR [1]: intercept	r=23.23	6.94		p=0.001
: predictor	r=-2.16	2.22	r=-0.09	p=0.365
hCG MoM				
Pearson's	r=-0.03	0.08		p=0.665
MR [1]: intercept	r=13.92	0.97		p<0.001
: predictor	r=-0.26	0.57	r=-0.03	p=0.65
PAPP-A MoM				
Pearson's	r=-0.04	0.08		p=0.589
MR [1]: intercept	r=14.18	1.16		p<0.001
: predictor	r=-0.49	0.82	r=-0.04	p=0.56
Composite measures				
Free Estradiol Index				
Pearson's	**r=0.22**	**0.09**		**p=0.019**
MR [1]: intercept	**r=12.81**	**4.63**		**p=0.007**
: predictor	**r=1.15**	**0.5**	**r=0.24**	**p=0.022**
Free Testosterone Index				
Pearson's	r=0.11	0.09		p=0.258
MR [1]: intercept	r=13.33	5.16		p=0.145
: predictor	r=5.80	3.91	r=0.14	p=0.124
Steroid Factor				
Pearson's	r=0.18	0.09		p=0.061
MR [1]: intercept	r=16.75	0.09		p<0.001
: predictor	r=0.77	0.55	r=0.17	p=0.085

3.3.4 Association of hormones with infant Q-CHAT score

Log-transformed concentrations of hormone levels were studied in relation to infant Q-CHAT with multiple linear regression models that controlled for birth weight, maternal PCOS, maternal AQ and infant age at the time of Q-CHAT, adjusted for gestational age at birth. The interaction between hormone concentration and infant sex was also used as a variable in the same model (Table 3.3). Infant sex moderated the association between maternal estradiol levels and QCHAT scores, with a positive association for Q-CHAT in males but not females (hormone-by-sex: semipartial r=0.23, p=0.036) (Tables 3.3, Figure 3.4B).

Standardised hCG MoM values were significantly associated with Q-CHAT scores, with a significant moderating effect for infant sex (hormone-by-sex: semipartial r=-0.26, p=0.005). This effect followed the opposite pattern to estradiol, with a positive relationship in females but not males (Table 3.3).

All multiple regression models that yielded significant results (for both AQ and Q-CHAT) passed tests on the assumptions of homoscedasticity and normality of residuals (full model results: Appendix Tables 3.6 - 3.8).

Finally, a sensitivity analysis for the effects of sex steroids was conducted in a subset of the cohort, in which the n=4 women who had conceived via IVF were excluded (Appendix Table 3.9). There was a consistent trend with the previously reported results for the entire cohort but the associations were marginally non-significant in this subset (AQ-FEI: p=0.065 / QCHAT-E2: p=0.052).

Table 3.3: Multiple regression models for predicting Q-CHAT scores and accounting for a hormone-by-sex interaction, further controlled for maternal AQ, a diagnosis of PCOS, maternal age, birth weight and infant age at Q-CHAT assessment-adjusted for gestational age at birth.

	QCHAT			
	Standardised Coefficient	**Standard Error**	**Partial Correlation Coeff.**	**p - value**
Estradiol				
MR [1] intercept:	r=114.20	35.21		p=0.002
hormone:	r=-4.00	2.311	-0.19	p=0.087
hormone-by-sex:	**r=8.27**	**4.08**	**0.23**	**p=0.036**
Testosterone				
MR [1]intercept:	r=80.01	29.07		p=0.007
hormone:	r=-1.95	2.12	-0.10	p=0.362
hormone-by-sex:	r=0.99	3.28	0.03	p=0.765
DHEAS				
MR [1] intercept:	r=90.03	29.68		p=0.003
hormone:	r=-2.33	1.58	-0.17	p=0.144
hormone-by-sex:	r=1.36	3.22	0.05	p=0.674
Progesterone				
MR [1]intercept:	r=84.06	32.16		p=0.011
hormone:	r= -3.70	4.65	-0.18	p=0.429
hormone-by-sex:	r=11.64	6.86	0.21	p=0.095
hCG MoM				
MR [1]intercept:	r=34.2	20.61		p=0.10
hormone:	**r=2.95**	**1.07**	**0.25**	**p=0.007**
hormone-by-sex:	**r=-4.34**	**1.53**	**-0.26**	**p=0.005**
PAPP-A MoM				
MR [1]intercept:	r=46.04	21.13		p=0.031
hormone:	r=1.69	1.45	0.11	p=0.245
hormone-by-sex:	r=-1.37	2.29	-0.06	p=0.552
Composite measures				
Free Estradiol Index				
MR [1]intercept:	r=82.42	29.02		p=0.006
hormone:	r=-0.969	1.37	-0.02	p=0.481
hormone-by-sex:	r=1.989	1.68	0.11	p=0.240
Free Testosterone Index				
MR [1]intercept:	r=77.86	29.16		p=0.009
hormone:	r=2.50	8.78	r=0.03	p=0.777
hormone-by-sex:	r=-9.53	12.15	r=-0.09	p=0.417
Steroid Factor				
MR [1]intercept:	r=85.95	29.01		p=0.004
hormone:	r=-1.95	1.19	r=-0.19	p=0.108
hormone-by-sex:	r=2.80	1.81	r=0.20	p=0.081

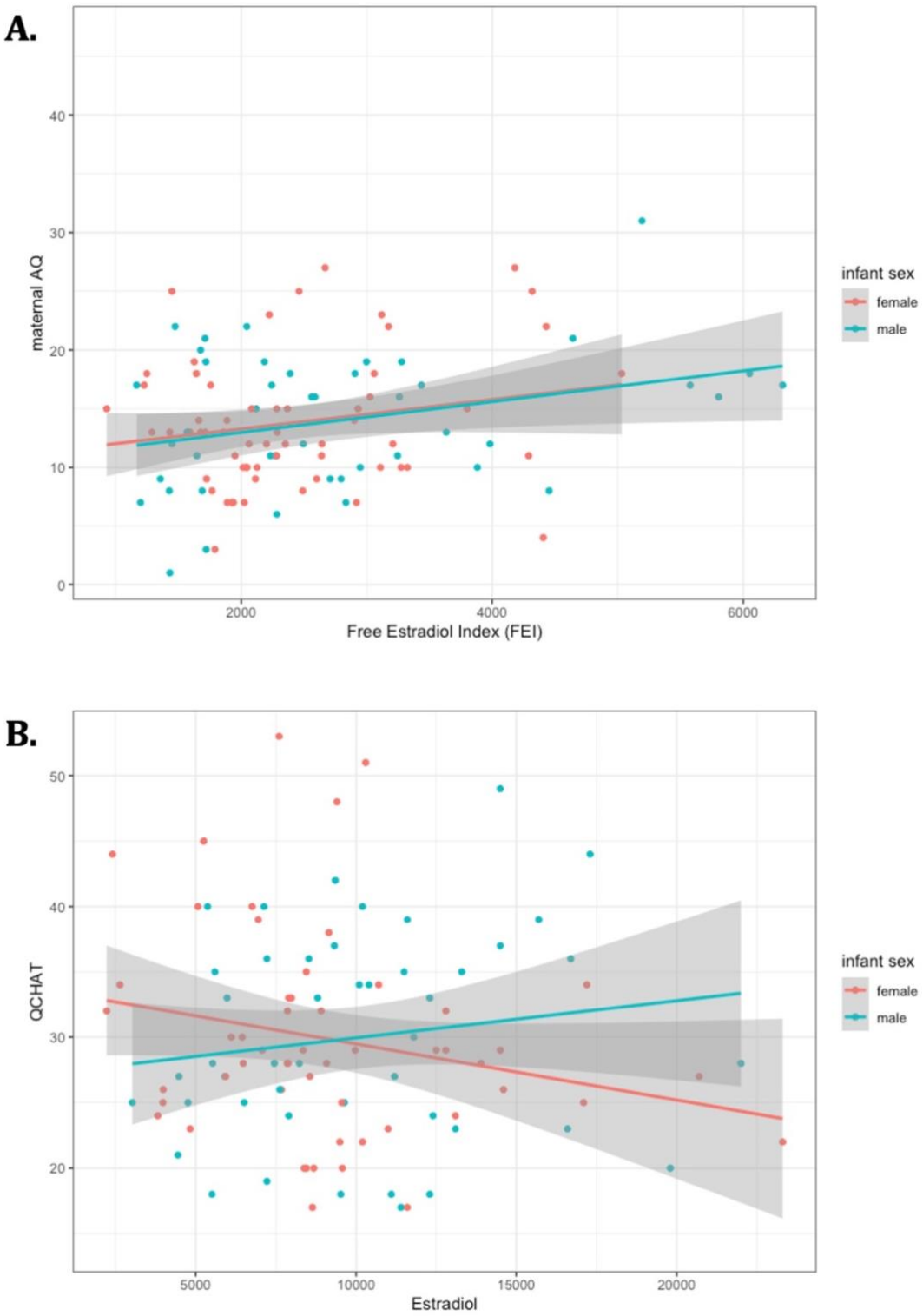


Figure 3.4: Scatterplots with linear fit-models for (A) the association between maternal free estradiol (FEI) and AQ score, and (B) the association between maternal estradiol and Q-CHAT; separate linear models are presented for each sex and show significant interactions with infant sex.

3.4 Discussion

Study 2 is the first clinical longitudinal study to report on the endocrine profile of pregnant women and how this relates to their own autistic traits and to the autistic traits of their infants. First, it was found that the fraction of free circulating estradiol (FEI) during pregnancy correlated positively with the autistic traits of pregnant neurotypical women. Second, maternal estradiol was associated with infant autistic traits (as measured by the Q-CHAT) in a sex-dependent way, with a positive correlation in males but not females. Third, the opposite was true for standardised hCG MoM values, where a negative correlation with Q-CHAT scores in males was noted. These associations with early neurodevelopment were independent of the mother's AQ score or PCOS status, as well as other infant characteristics such as birth weight or age at the time of assessment. Finally, this is the first longitudinal study to show a significant positive correlation between maternal and infant autistic traits, as measured by the AQ and Q-CHAT respectively.

The Q-CHAT is a relatively new measure of autistic traits in toddlers, and has been validated in independent cohorts around the world (Allison et al. 2008; Ruta et al. 2019; Roman-Urrestarazu et al. 2020; Allison et al. 2021). At this early developmental stage, it is generally challenging to note that the majority of the children who will later be diagnosed with autism. However, it is important to clarify that the Q-CHAT has fared better than the more widely used M-CHAT in this regard (Yuen et al. 2018). Another advantage of the Q-CHAT over the M-CHAT is that it dimensionalises autistic traits, as it is graded on a Likert scale and therefore allows for greater variance in estimating each trait. Specifically for this line of research, the Q-CHAT is also useful as it also captures the small sex differences in infant milestones in sufficiently large cohorts (Allison et al. 2021). In this study, the Q-CHAT was mostly rated by the participating mothers, so a correlation between their autistic traits (on the AQ) and the Q-CHAT could be attributed to heritable factors, but also to rater bias. For this reason, maternal AQ was controlled for in the multiple regression models pertaining to the infants.

The association between free circulating estradiol and maternal AQ was independent of maternal age or a diagnosis of PCOS. In addition, women with clinical history of excess body hair growth in more than one area of their body also had significantly higher autistic traits scores, as well as higher levels of circulating estradiol and FEI (Figure 3.2). This is

consistent with previous studies that reported higher rates of steroid-exposure related symptoms, such as hirsutism in autistic women (Pohl et al. 2014). The correlation between FEI and AQ was independent of PCOS diagnostic status, and women diagnosed with PCOS did not have higher AQ scores (Table 3.1). This may be due to reduced statistical power (n=13 with PCOS diagnosis) or the fact that all the women in this cohort had no autism diagnosis themselves and were without significant challenges in fertility, contrary to previous studies that found a link between PCOS and autism (Cherskov et al. 2018).

There was no association between testosterone levels or the FTI and maternal autistic traits in this study. Testosterone correlated positively with estradiol (Appendix Table 3.3) but did not differ between women with and without PCOS or correlate with a clinical history of hirsutism. Previous studies have also shown that circulating testosterone levels measured at a single time-point do not always correlate closely with their associated clinical parameters, such as hair growth (Pasquali et al. 2016). Particularly during pregnancy, estradiol levels may be more clinically informative, as testosterone is rapidly aromatised into estradiol by the placenta. Estradiol could then be interpreted as the end-product of wider steroidogenesis and may be a better biomarker of the 'steroidopathy' previously indicated by epidemiological studies of autistic women (Pohl et al. 2014).

This is also the first study to investigate circulating hormones in maternal serum in relation to autistic traits in their infants measured between 18 and 20 months of age via the Q-CHAT. This questionnaire was developed to enable parents to quantify autistic traits, moving away from a restrictive binary format of items into a Likert format. To date, the Q-CHAT has been validated in several studies. Interestingly, the Q-CHAT also shows sex differences in early autistic traits, with males scoring significantly higher than females in larger cohorts (Allison et al. 2008; Ruta et al. 2019; Roman-Urrestarazu et al. 2020; Allison et al. 2021).

Study 2 found significant moderation effects of infant sex on the associations between hormones and neurodevelopment. Specifically, increased maternal estradiol was more predictive of Q-CHAT scores of males than females (Figure 3.4, Table 3.3). This was independent of potential confounding variables, such as maternal age, diagnosis of PCOS and maternal autistic traits. Contrary to the results with the AQ, free estradiol levels (FEI), as estimated via serum SHBG, were not predictive of Q-CHAT scores. SHBG is a peptide

and it does not cross the placenta as easily as steroids, as shown in rare cases of partial deficiency in mothers but not their fetus (Hogeveen et al. 2002). Maternal SHBG may therefore not accurately capture the bioavailability of steroids in the fetal circulation or accurately predict potential effects on infant neurodevelopment.

Further research is needed to understand the interaction between maternal estradiol levels and fetal sex in predicting Q-CHAT scores. As with the observed male bias in autism diagnoses, the lack of an association between hormone levels in females may reflect behavioural differences that are not adequately captured by this particular instrument (Lai and Szatmari 2020). Alternatively, this interaction may represent evidence of differential liability to prenatal hormone levels, whereby males are affected more than females by the same maternal endocrine factors. Sine males are undergoing an additional increase in steroid levels during mid-pregnancy, due to the activation of the testes, this interaction could also be attributed to the added effect of elevated steroids in the fetal circulation of males (Welsh, Suzuki, and Yamada 2014). While fetal steroids were not been measured directly in this study, previous comparisons in humans showed that estradiol levels correlated between maternal serum and amniotic fluid, in both the 2nd and 3rd trimester (van de Beek et al. 2004). The same was found for hCG levels in the 2nd trimester (Steier, Myking, and Bergsjø 1999). In contrast, increases in androgen levels of male pregnancies, were not detectable in maternal serum (van de Beek et al. 2004). Therefore, the interaction of sex with estradiol levels in predicting Q-CHAT scores, in this study, could potentially be attributed to the additive effect of elevated androgens in the fetal circulation of males, which may not have captured by assaying maternal serum, rather than amniotic fluid (Auyeung et al. 2010).

The findings with hCG mirror those for estradiol, showing a negative association with Q-CHAT scores in females but not males. hCG is produced by the developing trophoblast cells in the placenta and regulates early implantation as well as steroid production (Canfield et al. 1987). hCG levels also show baseline sex differences in typical cohorts as early as the first half of pregnancy (Adibi et al. 2015). In the current cohort, hCG was measured late in the 1st trimester, during the first ultrasound visit, as part of the screening programme for Down's syndrome. This is part of routine prenatal screening in the UK to test for placental dysfunction that can often be indicative of genomic instability due to aneuploidies (Wright et al. 2010). In cases of clinically diagnosed autism, both very low

and very high levels of hCG have been found in maternal serum, leading to a "U-shaped" association when studying both males and females (Windham et al. 2016). Furthermore, autistic traits in the children have been associated with the severity of nausea and 'morning sickness' during pregnancy; symptoms that have also been linked to high hCG levels (Whitehouse et al. 2018; Goodwin et al. 1992). Further studies into placental functionality could offer insight into these observations, the role of sex, and more specifically, whether the observed interaction effect for Q-CHAT score is part of an adaptive response that is more pronounced in females.

3.4.2 Limitations

Limitations of the current study include the relatively small sample size, as well as potential ascertainment bias given the voluntary process of recruitment. The participating women in the study may represent a subset of the wider population, in terms of personality traits, as they consented to a longitudinal study with a two-year timeline, to an additional ultrasound visit for their baby and to the prenatal screening tests in the first place. This willingness to participate in prenatal testing has been profiled and shown to differ between cultures, as well as according to individual circumstances, such as the nature of the condition (pregnancy onset vs childhood onset) or having a previous child with a similar condition (Gottfredsdóttir, Björnsdóttir, and Sandall 2009; A. E. Raz, Amano, and Timmermans 2019). Nevertheless, the range of maternal AQ scores was within the normative observations of volunteers in previous studies. In addition, it is important to consider that AQ and Q-CHAT scores were both rated by the mother herself. This was addressed by controlling for maternal AQ when studying associations between hormones and infant Q-CHAT scores. The items of the AQ-Adult and Q-CHAT are also substantially different, with the latter dealing with behavioural and developmental milestones that are specific to infants rather than to interests and personality traits that are more evident in adulthood. Furthermore, the findings of the current study may have been inflated by Type I errors, as p-value thresholds were not corrected for multiple testing. However, the findings on estradiol were consistent for both maternal and infant outcomes (Figure 4), were reflected in clinical hirsutism differences (Figure 2), and are in accordance with those observed in other studies of clinical autism (Baron-Cohen et al. 2019; Bilder et al. 2019). The high degree of correlation between many of the assessed

hormones (Figure 3) also indicates a common functional and regulatory framework. The association tests for individual hormones may then, not be entirely independent, but instead affected by a common steroidogenic factor as previously reported (Baron-Cohen et al. 2015). Replication of these findings in a larger, independent cohort is warranted to confirm their validity. In addition, a larger cohort could provide sufficient statistical power to allow a sex-stratified analysis, which could further explain the observed interaction and differing effects of maternal hormones on infant autistic traits (Figure 3.4B). Finally, to improve on these population-level associations, multiple-of-median (MoM) values for estradiol and testosterone should also be developed and studied, as they are more specific to confounding parameters (e.g. gestational age, ethnicity etc) and are better suited for individual prognosis.

3.4.3 Conclusions

Study 2 is the first longitudinal study to report associations between maternal steroidogenic factors and the autistic traits in both the mother and her infant, with significant moderating effects of sex being noted for the latter.

In the following chapters, additional research is presented in order to establish how maternal steroidogenesis may affect fetal neurodevelopment, and how these processes interact with genetics to disproportionately increase the liability for autism and autistic traits in males.

Chapter 4
Study 3 - Sex and the placenta

Summary of Study 3

Placental complications are more frequent in pregnancies of males, as well as of children that later receive an autism diagnosis. In Study 3, concentrations of angiogenesis-related markers, placental growth factor (PlGF), soluble fms-like tyrosine kinase (sFlt-1) and plasminogen activator inhibitor (PAI-2), were assessed in maternal plasma of expectant women in the late 1st (mean= 13.5 weeks gestation) and 2nd trimesters (mean=20.6 weeks gestation), as part of the Generation R Study, in Rotterdam. Subsequent assessment of autistic traits in the offspring at age 6 was performed with the 18-item version of the Social Responsiveness Scale (SRS). Diagnosis of autism was based on medical records. Z-scores of SRS scores were examined in association with the log-transformed concentrations of placental proteins, in sex-stratified univariate and multiple regression models that adjusted for various covariates. Maternal placental protein concentrations were further compared between children with and without a later autism diagnosis. PlGF was significantly higher in the pregnancies of males and increased faster from 1st to 2nd trimester compared to pregnancies of females. PlGF levels in the 2nd trimester, as well as the rate of PlGF elevation between measurements, both correlated to SRS scores in females (n=1804), with a similar trend for the latter in males (n=1665), in multiple regression models adjusting for various covariates. Autistic males (n=56) had significantly lower sFflt-1 levels compared to undiagnosed males (Cohen's D=0.28, p=0.027). In conclusion, male-type shifts in the levels of these markers, namely high PlGF and low sFlt-1, are associated respectively with higher SRS scores in females and a diagnosis of autism in males. Sex differences in placental adaptive mechanisms may be contributing to sex differences in neurodevelopmental liability.

4.1 Introduction

As it was discussed in Chapter 1, sex differences in prenatal physiology extend beyond steroid level differences and include tissues such as the placenta (section 1.5, Chapter 1). In Studies 2 and 3, prenatal estrogens were found respectively to be associated with autism in males and with autistic traits in mothers (as a free fraction) and their female infants. Prenatally, estrogens and other steroids are derived from various endocrine sources that are mainly regulated by the placenta. In addition to aromatising androgens to estrogens, 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 (Rosenfeld 2020; Nugent and Bale 2015; Santos et al. 2020).

In autism, atypical placental morphology indicating excess proliferation (Straughen et al. 2017; Anderson et al. 2007), increased placental inflammation and increased placental size (Park et al. 2018) 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 (Maher et al. 2020; 2018). Therefore, the placenta may be mediating sex differences in neurodevelopmental and autism likelihood, since male placentas produce more steroids, and are more prone to complications, such as early miscarriage, pregnancy-induced hypertension and spontaneous preterm birth (Gong et al. 2018; Orzack et al. 2015; Verburg et al. 2016).

Study 3 uses data from 'Generation R', 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 in terms of autistic traits (e.g., on the Social Responsiveness Scale - SRS), autism diagnoses, as well as 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 (Brown et al. 2014). PlGF and sFlt-1 have opposing

regulating properties on angiogenesis, via activation and suppression of VEGF-related signalling respectively. PAI-2 is synthesised by the mature trophoblast and is thought to regulate coagulation in the newly formed blood vessels of the placenta. These proteins have been proposed by several research groups as potential biomarkers for placental dysfunction, such as in cases of gestational hypertension and preeclampsia (McLaughlin et al. 2021).

However, it has not yet been examined if sex differences in these placental markers are linked to established sex differences in neurodevelopmental outcomes, such as autism spectrum traits (Bale 2016).

In this Chapter, these placental proteins are studied in terms of baseline sex differences, which are then associated with the development of autistic traits in children of the 'Generation R' birth cohort.

4.2 Methods

4.2.1 The cohort

Study 3 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 (Blanken et al. 2018). 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 also being followed-up regularly, with both in-person and questionnaire-based measures of their overall development.

For this analysis, only singleton live births were initially included (n=9506) (Figure 4.1). 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 Appendix Table 4.1. With regards to ethnicity, participating mothers and fathers reported it based on classifications recommended by 'Statistics Netherland' and this was further divided into groups for the purposes of statistical analysis (Appendix 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.

4.2.2 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, via immune electrochemiluminescence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, The Netherlands) in ng/ml and pg/ml respectively. Concentrations of PAI-2 in ng/ml were only measured in the first time-point via enzyme-linked immunosorbent assay. Further details on the protocols of recruitment and sample processing have been previously reported (Brown et al. 2014).

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 (M. A. Brown et al. 2001). 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") referred to infants of lower birth weight than a cut-off specified by 1.5 times the lower bound of the interquartile range of the entire cohort. These pregnancy complications (preeclampsia, PIH, spontaneous preterm birth and SGA) were classified as relating to the placenta.

4.2.3 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 (Blanken et al. 2018; Vinkhuyzen et al. 2018). 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 (Kooijman et al. 2016).

4.2.4 Autism diagnoses

All diagnoses of the children were made in the community by specialised healthcare professionals and according to the criteria the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV/5 or the International Classification of Primary Care (ICPC). Linkage to the children's 'Generation R' records was achieved following targeted contact of the families' dedicated general practitioners, who are required by the Dutch healthcare regulatory authorities to collect all records and assessments of their patients within the practice or conducted by specialised services, as in the case for autism diagnoses. General practitioners were contacted in order to submit these records in a targeted drive that prioritised children, who had scored high in questionnaires of neurodevelopmental deficits during the study (the CBCL and SCQ - with sex-specific thresholds), as well as children whose parents had reported that the child had undergone a diagnostic assessment for autism, at any point during their participation in the study. This contact drive and obtaining of specialist records which confirmed diagnoses, took place before study participants had reached 9 years of life, with the a mean age of diagnosis at 6 years of age, as described in previous Generation-R publications (Blanken et al. 2018).

4.2.5 Statistical Analysis

Baseline cohort 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 specify 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.

To facilitate statistical comparisons with linear regression, distributions of plasma-derived placental markers were log-transformed to reduced pronounced skewness. The transformed concentrations for sFlt-1 and PlGF were compared between the sexes, via

both pairwise Mann-Whitney U-tests, as well as via multiple linear regression, with fetal sex at birth as the predictor, and further controlling for gestational age at the time of plasma collection and for placental weight at birth.

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. This was done to measure the rate of change, similarly to the computation of growth velocities in previous autism studies (Regev et al. 2021), as well as to standardise more effectively for gestational age variance between the time-points.

Weighted SRS scores of the entire cohort also exhibited significant skew (3.08). To address this and facilitate statistical testing, z-scores were computed according to the properties of their distribution in the entire cohort (from here on "SRS scores"). 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).

Prior to regression analyses, the log-transformed concentrations of the placental proteins were first adjusted for the effects of gestational age and placental weight at birth, via linear regression. The residuals were then added to the intercept and further examined in association with SRS Scores. This analysis was stratified by sex and every placental variable (including the rate of PlGF elevation - 'PlGF-e') was studied separately in two multivariable regression models. Model 1 was controlled for the age of the child at the time of SRS scoring. Model 2 was further controlled for the following covariates: maternal age, maternal BMI in the beginning of the pregnancy, maternal ethnicity, birth weight adjusted for gestational age and maternal education level.

Nominally significant results in both models were further scrutinised in sensitivity analyses, that restricted the cohort to the following categories, excluding potential outliers for both autistic traits and placental function: First, in pregnancies of mostly European maternal ethnicity, given observed differences in SRS scores of potentially cultural origin (Bölte, Poustka, and Constantino 2008). 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 (Appendix 4). Finally, the levels of placental protein markers were compared between pregnancies of males diagnosed with autism and those without a diagnosis via Mann-Whitney U-tests.

4.3 Results

4.3.1 Neurodevelopmental outcomes

The current analysis was restricted to a subset of the Generation R cohort (n=7,293), in which placental marker measurements were available in maternal plasma at two time-points of pregnancy (Figure 4.1). A further subset of them also had available scores for autistic traits (n=3,469), as measured on the SRS at age 6 (mean age=74 months, SD=5.8 months). In this subset of the Generation R cohort, n=56 males and n=9 females had been diagnosed with autism by the time this study commenced. Autistic traits, as scored on the SRS, differed significantly between the sexes, with males scoring significantly higher than females (Figure 4.2A). SRS Scores also correlated to the age of the child at the time of assessment, as well as and to the age of the mother (Appendix Table 4.1). Further comparative assessment of SRS Scores with regard to cohort characteristics and demographics has been previously published elsewhere (Amiri et al. 2020).

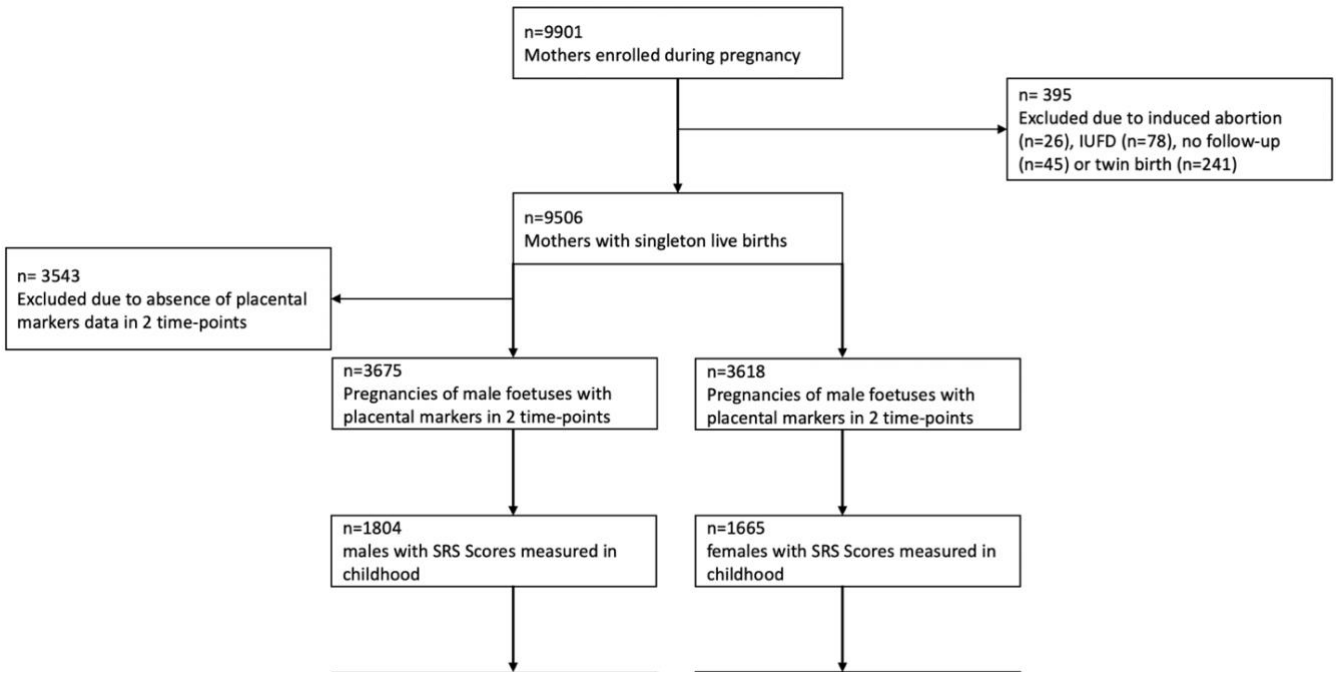


Figure 4.1: Flowchart of the study with the sample sizes used for comparison of placental markers in association with autistic traits and a diagnosis of autism.

4.3.2 Placental markers in maternal plasma

Placental markers showed varying degrees of change between the two time-points of measurement 1st and 2nd trimesters. PAI-2 was only measured in the 1st time-point. sFlt-1 only 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 all placental-derived proteins correlated significantly to each other, both 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 to each other in varying degrees (2nd trimester PlGF-sFlt-1: Pearson's r=0.12, p<0.0001)(Appendix Figure 4.1), as well as to a variety of maternal characteristics (Appendix Table 4.2).

In the 1st trimester, as previously reported, all three placental markers were significantly lower in pregnancies of males (Brown et al. 2014). 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 (Figure 4.2B). These sex differences were significant in pairwise Mann-Whitney U-tests (Table 4.1) and in multiple regression models, that also controlled for gestational age and placental weight (Appendix Table 4.3).

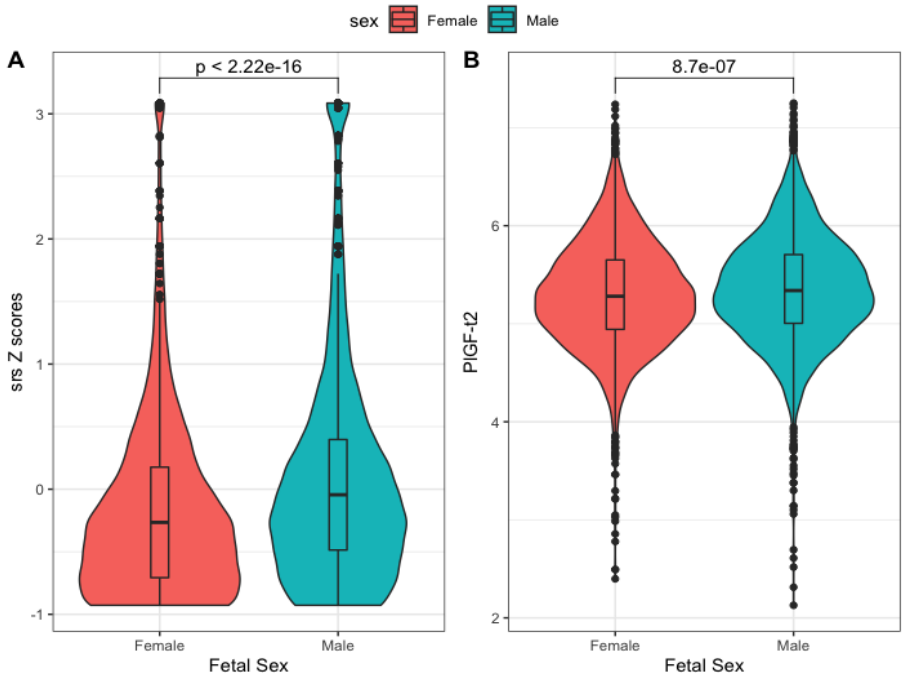


Figure 4.2: Males have significantly higher (A) autistic traits and (B) PlGF levels in maternal plasma at the 2nd trimester. P-values are of U-tests of SRS z-scores and PlGF concentrations respectively.

1st trimester	*N*	Mean	Mean Males	Mean Females	p - value for sex difference
PAI-2 ng/ml	5900	42.13	40.76	43.51	<0.0001
PlGF pg/ml	5963	59.46	58.87	60.06	0.001
sFlt-1 ng/ml	5951	5.82	5.58	6.07	<0.0001
2nd trimester					
PlGF pg/ml	7294	239.41	246.84	231.81	0.0001
sFlt-1 ng/ml	7292	6.11	5.82	6.38	<0.0001
Longitudinal					
PlGF - e	5250	25.89	26.74	25.02	0.007

Table 4.1: 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 elevation (PlGF-e) between them. P-values for sex-difference correspond to comparisons of the log-transformed values via U-tests.

4.3.3 Association of placental markers with neurodevelopmental outcomes

In sex-stratified analysis, higher PIGF levels were associated with more autistic traits (SRS scores) in females, when controlling for age at the time of assessment (Model 1: β=0.17, p<0.0001) and various other covariates, including maternal age, BMI and birth weight adjusted for gestational age (Model 2: β=0.09, p=0.004). The rate of change of PlGF elevation between time-points was also positively correlated to the autistic traits in females (Model 1: β=0.003, p=0.0008/ Model 2: β=0.002, p=0.01), with a notable trend in males (Model 1: β=0.003, p=0.02 / Model 2: β=0.002, p=0.09). There was no association of PAI-2 or sFlt-1 levels at any single time-point, with the autistic traits of either males or females (Table 4.2).

	MALES				*FEMALES*			
1st trimester	N	β	p	Model Adj-R^2	N	β	p	Adj-R^2
PAI-2								
Model 1	1478	-0.005	0.952	0.012	1405	0.004	0.958	0.004
Model 2		0.078	0.132	0.088		0.082	0.064	0.094
PIGF								
Model 1	1504	0.040	0.518	0.012	1408	**0.153**	**0.001**	0.013
Model 2		0.040	0.241	0.090		**0.098**	**0.000**	0.094
sFlt-1								
Model 1	1502	0.050	0.328	0.013	1408	0.033	0.434	0.005
Model 2		0.063	0.120	0.087		0.032	0.320	0.094
2nd trimester								
PIGF								
Model 1	1804	0.046	0.410	0.012	1665	**0.171**	**<0.0001**	0.018
Model 2		0.051	0.171	0.085		**0.087**	**0.004**	0.084
sFlt-1								
Model 1	1802	-0.015	0.740	0.012	1665	-0.0001	0.999	0.004
Model 2		-0.001	0.977	0.085		-0.0142	0.588	0.081
Longitudinal								
PIGF-e								
Model 1	1367	**0.003**	**0.021**	0.015	1260	**0.0033**	**0.001**	0.011
Model 2		0.002	0.087	0.097		**0.0024**	**0.011**	0.095

Table 4.2: Association of z-scores of child SRS Scores with placental protein concentrations in maternal plasma, adjusted for gestational age at time of measurement and placental weight. 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, birth weight adjusted for gestational age and maternal education level.

4.3.4 Sensitivity & Mediation analyses

Sensitivity analyses were performed in subsets of the original cohort, by excluding potential outliers for autistic traits (non-European ethnicities or autism diagnoses) or for placental markers levels (pregnancies with placental complications) (Appendix Table 4.4). These confirmed that PlGF levels in the 2nd trimester, as well as the rate of PlGF-elevation continued to be significantly associated with autistic traits in females.

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, at both time-points of measurement. This effect was negative in the 1st trimester (ACME: -0.006, p=0.006) and positive in the 2nd trimester (ACME: 0.005, p=0.032), mirroring the reversal of baseline sex differences in PlGF levels in the second time-point (Table 4.1). The rate of PlGF-elevation also significantly mediated part of the association of sex with SRS Scores (ACME: 0.004, p=0.036). This was not found for other variables with pronounced sex differences, such sFlt-1 levels or PAI-2.

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
PAI-2	-0.005 (-0.01 - 0.00)	0.15	0.26 (0.19 - 0.32)	<0.0001
PlGF	**-0.006 (-0.01 - 0.00)**	**0.006**	0.26 (0.19 - 0.33)	<0.0001
sFlt-1	-0.004 (-0.01 - 0.00)	0.29	0.25 (0.19 - 0.32)	<0.0001
2nd trimester				
PlGF	**0.005 (0.00 - 0.01)**	**0.032**	0.24 (0.117 - 0.31)	<0.0001
sFlt-1	0.001 (-.00 - 0.01)	0.63	0.25 (0.18 - 0.32)	<0.0001
Longitudinal				
PlGF - elevation	**0.004 (0.00 - 0.01)**	**0.036**	0.24 (0.18 - 0.30)	<0.0001

Table 4.3: Mediation of placental markers on sex differences in autistic traits (SRS scores). Sex differences in prenatal PlGF levels mediate a small part of the sex differences in SRS scores at age 6.

4.3.5 In autism cases

Case-control comparisons between diagnosed (n=56) and undiagnosed males (n=3619) showed that placental proteins in maternal plasma of the 1st trimester did not differ between them. In the 2nd trimester, sFlt-1 levels were significantly lower in male pregnancies of children later diagnosed with autism (Cohen's D=0.28, p=0.027)(Appendix Figure 4.3). This was further confirmed in a multiple logistic regression model of the entire cohort (both male and females). This model controlled for birth weight and tested for an interaction between fetal sex and sFlt-1 levels for autism status, which was also significant (β=1.66,p=0.045) in addition to the effects of sex and sFlt-1 (Appendix Table 4.5).

1st trimester	*Mean no-diagnosis (n=3619 males)*	*Mean Autism (n= 56 males)*	*p-value of group comparison*
PAI-2 ng/ml	40.77	40.35	0.50
PlGF pg/ml	58.95	54.63	0.91
sFlt-1 ng/ml	5.59	4.90	0.14
2nd trimester			
PlGF pg/ml	247.15	226.42	0.45
sFlt-1 ng/ml	**5.84**	**4.83**	**0.027**
Longitudinal			
PlGF - elevation	26.77	25.52	0.97

Table 4.4: Comparison of placental protein concentrations in maternal plasma between males diagnosed with autism (n=56) and males without a diagnosis (n=3619). P-values correspond to Mann Whitney U-tests. Placental markers were previously log-transformed and adjusted for their associatinos with gestational age at measurment and placental weight

4.4 Discussion

Study 3 investigates sex differences in placental markers of angiogenesis and their association with neurodevelopmental outcomes, as well as a diagnosis of autism in childhood, in a large prospective, population-based cohort.

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. Although these markers have been largely studied in association with placental and cardiovascular health, they may still be relevant for neurodevelopment. For example, PlGF has been shown to induce proliferation of neuronal Schwann cells, to facilitate cortical expansion (Dewerchin and Carmeliet 2012), and increase the permeability of the maternal blood brain barrier in rodents (Schreurs et al. 2012).

These two placental markers were previously studied in the late 1st trimester in the same cohort, and found to be significantly lower in the pregnancies of males (Broere-Brown et al. 2016). 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 trimesters. This is consistent with previous findings in an independent cohort, in which changes in PlGF were modelled in several time-points (Sovio et al. 2018).

In cellular and human studies outside of pregnancy, PlGF levels have been found to correlate to the levels of steroid derivatives and DHEAS (Pertegal et al. 2015; Lowin et al. 2012). DHEAS is also significantly higher in the placentas of males, as shown via RNA-Seq in a large clinical cohort (Gong et al. 2018). The rapid increase of PlGF into the 2nd trimester could then be attributed to the effects of fetal androgens, which are rapidly elevated in males during mid-pregnancy, following the activation of the testes (Welsh, Suzuki, and Yamada 2014).

Clinically, very low concentrations of PlGF are considered a biomarker of preeclampsia (McLaughlin et al. 2021). This is true in both males and females 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 was

previously shown that PlGF concentrations correlate to infant growth rates of body weight and head circumference, which are also higher in males, compared to females (Broere-Brown et al. 2016; Bergen et al. 2015).

Study 3 shows, for the first time, that sex differences in PlGF levels are also linked to neurodevelopment. 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', given males' higher baseline PlGF levels in the 2nd trimester. In sensitivity analyses, the association persisted in females, when autistic people or complicated pregnancies were excluded from the cohort (Appendix 4 Table 4).

Large epidemiological studies have previously linked placental dysfunction to a later diagnosis of autism (Maher et al. 2020). Smaller clinical cohorts have also shown differences in the morphology and shape of placentas in autism, compared to controls (Anderson et al. 2007; Park et al. 2018). However, 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 (Schreurs et al. 2012), and potentially prolong exposure of the fetal brain to growth factors and steroids (Auyeung et al. 2009). 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 (Vacher et al. 2021). More research would be needed in both humans and experimental models, in order to understand this phenomenon.

In addition, a comparative analysis of placental marker concentrations was conducted between cases and controls for a diagnosis of autism. Compared to the previous analysis on traits, the statistical power was greatly reduced as n=59 males and only n=9 had been reliably certified to have received a diagnosis of autism by approximately 9 years of age, given the ongoing prospective nature of the cohort. No significant differences were found

in PlGF levels. However, the levels of sFlt-1 were significantly lower in autistic males, than undiagnosed males. Males in general had significantly lower levels of sFlt-1 than females; so a pattern emerges that mirrors the one seen with PlGF and autistic traits in females. This case-control comparison between autistic and undiagnosed children was restricted to males, given the low numbers of females diagnosed with ASD. This may be the reason why PlGF differences were not detected in this male-only comparison, given their higher baseline PlGF levels compared to females. In-vitro experiments have shown that sFlt-1 is downregulated by hypoxia in cultured endothelial cells (Ikeda et al. 2011). Additional studies would be necessary to replicate this finding in an independent cohort, in autistic females and to investigate further the clinical relevance of low sFlt-1 levels in pregnancies of individuals who are later diagnosed with autism.

Taken together, these findings could be interpreted as evidence of a 'male-type' shift in connection with both autistic traits (high PlGF) and an autism diagnosis (low sFlt-1). Similar male-type patterns have been noted in autistic females' personality traits, facial structure, and brain structure (Greenberg et al. 2018; Tan et al. 2017; Lai et al. 2013). Autistic females also have higher prevalence in steroid-related symptoms and conditions (including PCOS and metabolic dysfunction) (Pohl et al. 2014; Cherskov et al. 2018; Simantov et al. 2021). In turn, these conditions have been variably linked to the effects of the placenta, which is hypothesised to 'condition' the developing endocrine and nervous system of the fetus (Risal et al. 2019). Therefore, pregnancies that lead to autism or higher autistic traits, may be linked to more 'male-type' shifts in prenatal physiology that extend beyond PlGF and may include higher steroid levels (Studies 1 and 2)(Auyeung et al. 2009), but also other factors relating to lifelong health and disease.

With regards to limitations, the change in PlGF levels was only modelled based on two time-points, assuming a linear rate of change for both sexes and for all gestational stages. Additional time-points may be needed for more accurate modelling and to study catch-up effects in females, as indicated by a previous study (Sovio et al. 2018). More research is also necessary to further understand the mechanisms by which fetal sex interacts with the placenta, as well as other environmental and genetic risk factors for autism. Finally, it is important to note that this study only examined population-level associations via z-scores. To increase the clinical and prognostic value on the individual level, multiple-of-

median (MoM) values should be utilised instead, which could be standardised to sex, gestational age and national trends.

In conclusion, this study has shown that sex differences in placental functionality are associated to autism-related outcomes in childhood. In the next chapter, the investigation of sex differences in hormones and growth patterns is expanded into early infancy.

Chapter 5
Studies 4 - 6 : Sex and infant development

Summary of studies 4, 5 and 6

Prior research has focused on prenatal sex steroid levels and their association with autistic traits, in order to explain the sex ratio in diagnoses. Perinatal physiology also differs between the sexes, in terms of:

1. The levels of postnatal testosterone during 'mini-puberty'

2. The rate of perinatal brain growth

3. The volume of specific brain regions that undergo sex differentiation.

These three factors are analysed here, in three studies, using the same cohort of infants. Autistic traits and autism likelihood, based on family history, are also included as outcomes.

The 'Cambridge Human Imaging and Longitudinal Development Study' (CHILD) is a prospective enriched cohort study in Cambridge, UK that includes both prenatal brain imaging (n=41, mean=31.98 weeks gestation), as well as postnatal brain imaging (n=27, mean=10.8 weeks after birth) of the fetus/infant. A subset of the cohort also includes children with a family history of autism (diagnosed parent or sibling, n=11 at fetal stage, n=7 at infant stage). Salivary testosterone levels were also assessed at two time-points, soon after the 2nd and 6th month of life. Autistic traits were scored on the Quantitative Checklist of Autism in Toddlers (Q-CHAT) after the children were 18th months of age.

-Study 4: Mini-puberty Salivary testosterone levels were significantly higher during 'mini-puberty' in the 2nd and 3rd month of life, than after the 6th month of life, for both males and females. There was no significant sex difference at either time-point. Log-transformed testosterone levels were not associated with autistic traits (Q-CHAT). There was no interaction effect with infant sex, autism family history or baseline testosterone levels after mini-puberty (at >6 months of age).

-Study 5: Perinatal Brain Growth

Whole-brain metrics (total volume and surface area) and their perinatal rate of change were all significantly higher for males, after controlling for birth weight differences and other covariates. They also negatively correlated to autistic traits, in pairwise analyses and multiple regression models. Their perinatal rate of change also interacted with sex in this association, affecting males more than females. Infant testosterone levels did not mediate the negative association between whole-brain metrics and later autistic traits, but family history of autism interacted significantly with this.

-Study 6: Regional sex differences in infant neuroanatomy

Several brain regions (n=32) showed significant sex differences in volume, after controlling for both birth weight, head circumference and correcting for multiple testing. Of these regions, the volume of four was associated with autistic traits in infants: the middle temporal pole, the fusiform gyrus, the thalamus and the midbrain.

In conclusion, perinatal brain growth interacted with sex, but not with postnatal testosterone, in its association with autistic traits in the second year of life. Reduced brain growth in females and infants with a family history of the condition was the main driver of this effect.

5.1 Introduction

As summarised in Chapter 1, autism is a neurodevelopmental condition that combines difficulties in social interaction and communication, with restricted interests and repetitive behaviours. While a lot of cognitive research has been dedicated to delineating the patterns in behavioural profiles, perfecting diagnostic instruments and studying comorbid conditions (physical or psychogenic), arguably fewer studies have focused on the neurodevelopmental underpinnings of the condition; namely the earliest patterns of autism-linked brain development. Additional study is required to understand when autism starts and how it differs to the normative process of brain development.

Based on twin-heritability estimations, to sequencing and finally recent genome-wide-association studies that capture common variance, there is now consensus that most of the liability in autism (diagnosis or traits) can be attributed to genetics (Grove et al. 2019; Gaugler et al. 2014). If the developmental effects of autism-related genetic variance are better understood, then potential physiological biomarkers for autism could theoretically be found as early as conception. This line of research is inevitably faced with the inherent challenges in studying the human brain in early infancy and pregnancy.

Nevertheless, a diagnosis of (non-syndromic) autism remains behavioural and is only possible after 18-months of age, when specific neurodevelopmental behaviours emerge in toddlerhood, pertaining to social attention, language use and play. The Q-CHAT is a psychometric measure, rated by a parent or care-giver, that was developed to capture these traits in an additive way, creating a continuous spectrum (Allison et al. 2008). After the Q-CHAT was first proposed, several studies have confirmed its validity in predicting a later autism diagnosis in children via gold-standard neurodevelopmental assessments by specialists (i.e., based DSM-IV/5 or ICD-10 criteria). Validation studies for the Q-CHAT have been conducted in different populations of different ethnicities, with consistent results (Ruta et al. 2019; Roman-Urrestarazu et al. 2020; Allison et al. 2021).

Interestingly, a sex difference in Q-CHAT scores has been noted in many of these studies, with males scoring higher in terms of autistic traits at 2 years of age. This is consistent with early sex differences in several socio-cognitive milestones, such as language development (Schaadt, Hesse, and Friederici 2015; Etchell et al. 2018), as well as in autistic traits in childhood and adulthood, which have been consistently found to be

higher in males. This could be attributed to potential biases in terms of gendered behaviours that are not well characterised in young females (Lai and Szatmari 2020). However, baseline sex differences in physiology may still be mediating a male liability to autistic traits and eventually autism (Baron-Cohen et al. 2011). These sex differences in physiology may be preceding the emergence of autistic traits and be detectable prenatally or in early infancy.

Perinatal physiology may interact with fetal/infant sex via differences in steroid hormone levels, placental function, and the rate of growth of the body and brain. With regards to the latter, the 'brain overgrowth theory' has been put forward, based on a series of findings. This postulates that the brain in autistic people is characterised by aberrant proliferation and/or reduced apoptosis during putative points of perinatal development. Multiple studies have identified differences in brain structure between autistic and neurotypical children at two years or older that are consistent with the "brain overgrowth" theory (Hazlett et al. 2011; Fingher et al. 2017; Xiao et al. 2014; Courchesne et al. 2007), with one study even suggesting that autism-related structural deviations may be present as early as 6 months of age (Hazlett et al. 2017).

However, these studies often fail to investigate baseline sex differences and particularly their interaction with brain size, in terms of predicting autism or autistic traits. This interaction has mostly been studied in the brains of adults with autism, where it was shown that autistic people show more 'male-like' patterns in brain structure, irrespective of their sex (Lai et al. 2013). A longitudinal study in children with autism (ages 1.5 to 5 years old) reported that females with the condition exhibited a greater deviation, in terms of brain overgrowth than their male peers, but this analysis did not implicate specific regions of functional relevance (Schumann et al. 2010).

Sex-differential growth trajectories though are a consistent feature in early development and even younger ages. The most consistent finding is that male infants show larger total brain volumes than females from birth onwards (Benavides et al. 2018; Gilmore et al. 2007), and that total brain volumes on average grow at a faster rate in males during infancy (Holland et al. 2014). Prenatally, sex differences in brain structure and growth are less clear. Although some studies have found subtle sex differences in fetal brain diameter and ventricular volume, others have failed to find any sex differences in brain structure (Andescavage et al. 2017; Kyriakopoulou et al. 2017; Scott et al. 2011) This

indicates that the developmental period immediately after birth may be the earliest time-point to study sex differences in brain structure and their association with autism.

Finally, early infancy is also characterised by a brief increase in steroid hormones and particularly testosterone at around 2 months of age; a period termed 'mini-puberty'. This appears to be specific to males and is thought to facilitate meiosis and the maturation of spermatocytes. The neurodevelopmental significance of this phenomenon is unclear. Association studies have found links between steroid levels during mini-puberty and language development (Schaadt et al. 2015). But the association was not significant in studies of autistic traits, as measured on the Q-CHAT, in two independent studies. The first measured steroids at a later time-point than the predicted peak (at 8 months of age) and controlled for any sex differences in a linear regression model (Auyeung et al. 2012). In the second study, the authors were able to measure testosterone in saliva earlier (mean age = 7.8 weeks) but opted for a sex-stratified analysis, potentially reducing power (40 males, 47 females)(Kung et al. 2016). Neither of these studies included more than one time-point of steroid measurement, or tested for an interaction with infant sex, rather than control or stratify for sex.

To address many of the gaps in research, the CHILD was set up. The design of this cohort is unique in the autism literature. First, it is longitudinal (Figure 5.1), monitoring individuals from the first trimester post-conception, until their second year of infant life, at multiple time-points. Second, it includes two brain scans, both prenatally (in the late second trimester) and after birth (at 2 months of age). Third, it includes two measurements of salivary hormones, to better capture the peak of mini-puberty. Fourth, it measures autistic traits as an outcome in late infancy (via the Q-CHAT) but also includes a subset of participants from families at high-likelihood for autism (presence of a diagnosed parent or sibling) (Figure 5.1).

Therefore, the studies based on this cohort are well-placed to uncover differing trends in brain development and link them to infant autistic traits, as well as check for interactions with fetal sex and familial autism likelihood. Given the relatively small sample size ($n<41$), this study should be considered as an exploratory, "pilot" project, therefore findings need to be replicated in subsequent projects.

With regards to this chapter, the following hypotheses will be investigated:

1. Study 4: That testosterone levels during mini-puberty correlate to infant autistic traits, and this association interacts with baseline sex differences in testosterone levels.
2. Study 5: That brain growth correlates to infant autistic traits and this association interacts with baseline sex differences in brain growth.
3. Study 6: That this interaction between sex, brain growth and autistic traits is driven by brain regions that have been previously implicated to neurocognitive milestones, such as social attention, language etc.

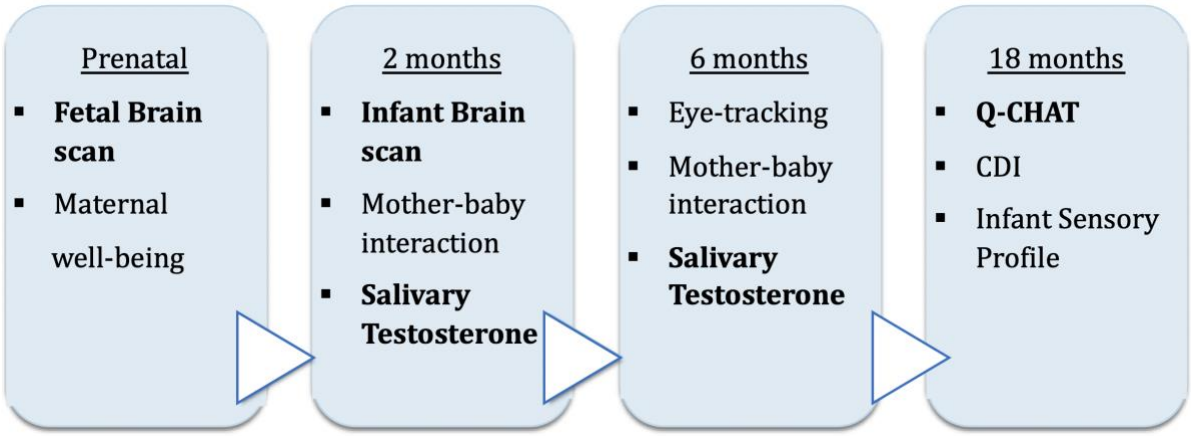


Figure 5.1: CHILD Study Flowchart. Measures in this chapter in bold.

5.2 Methods

5.2.1 Participants

Pregnant women were recruited to take part in CHILD at the Rosie Maternity Hospital, in Cambridge, via advertising material or in-person discussions in the prenatal ultrasound unit, during their first or second pregnancy monitoring appointment. An additional subset of the cohort consisted of pregnant women with a family history of autism, having themselves received a diagnosis of autism, their partner, or a previous child. These "high-likelihood" participants were recruited through the Cambridge Autism Research Database, support groups across the UK, social media that are specific to the condition, and adverts placed on magazines. Only women with a singleton pregnancy were eligible to take part in the study and those who reported no consumption of alcohol or smoking, in the initial screening form for study recruitment.

Participants agreed to take part in two MRI brain scans of their child's brain, prenatally during 30-33 weeks of pregnancy (fetal stage) and postnatally at 8-12 weeks after birth (infant stage). Infant scans were conducted during natural sleep with no sedation used.

A total of 43 participants underwent scanning at 30-33 weeks of pregnancy. Structural data was not obtained from two participants due to movement artefacts, for a total sample size of n=41 brain scans at the fetal stage, of which n=11 at high-likelihood for autism. Two typical and one autism high-likelihood infants could not be followed-up for the infant scan. Scanning was therefore conducted with 40 participants at the postnatal stage. Structural data was not obtained from one autism high-likelihood participant, due to movement artefacts. Moreover, data was not obtained from 11 participants (of whom one was in the high-likelihood group) due to the infant waking and showing distress in the MRI scanner or due to the inability of the infant to achieve sleep. Therefore, at the infant stage, structural MRI scans were, available for 27 participants, of which 7 were at high familial likelihood for autism. None of the infants included in the study had been born preterm (<37 weeks gestation at birth).

Consent was also obtained for specific linkage to all pregnancy-related clinical data. Birth-related variables, such as birth weight and gestational age at birth, were obtained via access on the CUH Trust's clinical records system (EPIC), by specialist clinicians and anonymised immediately after extraction.

5.2.2 MRI Acquisition and Pre-processing

All MR images were obtained at the Paediatric Scanning Unit of the Rosie Hospital, Cambridge, UK with a 1.5 Tesla GE scanner. Structural images were acquired using the balanced steady-state gradient echo sequence FIESTA (Fast Imaging Employing Steady-state Acquisition). The following parameters were used: repetition time of 3.5ms, echo time of 1.4ms, a flip angle of 55°, 172 slices with a slice thickness of 1mm, voxel size of 1.875 x 1.875 x 1mm3, field of view of 256 x 256. BOLD sensitive echo-planar images were acquired using the following parameters: repetition time of 2000ms, echo time of 40ms, a flip angle of 90°, 3150 slices with a slice thickness of 4mm, voxel size of 5.625 x 5.625 x 4mm3, field of view of 64 x 64.

5.2.3 Structural image pre-processing

Fetal brain images were manually reoriented using the Reorient tool in ITK-SNAP (Yushkevich et al. 2006) as *in utero* head movement led to mismatches between image orientation codes and the actual orientation of the fetal brains. This step was not necessary for infant structural images as infants were scanned during natural sleep with cushion padding either side to minimise movement within the scanner. Spatial origin was set to the Anterior Commissure fibre tract permitting accurate co-registration of reoriented images. All images were then manually skull stripped with the resulting ROI extending to the skull/CSF interface and to the base of the Cerebellum. Using a gender balanced random sample of subjects with both fetal and infant images a study specific structural template was generated using Advanced normalisation tools (ANTs; Avants et al. 2009).

The STA31 template, part of a spatiotemporal MRI atlas for segmentation of early brain development (Gholipour et al. 2017) together with all fetal and infant structural images were registered to the study specific template using ANTs (Avants et al. 2009). So as not to introduce interpolation artefacts, STA31-specific-template and subject-specific-template spatial warps were combined into a single inversely symmetrical STA31-to-subject transformation. As it was not possible to directly segment brain images into grey and white matter, due to the lack of grey/white matter contrast during this developmental period, STA31 parcellations were re-sampled into subject space to

indirectly assess grey matter properties within the brain. The sum of combined re-sampled STA31 segmentations and parcellations was then used to estimate total brain volume from the structural images.

5.2.4 Salivary Hormones

Saliva was collected in the form of passive drool from the infants' mouth immediately before their postnatal brain scan, at approximately 2 months of age (mean=10.8[1.9] weeks). This was achieved with a specialised and validated method from Salimetrics, Ltd., using their Infant Collection Swab (SIS). This consists of an absorbent polymer that absorbs the infant's saliva, paired with a single-use, single-swab collection tube, in which the swab is placed. Two swabs were used per infant, when possible. Three infants did not tolerate a second swab. Swab-tube pairs were placed in a freezer at 4 degrees Celsius, immediately after collection. Within one hour, these were centrifuged to achieve gradual draining and collection of liquid saliva. They were kept at 3 degrees throughout. The resulting sample was then aliquoted into a cryovial and frozen at -80C. In a few cases (n<4), admixture with tears could not be excluded, given the infant's distress at the time of sample collection.

The same collection protocol was applied in a second in-person clinical appointment, when the infants were approximately 6 months old (mean=26.5[1.7] weeks). For this subset of samples, collection swabs were immediately frozen at -80C. These were thawed and centrifuged after a period of one year in storage at -80C.

5.2.5 Hormonal assays for testosterone

An Immunoassay kit developed and provided by Salimetrics, Ltd was used to measure testosterone concentrations in saliva. This has been validated in samples collected via the Infant Collection Swab, as well as in adults. Duplicate assays were conducted for each participant's pooled saliva sample (derived in most cases by two swabs used in quick succession). Final values were the average of the two assays. A quality control step involved conducting a third assay, in case concertation values of the two initial arrays, were not within 10% of each other. The average was then computed, based on the two assays that were closer together. All assays were conducted by specialist staff at the Anglia Ruskin Biomarkers Laboratory, which had been accredited by Salimetrics for this type of analysis.

5.2.6 Autistic Traits

Following informed consent at the point of recruitment, and again before their child's postnatal brain scan via MRI, participating mothers were invited via email to complete a series of online questionnaires on their child's development. These were provided via the University's Qualtrics platform. Autistic traits were the first item to be completed, by filling in the Qualitative Checklist of Autism in Toddlers (Q-CHAT) (Allison et al. 2008). Individualised links to Qualtrics, were sent immediately after 18 months had passed, since the child's birth. Anonymised data was then copied onto RedCap; an online platform for secure storage and data analysis, used by research staff and students of the University of Cambridge (Harris et al. 2019).

5.2.7 Statistical Analyses

5.2.7.1 Cohort and Outcome variables

All variables for the age at the time of postnatal assessments (e.g., age when Q-CHAT was completed) were adjusted for gestational age at birth. For example, an infant being born 2 weeks preterm, would result in all postnatal age variables being reduced by 2 weeks. None of the infants were born before 37 weeks gestation. This was done to better reflect the maturation of physiology since conception, rather than birth, as well as control for complications, such as being born preterm, when comparing infants in multiple regression models. For individuals where gestational age at birth was missing from birth records, it was assumed that the neonate had achieved term gestation (40 weeks).

Prior to regression analyses, skewness of the distribution of Q-CHAT scores (the outcome variable) was reduced by reducing extreme outliers (n=2) to a value within an interval of three times the top limit of the interquartile range (Q-CHAT=50.75).

5.2.7.2 Study 4: Mini-Puberty Analysis

Salivary testosterone levels at the 2nd month of life and 6th month of life were log-transformed to reduce skew in their distributions. Comparison between groups (sex or autism likelihood) were conducted with pairwise Student's t-tests. Associations with demographic variables (e.g., birth weight, age at sampling) were investigated by

calculating the Pearson's correlation coefficient between them and the transformed testosterone concentrations.

For Study 5.1, three multiple regression models were used to test for an association of testosterone levels during mini-puberty with the autistic traits of the infants at 18 months of age (measured on Q-CHAT); first by controlling for sex (Model 1), second by modelling an interaction with infant sex (Model 2) and third by adding the second testosterone measurement (Model 3).

5.2.7.3 Study 5: Whole-Brain Analyses

Two whole-brain variables were analysed for Study 5.2: total brain volume and total surface area, which were computed by adding the corresponding values for the left and right hemispheres, at the fetal and infant stage. Longitudinal composite measures were also computed for the growth between the fetal and infant stages by the following formulas for each fetus/infant:

For total volume (TV) change:

(TV at infant - TV at fetal) / ((age at postnatal scanning + gestational age at birth) - gestational age at prenatal scanning)

For surface area (SA) change:

(SA at infant - SA at fetal. stage) / ((age at postnatal scanning + gestational age at birth) - gestational age at prenatal scanning)

To investigate sex differences in brain structure in the typical group, both pairwise group comparisons (via Student's t-tests) and multiple regression models were used. The latter had whole-brain measures as the dependent variable and sex as the independent variable, further controlling for birth weight and age at time of scanning (for the non-longitudinal variables).

For subsequent analyses, only whole-brain measurements that showed significant sex differences were included (TV and SA at the infant stage and the longitudinal composite measures).

To investigate association with autistic traits (Study 5.2), these whole-brain measures were tested in multiple regression models, with Q-CHAT scores as the dependent variable, and further controlling for the following covariates: birth weight, sex, age at time of Q-CHAT (in months - adjusted for gestational age at birth). An interaction term was also included in the models, to investigate a brain-by-sex interaction in predicting autistic traits Bootstrap analysis was also conducted, by randomly removing data points, in order to check if the effect was reversed and therefore spuriously driven by outliers.

To study the role of autism family history on the association of autistic traits with whole-brain measures, separate multiple regression models were used that added an interaction term between the two (whole-brain measures-by-autism family history). These were also controlled for sex, birth weight and age at the time of Q-CHAT assessment (adjusted for gestational age at birth).

Finally, to investigate if the association between whole-brain variables and autistic traits, was mediated by steroid levels, a mediation analysis was performed. Log-transformed salivary testosterone concentrations, at the first time-point of measurement (2nd month postnatally) were used as the mediator and autistic traits as the outcome. Only whole-brain parameters that were shown to interact with sex in the previous multiple regression models were entered into this analysis, to reduce multiple testing.

5.2.7.4 Study 6: Analysis of sex differences and related regions

For Study 5.3, specific regions were sought that may be driving the associations observed in Study 5.2. This analysis was performed with brain-scan data corresponding to the infant stage only, as sex differences at the fetal stage were not statistically significant for whole-brain metrics.

Volume measurements of n=58 brain regions for each hemisphere were calculated, according to the parcellation method of the brain of infants, described previously (Gholipour et al. 2017).

Sex differences were then investigated via multiple regression models, with each region as the outcome and controlling for age at the time of the scan and overall size differences

via birth weight and head circumference. A False Discovery Rate (FDR) was applied on the p-values for sex differences, stemming from these models.

Regions that showed significant sex differences were then tested for an association and an interaction with infant sex, in predicting later autistic traits (Q-CHAT scores). Multiple regression models for this were further controlled for birth weight, head circumference and age at the time of Q-CHAT. A False Discovery Rate (FDR) was applied on the p-values for regional brain volume effects on autistic traits, stemming from these models.

Regions that showed sex significant differences, as well as a significant association with Q-CHAT scores, were further tested for an interaction effect with the familial likelihood for autism, in predicting autistic traits.

5.3 Results

5.3.1 Study 4: Mini - puberty and autistic traits

5.3.1.1 Hormones at mini-puberty

Testosterone levels in saliva were detected in both males and females in both time-points (1st time-point: n=33, at mean=10.8[1.9] weeks)(2nd time-point: n=34, at mean=26.5[1.7] weeks). These were marginally higher in males at both time-points (Figure 5.2), but this was not statistically significant when these were compared via Student's t-test, potentially due to the low sample size (t-test at 2 months: t=-1.37, p=0.143/ t-test at 6 months: t=-1.65, p=0.109). In addition, there were no group differences in testosterone levels between children with a family history of autism and those without, in pairwise comparisons (t-test at 2 months: t=-1.364/ t-test at 6 months: t=-1, p=0.327), as well as in linear regression models that controlled for sex. Linear regression models also did not reveal any associations between testosterone and cohort covariates, such as birth weight, maternal age or the exact age at time of saliva collection.

Figure 5.2: Testosterone levels at 2 (left) and 6 months (right) did not show significant sex differences but showed a consistent trend for higher levels in males.

When comparing hormone levels between time points, testosterone after the 2nd month did not correlate with testosterone levels after the 6th month, but this was only marginally non-significant in females, in sex-stratified analyses (males: Pearson's r=0.24, p=0.33 /females: Pearson's r=0.53, p=0.052).

Testosterone levels were significantly higher at the first time-point (after 2 months), compared to the second (after 6 months), in both males (t=4.87, p<0.0001) and females (t=3.69, p=0.001). The highest testosterone levels corresponded to 11 to 12 weeks of age (corrected for gestational age at birth) (Figures 5.3 and 5.4).

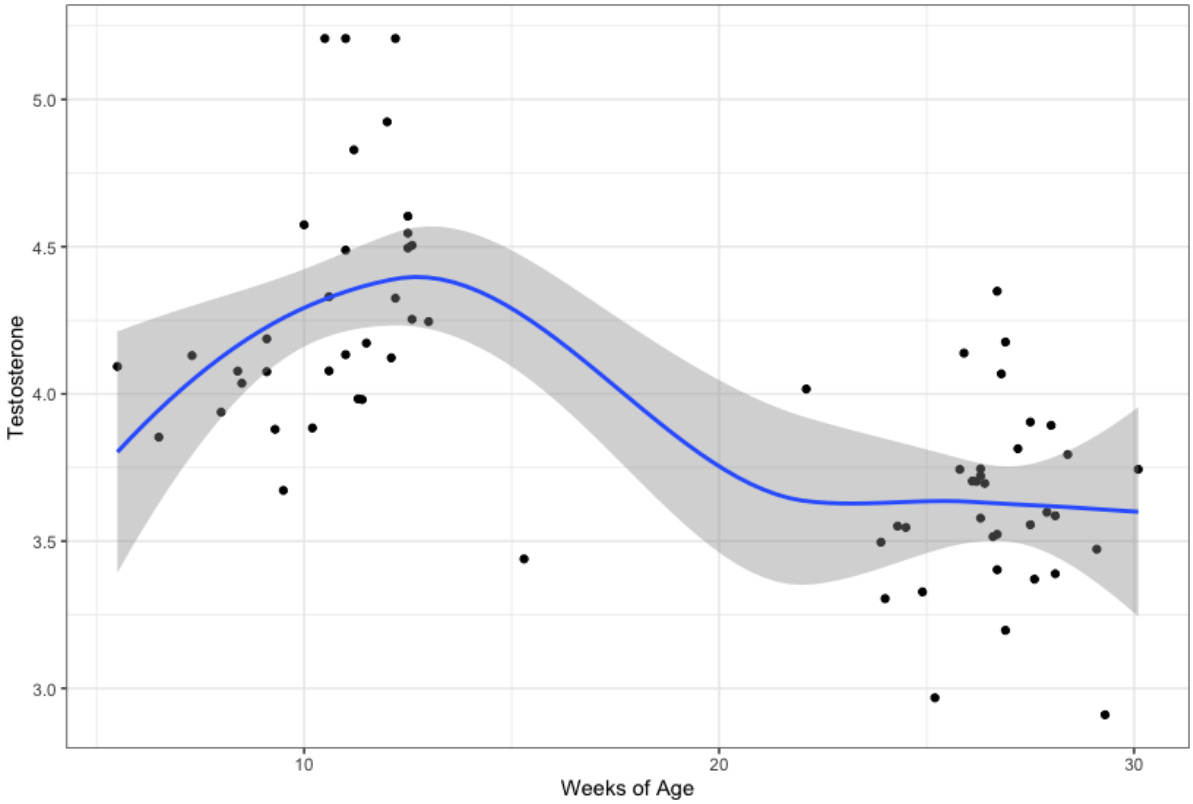


Figure 5.3: Testosterone levels(log-transformed), plotted for weeks of age (adjusted for gestational age at birth). Curve is loess fit for both time-points.

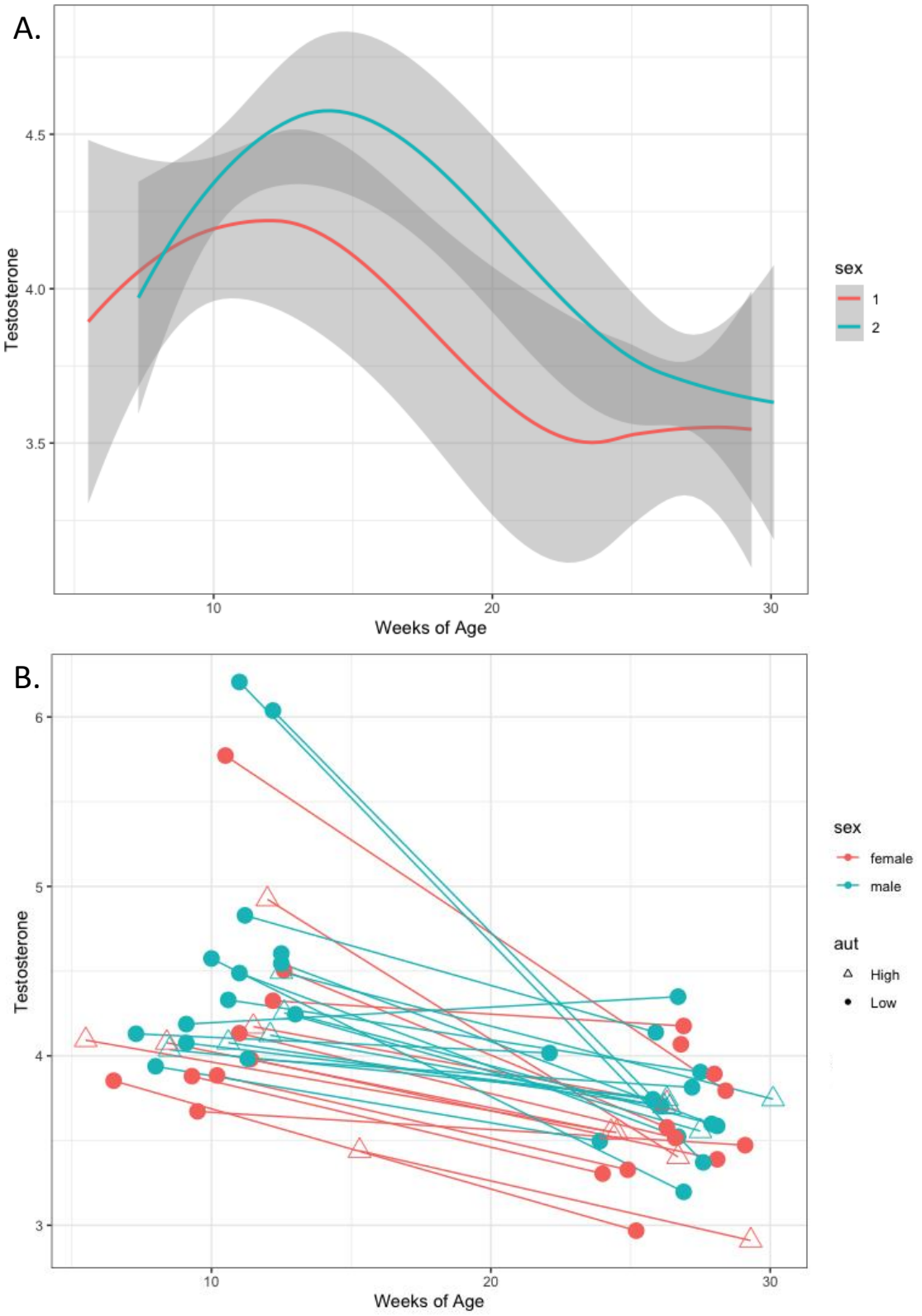


Figure 5.4: Testosterone levels for age since birth (in weeks). (A) Loess curves for each sex indicate a mini-puberty elevation for both males and females (B) Paired concertation values for each participant, for sex (1: female, 2: male) and familial likelihood.

5.3.1.2 Autistic Traits

Q-CHAT scores were available for n=36 infants, as rated by their parents. There was no significant difference between males (n=19) and females (n=17) (Student t-test: t=0.91, p=0.372), but infants of families with a history of autism (parent or sibling) scored significantly higher (n=10, mean= 41.6, SD=10.1) than infants with no history of the condition (n=26,mean=28.9, SD=5.7, t-test=2.65, p=0.0242).

5.3.1.3 Correlation with Q-CHAT

Testosterone levels did not correlate to infant autistic traits in pairwise Pearson's correlation coefficient (r=-0.09, p=0.649).

Three multiple regression models were used to test for an association of testosterone levels during mini-puberty with the autistic traits of the infants at 18 months of age; first by controlling for sex (Model 1), second by modelling an interaction with infant sex (Model 2) and third by adding the second testosterone measurement (Model 3) (see Table 5.1). The age at the time of Q-CHAT and family history of autism were included in all models as covariates. There was no statistical association of testosterone levels with infant autistic traits in any of the models. Infant sex or baseline testosterone (at 6 months) also did not have any statistically significant effects in predicting Q-CHAT scores. The only variable that did meet significance was family history of autism, which was significant in all of the models.

Table 5.1: Multiple regression models of log-transformed testosterone levels (T) to autistic traits (Q-CHAT scores).
"2m": time-point 2 at months of age, "6m": time-point at 6 months of age

	T - 2m	***age at Q-CHAT***	***sex***	***Autism history***	***T by sex***	***T - 6m***	***T-2m by T-6m***
Model 1	β=1.14	β=-0.40	β=-1.70	**β=-9.49**			
	p=0.638	p=0.516	p=0.595	**p=0.005**			
				Adj. R^2=0.191			
Model 2	β=1.39	β=-0.39	β=0.104	**β=-9.46**	β=-0.42		
	p=0.721	p=0.533	p=0.996	**p=0.007**	p=0.933		
					Adj. R^2=0.158		
Model 3	β=21.16	β=-0.32	β=-2.86	**β=-9.32**		β=27.19	β=-5.68
	p=0.562	p=0.631	p=0.463	**p=0.010**		p=0.546	p=0.580
							Adj. R^2= 0.115

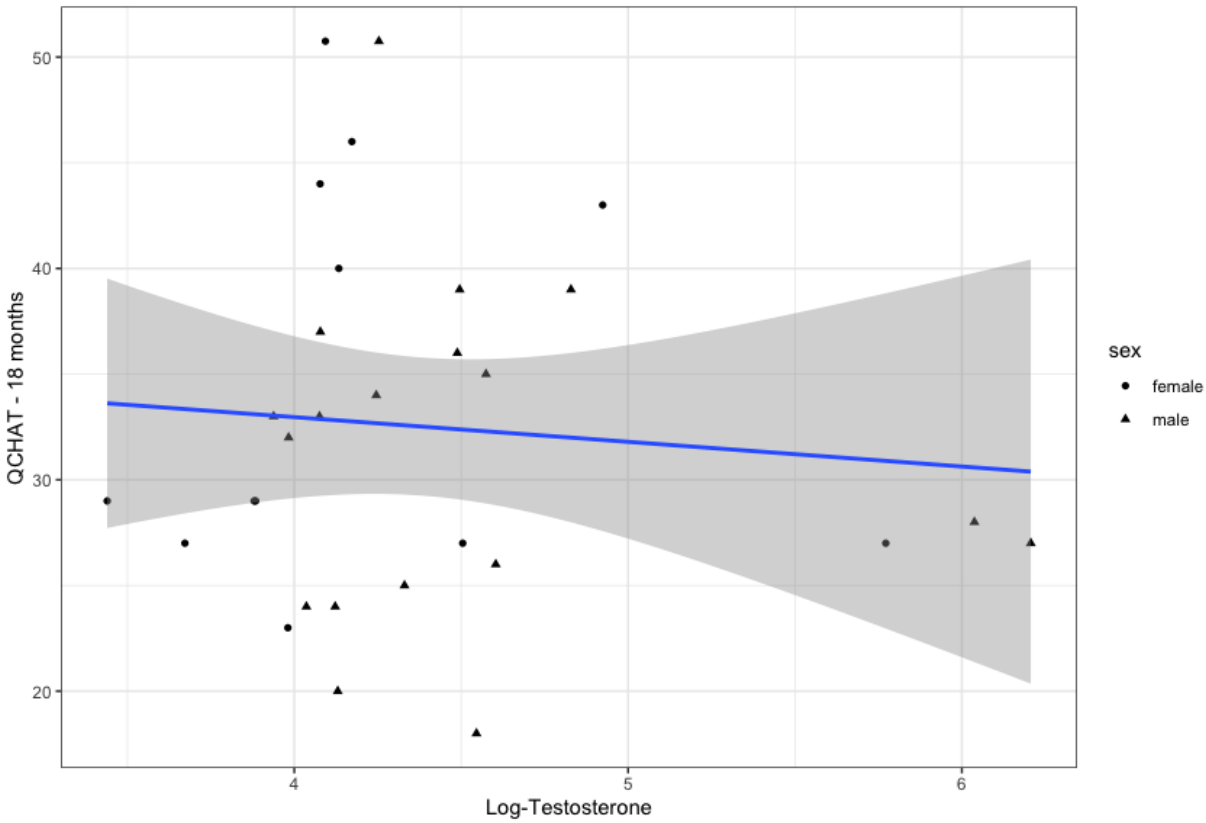


Figure 5.5: Testosterone levels (log-transformed) plotted for Q-CHAT scores. Linear regression fit is for both males and females.

5.3.2 Study 5: Brain growth and autistic traits

5.3.2.1 Structural MRI

Two whole-brain measures, total volume (TV) and surface area (SA), were obtained from structural MRI scans, prenatally (mean=31.98 weeks gestation, SD=1.52) and postnatally (mean=10.8 weeks after birth, SD=1.97) for each brain hemisphere. Interhemispheric correlations were very high (Pearson's r>0.95, p<0.00001) and values for left and right hemispheres were added to account for the whole brain in subsequent analyses.

Fetal and infant whole-brain measures correlated significantly to each other and to a similar extent, for both total volume and total surface area (TV: Pearson's r=0.51, p=0.009 / SA: Pearson's r=0.56, p=0.003).

When comparing males and females, whole brain measures were significantly larger in males postnatally, but not prenatally. Growth velocities between time-points (adjusted for age) for both TV and SA, were also larger in males. These differences were found in pairwise Student's t-tests, as well as in multiple linear regression models that controlled for birth weight (adjusted for gestational age at birth).

Table 5.2: Sex differences in whole-brain measures. TV: total brain volume, SA: surface area, MR: multiple regression model with sex and birth weight as independent variables

	Mean females (SD)	*Mean males (SD)*	*t- test*	*MR - birth weight*	*MR - sex*
Fetal					
TV	153732 (18769)	15690.7 (24621.4)	t=-0.47 p=0.643	β= 335.4 p=0.353	β=-1424.3 p=0.85
SA	22324.32 (1969.8)	22750.31 (2488.67)	t=-0.61 p=0.546	β=32.86 p=0.372	β=-45.25 p=0.953
Infant					
TV	351134.6 (37936.6)	408381.0 (50309.7)	**t=-3.34** **p=0.003**	β=955.5 p=0.298	**β=47531** **p=0.023**
SA	40809.37 (2830.4)	45284.64 (3778.2)	**t=-3.50** **p=0.002**	β=59.16 p=0.392	**β=3873.8** **p=0.015**
Longitudinal					
TV change	11640.08 (1883.9)	13806.99 (2064)	**t=-2.74** **p=0.012**	β=-75.83 p=0.066	**β=2842.3** **p=0.0025**
SA change	1091.111 (208)	1240.812 (167.3)	t=-1.94 p=0.07	**β=-8.817** **p=0.020**	**β=228.23** **p=0.006**

To study the interaction between sex differences, brain growth and autism, the following association analyses were restricted to measures that differed by sex (postnatal and longitudinal).

5.3.2.2 Association with infant autistic traits

Brain parameters that showed sex differences were further examined for their association with infant autistic traits, estimated via the Q-CHAT at 18 months of age. Multiple linear regression models accounted for an interaction with infant sex and controlled for birth weight differences (adjusted for gestational age at birth) and the age at the time of Q-CHAT. All brain parameters that were larger in males, were also correlated to Q-CHAT scores. These included whole brain measures postnatally in the 2nd month of life (total volume and surface area), as well as longitudinal composite measures of brain growth from fetal and infant brain scans (TV change and SA change). Only the longitudinal measures showed a significant interaction with fetal/infant sex, whereby a stronger effect was noted in males but not females. This was not significant when only neonatal brain data was assessed, showing both males and females contributed to the observed negative association of brain volume with Q-CHAT scores. Birth weight (adjusted for gestational age at birth) was significantly and positively associated with Q-CHAT scores in all the models.

Table 5.3: Multiple regression models of the association between whole brain measures and autistic traits (outcome variable, measured on Q-CHAT). TV: total brain volume, SA: surface area.

	Brain variable	*Sex*	*Brain by sex*	*Birth Weight*	*Age at Q-CHAT*
Infant TV	**β=-0.0002**	β=-41.878	β=0.0001	**β=0.438**	β=0.0821
	r=-0.45	r=-0.24	r=0.22	**r=0.42**	r=0.02
	p=0.017	p=0.183	p=0.215	**p=0.023**	p=0.906
					Adj R^2=0.339
Infant SA	**β=-0.0022**	β=-65.331	β=0.0015	**β=0.405**	β=0.124
	r=-0.44	r=-0.24	r=0.23	**r=0.39**	r=0.03
	p=0.018	p=0.172	p=0.191	**p=0.035**	p=0.861
					Adj R^2=0.326
TV change	**β=0.004**	β=48.445	**β=-0.005**	**β=0.593**	β=-0.609
	r=0.34	r=0.34	**r=-0.41**	**r=0.47**	r=-0.15
	p=0.095	p=0.095	**p=0.049**	**p=0.028**	p=0.442
					Adj R^2=0.208
SA change	**β=0.042**	β=41.065	**β=-0.048**	**β=0.656**	β=-0.337
	r=0.43	r=0.31	**r=-0.42**	**r=0.51**	r=-0.08
	p=0.035	p=0.114	**p=0.037**	**p=0.014**	p=0.673
					Adj R^2= 0.272

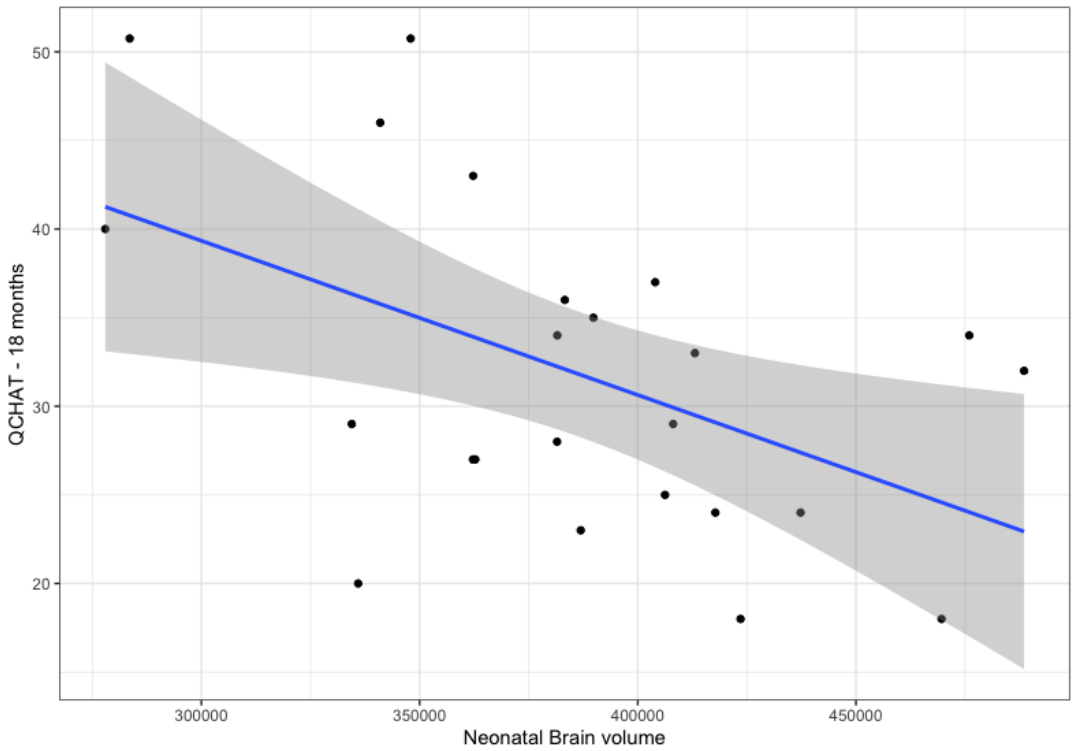


Figure 5.7: Total Brain volume (TV) in early infancy (2-months) correlates negatively with autistic traits after 18 months.

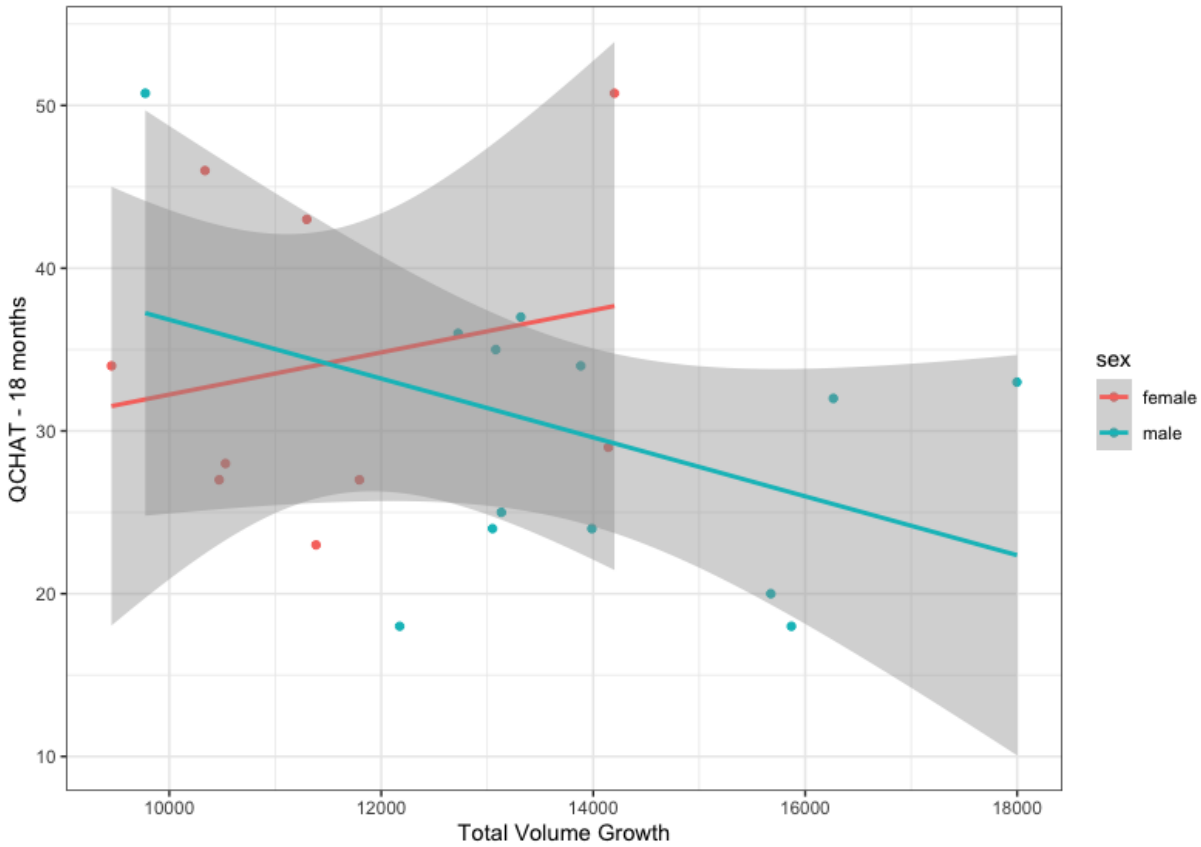


Figure 5.6: The correlation of longitudinal measures (volume change since infancy) to autistic traits, interacted with fetal/infant sex. Males with lower brain growth for their age, had higher Q-CHAT scores.

Infants of families with a history of autism (parent or sibling) had significantly higher Q-CHAT scores, so a further interaction analysis was conducted to investigate if this was also linked to the observed negative association between total brain volume measures and autistic traits. An interaction term was added to a multiple linear regression model that also controlled for infant sex, birth weight and age at the time of Q-CHAT. For all neonatal measures, this was significant, indicating that with individuals with a family history of autism were the main drivers of the observed negative association with infant Q-CHAT scores. This was not significant in the models assessing the longitudinal composite measures of brain growth.

Table 5.4: Multiple regression models of the association between whole brain measures and autistic traits), with the addition of familial likelihood as an interacting variable ('brain by autism'). TV: total brain volume, SA: surface area.

	Brain variable	*Autism in family*	*Brain by autism*	*Sex*	*Birth Weight*	*Age at Q-CHAT*
Neonatal TV	**β=-0.0002**	**β=-78.52**	**β=0.0002**	β=-1.962	β=0.263	β=0.494
	r=-0.54	**r=-0.47**	**r=0.43**	r=-0.08	r=0.22	r=0.12
	p=0.001	**p=0.003**	**p=0.0054**	p=0.5812	p=0.1252	p=0.4067
						Adj R^2=0.575
Neonatal SA	**β=-0.0028**	**β=-102.8**	**β=0.0023**	β=-1.256	β=0.228	β=0.290
	r=-0.51	**r=-0.43**	**r=0.41**	r=-0.05	r=0.19	r=0.07
	p=0.0022	**p=0.007**	**p=0.011**	p=0.743	p=0.196	p=0.629
						Adj R^2=0.534
TV change	β=-0.0026	β=-52.16	β=0.003	β=-6.482	β=0.118	β=-0.065
	r=-0.23	r=-0.33	r=0.27	r=-0.20	r=0.09	r=-0.02
	p=0.236	p=0.101	p=0.172	p=0.304	p=0.653	p=0.936
						Adj R^2=0.272
SA change	β=-0.004	β=-24.86	β=0.013	β=-8.072	β=0.241	β=-0.305
	r=-0.03	r=-0.16	r=0.10	r=-0.24	r=0.16	r=-0.07
	p=0.879	p=0.416	p=0.602	p=0.243	p=0.434	p=0.718
						Adj R^2=0.198

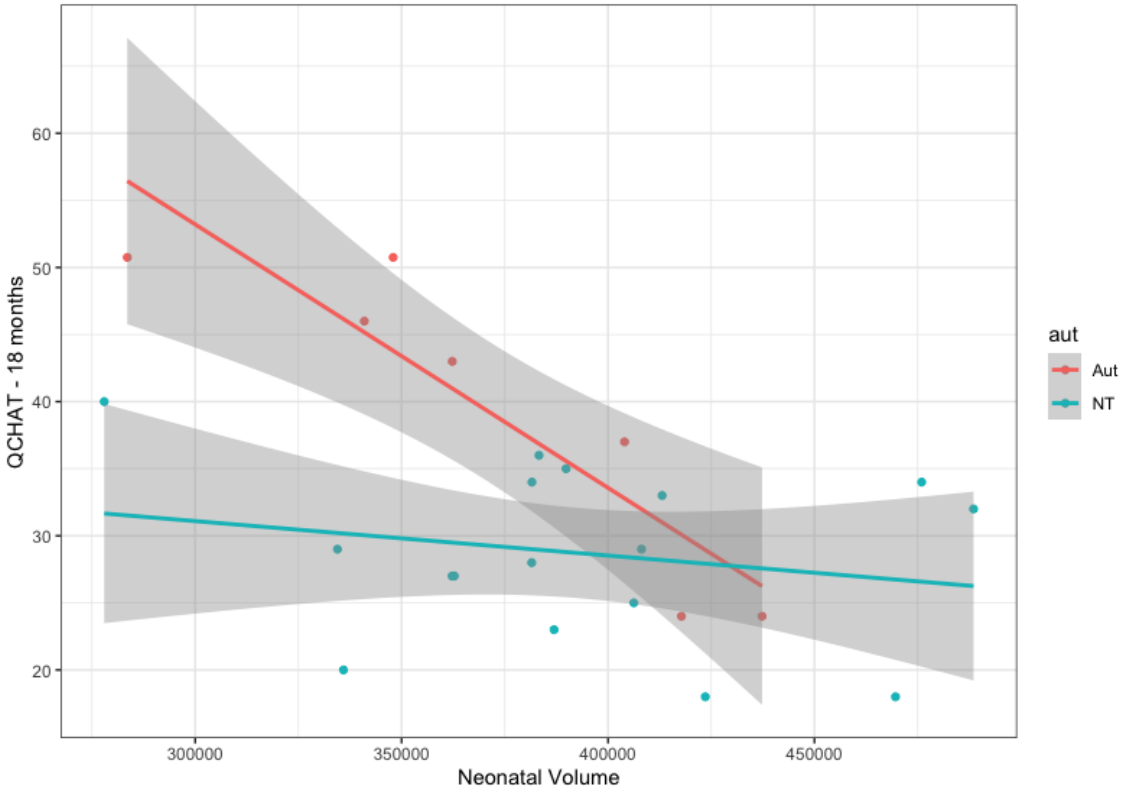


Figure 5.8: The correlation between whole brain measures to autistic traits significantly interacted with familial likelihood for autism.

5.3.2.3 Mediation Analysis for testosterone

A targeted mediation analysis was conducted to study if testosterone levels during mini-puberty mediated part of the statistical association of brain growth with later autistic traits. Only brain parameters that were shown to interact with sex (TV change and SA change) were entered into this analysis. No significant mediatory effect was found on autistic traits.

Table 5.5: Mediation analysis of salivary testosterone on the association between longitudinal brain growth measures and autistic traits. ACME: mediation effect, ADE: direct effect

	ACME		*ADE*	
TV - change	0.00004	p=0.94	-0.00107	p=0.36
SA - change	0.00012	p=0.94	-0.0056	p=0.66

5.3.3 Study 6: Sexually dimorphic brain regions and Q-CHAT

5.3.3.1 Structural sex differences in infant brain

Region-specific analyses were restricted to infant brain scans, because of their comparative reliability (relative to fetal brain scans) and the observed consistency of sex differences in whole brain measures, after controlling for birth weight and with multiple methods (Table 5.6). A region parcellation method based on a mask developed specifically for infants, included 58 regions, spanning cortical, subcortical regions, as well as the cerebellum, for each hemisphere. The average of left and right hemisphere for each region was then calculated and entered in subsequent analyses, given consistent interhemispheric correlations in measurements and in order to reduce multiple testing. Sex differences were then investigated via multiple regression models, with each region as the outcome and controlling for age at the time of the scan and overall size differences via birth weight and head circumference. A False Discovery Rate (FDR) was applied on the p-values for sex differences, stemming from these models.

A total of n=32 brain regions were significantly larger in males, after controlling for birth weight differences and age at the time of scanning (Figure 5.9), with the top 5 in terms of statistical significance being: the middle temporal pole, the cerebellum, the posterior cingulum, the lingual gyrus and the fusiform gyrus (Table 5.6).

Figure 5.9: Brain regions with significant size differences between males and female infants in CHILD, after controlling for head circumference, birth weight and correcting via FDR.

Table 5.6: Sex differences in brain regions, after controlling for covariates via multiple regression, sorted for statistical confidence. N=32 of the n=58 regions are significantly larger in males, after controlling for multiple testing via FDR.

	Sex	*Age at scan*	*Head circumference*	*Birth Weight*
Middle Temporal Pole *- β*	132.322	7.356	1.172	-0.033
p	<0.001	0.443	0.699	0.42
FDR - q	0.016			
Cerebellum				
β	775.023	233.94	21.152	0.13
p	0.001	0.001	0.306	0.64
FDR - q	0.021			
Lingual				
β	871.014	48.234	51.662	-0.013
p	0.001	0.489	0.027	0.965
FDR - q	0.021			
Posterior Cingulum				
β	151.704	14.434	3.712	-0.015
p	0.002	0.289	0.389	0.791
FDR - q	0.023			
Fusiform				
β	573.039	99.467	36.768	-0.264
p	0.002	0.06	0.031	0.238
FDR - q	0.023			
ParaHippocampal				
β	175.881	20.489	14.796	-0.123
p	0.003	0.211	0.008	0.089
FDR - q	0.025			
Medulla				
β	84.123	19.403	6.567	0.058
p	0.004	0.024	0.017	0.109
FDR - q	0.03			
Precuneus				
β	819.294	77.708	20.717	0.103
p	0.005	0.343	0.425	0.768
FDR - q	0.03			
Rolandic Operculum				
β	535.376	72.171	19.068	-0.083
p	0.004	0.179	0.261	0.717
FDR - q	0.03			
Calcarine *-*				
β	373.766	20.537	23.373	-0.037
p	0.007	0.599	0.07	0.828
FDR - q	0.03			

Frontal_Inf_Tri				
β	240.791	0.847	10.336	0.031
p	0.006	0.973	0.197	0.77
FDR - q	0.03			
Inferior Occipital				
β	283.021	41.672	9.937	-0.075
p	0.007	0.164	0.292	0.555
FDR - q	0.03			
Middle Occipital				
β	419.46	48.677	11.961	0.023
p	0.006	0.264	0.386	0.901
FDR - q	0.03			
Heschl's gyrus				
β	113.149	4.98	8.648	0.031
p	0.009	0.685	0.035	0.564
FDR - q	0.032			
Hippocampus				
β	142.042	25.794	8.298	-0.054
p	0.01	0.108	0.104	0.428
FDR - q	0.032			
Inferior Temporal				
β	481.378	54.083	29.146	-0.183
p	0.01	0.311	0.093	0.427
FDR - q	0.032			
Superior Temporal				
β	485.438	89.392	37.08	-0.267
p	0.008	0.094	0.033	0.239
FDR - q	0.032			
Angular				
β	262.31	20.875	18.387	0.078
p	0.01	0.476	0.057	0.537
FDR - q	0.033			
Middle Cingulum				
β	461.263	70.338	19.306	0.049
p	0.012	0.188	0.253	0.829
FDR - q	0.033			
Inferior Frontal Operculum *- β*	210.457	16.891	13.027	-0.002
p	0.011	0.477	0.093	0.987
FDR - q	0.033			
Hippocampal Commissure *- β*	54.52	12.75	2.331	-0.019
p	0.012	0.049	0.244	0.481
FDR - q	0.033			
Midbrain				
β	84.303	18.57	3.541	0.006
p	0.013	0.065	0.256	0.89

FDR - q	0.033			
Insula				
β	248.088	30.825	14.283	0.146
p	0.014	0.291	0.13	0.25
FDR - q	0.034			
Middle Temporal				
β	549.941	67.519	30.66	-0.11
p	0.015	0.301	0.145	0.694
FDR - q	0.035			
Corpus Callosum				
β	282.621	-0.079	21.436	0.135
p	0.02	0.998	0.064	0.377
FDR - q	0.044			
Superior Medio-Frontal *- β*	406.512	47.064	15.681	-0.1
p	0.023	0.367	0.346	0.656
FDR - q	0.044			
Superior Frontal				
β	460.807	57.823	14.029	-0.001
p	0.023	0.327	0.453	0.997
FDR - q	0.044			
Internal Capsule				
β	128.641	16.828	7.989	-0.011
p	0.023	0.309	0.135	0.875
FDR - q	0.044			
Superior Parietal				
β	355.135	62.568	11.5	-0.001
p	0.023	0.174	0.426	0.997
FDR - q	0.044			
Thalamus				
β	225.61	49.168	2.768	-0.071
p	0.02	0.09	0.757	0.561
FDR - q	0.044			
Precentral				
β	546.215	64.129	15.966	0.141
p	0.024	0.365	0.477	0.643
FDR - q	0.044			
Postcentral				
β	589.697	60.033	28.809	0.189
p	0.027	0.44	0.248	0.573
FDR - q	0.048			

5.3.3.2 Sex differentiation of the brain and later autistic traits

Multiple regression of brain region volume to autistic traits was conducted for each of the regions that showed significant sex differences at the neonatal brain scan (Table 5.7). Models also included an interaction term of region volume and infant sex, as well as covariates (age at the time of Q-CHAT and birth weight).

Of the 32 sexually differentiated regions in infants, 10 were also negatively associated with Q-CHAT scores at a nominal level of significance (Table 5.7). After application of a false discovery rate (FDR), the volume of four regions remained significantly associated with Q-CHAT scores: the middle temporal pole, the fusiform gyrus, the thalamus and the midbrain. The thalamus and middle temporal pole showed nominal trends ($p<0.1$) towards an interaction with infant sex, in predicting autistic traits in infants. These trends were in opposite directions (Table 5.7 and Figure 5.9).

In addition to sex, further analysis for these four regions, showed that the volume of the middle temporal pole, the thalamus and the midbrain also significantly interacted with the participants' family history of autism in predicting infant autistic traits. Infants at increased familial likelihood, also had lower volumes in these regions (measured at 2 months) and this corresponded to higher autistic traits, as shown on the Q-CHAT at 18 months (mid temporal pole interaction: $\beta=0.17$, $p= 0.01$ / thalamus: $\beta=0.04$, $p= 0.04$ / midbrain: $\beta=0.09$, $p= 0.02$). This interaction was not found for the volume of the fusiform gyrus (interaction: $\beta=0.01$, $p= 0.13$).

Table 5.7: Multiple regression models of infant brain volume to autistic traits (Q-CHAT) of all nominally significant regions. N=4 regions were significant, after controlling via FDR. 'Volume-by-sex' is the interaction term for each region's volume and infant sex. HC: head circumference

	Volume	*Sex*	*Volume - by - sex*	*Age at Q-CHAT*	*Birth Weight*	*HC*
Middle Temporal Pole - *β*	**-0.178**	-56.181	0.135	-0.078	0.004	0.323
p	**0.006**	0.098	0.066	0.935	0.526	0.455
FDR - q	**0.045**					
Fusiform						
β	**-0.024**	-37.738	0.010	-0.213	0.006	0.872
p	**0.004**	0.445	0.382	0.818	0.335	0.141
FDR - q	**0.045**					
Thalamus						
β	**-0.043**	182.575	-0.066	1.283	-0.002	1.015
p	**0.004**	0.048	0.052	0.191	0.719	0.034
FDR - q	**0.045**					
Midbrain						
β	**-0.141**	-23.465	0.025	0.641	0.008	0.814
p	**0.005**	0.706	0.648	0.486	0.152	0.078
FDR - q	**0.045**					
Superior Temporal						
β	-0.019	24.002	-0.007	-1.280	0.005	1.393
p	0.015	0.453	0.522	0.163	0.366	0.009
FDR - q	0.081					
Lingual						
β	-0.020	-30.644	0.009	0.085	0.006	0.843
p	0.016	0.476	0.364	0.933	0.306	0.137
FDR - q	0.081					
Middle Temporal						
β	-0.022	-23.299	0.009	-0.523	0.006	0.746
p	0.018	0.458	0.403	0.585	0.283	0.126
FDR - q	0.081					
Inferior Temporal						
β	-0.022	-2.260	0.002	-0.577	0.003	0.932
p	0.025	0.953	0.853	0.553	0.630	0.075
FDR - q	0.101					
Hippocampal Commissure - *β*	-0.197	-58.866	0.130	-0.017	0.008	0.419
p	0.028	0.212	0.220	0.987	0.208	0.425
FDR - q	0.101					
Insula						
β	-0.030	34.082	-0.012	-0.969	0.013	0.984
p	0.048	0.537	0.563	0.323	0.037	0.074
FDR - q	0.141					

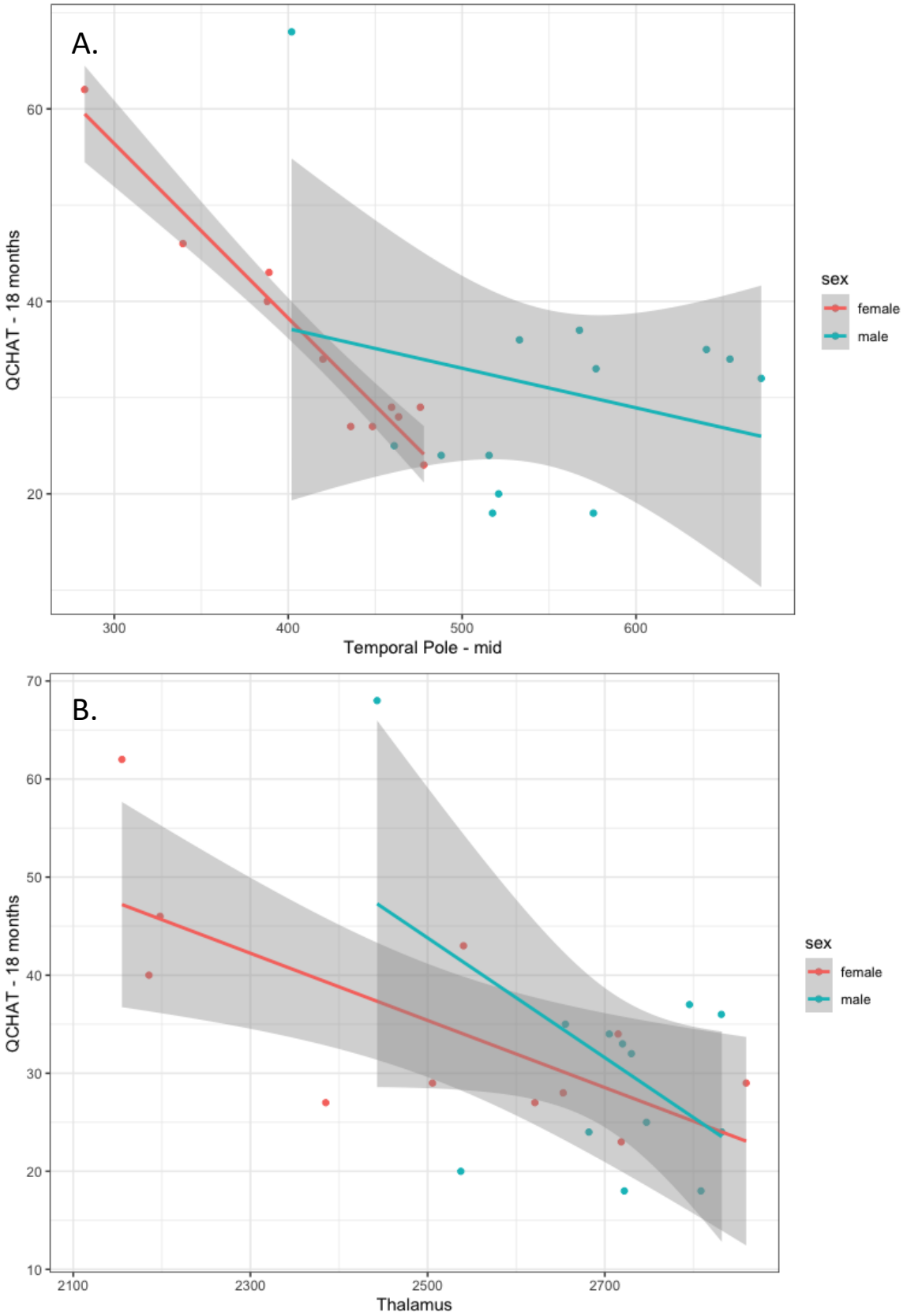


Figure 5.10: Regions that differed significantly between the sexes (A) middle temporal pole and (B) the thalamus, also correlated with autistic traits. A trend for an interaction with infant sex was noted for both (p<0.1), but in opposite directions.

5.4 Discussion

The CHILD study is the first longitudinal project in autism research with a focus on very early development, that includes both prenatal and postnatal brain scans, as well as the opportunity to study several factors that may be increasing the likelihood for autism in the children. These include steroid hormones levels in the infant ('mini-puberty'), perinatal brain growth and genetic likelihood based on autism family history. Therefore, this study allows for the investigation of interactive effects between these factors in early development and how these can, together, predict the emergence of autistic traits as early as 18 months of age.

This chapter focused on the potential role for biological sex and specifically on how baseline sex differences may be interacting with the factors mentioned above and leading to higher autistic traits. For clarity, the analysis has been separated in three separate studies and the discussion of the findings, follows the same structure:

5.4.1 Study 4: Mini-puberty

This study did not find any association between testosterone levels during mini-puberty and later autistic traits. This is consistent with previous studies that have investigated the same question (Auyeung et al. 2012; Kung et al. 2016). Compared to previous attempts, this study was the first to include (1) individuals at high familial likelihood for autism (2) measurement as early as the second month of postnatal life, which better captures the brief spike in steroid levels (3) two consecutive measurements (at mini-puberty and fourth months later at 'baseline'), in order to better describe this spike and ascertain, for the first time, the extent of this phenomenon in females using salivary testosterone, and finally (4) a statistical model that included an interaction with infant sex, rather than merely controlling for any sex differences that could be mediating both hormone level differences, and differences in autistic traits.

Despite these novel aspects of the study, no link was observed between testosterone levels and autistic traits, as described by the Q-CHAT questionnaire in early puberty. This is consistent with the null findings in previous studies that measured testosterone in infancy and at birth (in cord blood)(Auyeung et al. 2012; Kung et al. 2016; Jamnadass et al. 2015). On the contrary, studies that have examined steroids prenatally, in amniotic

fluid, have established links between them and infant autistic traits (Auyeung et al. 2010; Lutchmaya et al. 2002). These series of findings would indicate that prenatal, rather than postnatal, brain development is more susceptible to the neurodevelopmental effects of sex steroid hormones. This would place autism-related susceptibility at the same developmental window as sex differentiation; a notion that is consistent with the empathising-systemising theory of autism(Baron-Cohen 2002; Greenberg et al. 2018). This is because the 'masculinising' of both physiology and psychology has mostly been traced back to the 'prenatal masculinising window' in the 2nd trimester; a spike in steroid levels in the fetal circulation of males that follows the maturation of the fetal gonads (Welsh et al. 2014; Aydin et al. 2019). Nevertheless, organisational effects of postnatal testosterone cannot be excluded and indeed have been proposed to lead to more 'masculine' play behaviour in childhood in a recent study by one of the groups that found no link to autistic traits (Lamminmäki et al. 2012). The relative significance of prenatal, rather than postnatal surges in sex steroids in autism, would implicate steroid synthesis pathways that are specific to pregnancy, such as those involving the placenta. The placenta metabolises androgens rapidly to estrogens and also synthesises a variety of other steroids and neurotransmitter precursors throughout pregnancy. A link between placental steroid synthesis is further indicated by recent findings on associations of prenatal estrogens in humans and placenta-derived allopregnanolone in animals, with autistic traits (Tsompanidis et al. 2021; Vacher et al. 2021). Interestingly, in both organisms, the association significantly interacted with the sex of the offspring, with prenatal, placenta-related steroids affecting males and females differently. This could be related to baseline sex differences in placental functionality, which include both difference in steroid hormone levels, but also molecular differences in terms of X-chromosome inactivation and gene expression levels (Gong et al. 2018; Gonzalez et al. 2018).

This study confirms that salivary testosterone is elevated in females, during mini-puberty, albeit to a lesser extent than the one in males. Previous longitudinal studies of the phenomenon in females have relied on urinary secretion of the hormone (Lamminmäki et al. 2012). Only one study assessed the venous circulation of infants directly (n=32) and reported a gradual decrease of testosterone females since birth, rather than a transient elevation in the second and third month of life, as seen in males.

They reported much higher testosterone levels in males than females in the second month of life (more than a factor of 8x difference) (M. Becker et al. 2015). However, this study found considerable overlap in salivary testosterone levels between the sexes and no statically significant differences between males and females, at either the 2nd months or 6 months of age. A larger study would be needed, with the addition of an earlier time-point, to confirm if females too experience a transient increase in testosterone levels during their second month of life, rather than a gradual decrease since birth.

This study is limited considerably by its low sample size, compared to previously successful studies of mini-puberty. For example, with a sample size of n=77, researchers were able to uncover a correlation of steroid levels during this period, with the size of expressive vocabulary (Kung et al. 2016). Smaller cohort studies (n<30) have reported language-related associations, but only when they included physically measured outcomes, rather than parent-reported psychometric measures (e.g. phonetic articulation analyses of voice recordings or brain imaging via EEG) (Quast et al. 2016; Schaadt et al. 2015). The developmental window of mini-puberty may then better correspond to associated cognitive traits of autism, such as language development, rather than core features such as social (in)attention and repetitive behaviours.

In conclusion, Study 4 addresses many of the methodological insufficiencies of previously null studies and is still consistent with their lack of findings. A link between mini-puberty testosterone and autistic traits is unlikely based on our and others' findings but cannot yet be excluded given limitations in sample size of this study and the methodologies of previous studies.

5.4.2 Study 5: Perinatal Brain Growth

This study did not find evidence of excess brain growth in autism, nor an interaction with sex differences in this regard (i.e., high volume in males as well as high Q-CHAT scorers). On the contrary, the data indicate the opposite. Whole-brain measures (total volume and surface area) in infancy, as well as their change between late pregnancy and early infancy, were all negatively associated with autistic traits (Q-CHAT at 18 months). This effect was driven by individuals at high likelihood for autism, who had significantly higher Q-CHAT scores as a group. This association also interacted with underlying sex differences at the

infant stage, with males showing a more pronounced association between low brain volume in infancy and autistic traits. Prenatally, in the third trimester, whole brain metrics did not show significant sex differences and did not significantly predict later autistic traits.

This is the first time infant autistic traits, as measured in the Q-CHAT, are linked to structural brain differences obtained via MRI. Recent studies of autistic traits in childhood (Generation R - SRS) and in adulthood (with AQ Scores), have also reported negative correlations with brain volume and surface area, for multiple regions across the entire brain (e.g, frontal, temporal, parietal and occipital regions) but didn't report whole-brain correlations (Alemany et al. 2021; Schröder et al. 2021). A recent longitudinal MRI study of autistic individuals also did not found evidence that was consistent with "brain overgrowth" in childhood (Lee et al. 2021). One of the largest cross-sectional studies in autism (ages 2 to 64 years old) found instead a combination of increased cortical thickness (frontal regions), as well as lower cortical thickness (temporal regions), with peak differentiation of brain structure during adolescence (Rooij et al. 2018).

Besides brain imaging via MRI, evidence of biometric differences in head circumference are also mixed, with both smaller and larger measurements than the typical population, for both autistic traits in childhood (Generation R - Social Responsiveness Scale) and for clinically diagnosed autism (Blanken et al. 2018). Clinical cases of microcephaly or hydrocephalus, as well as genetic mutations relating to both, are all enriched in neonates that are later diagnosed with autism (Fombonne et al. 1999; Nebel et al. 2015).

Since the CHILD is an enriched cohort for familial likelihood, the findings of this study may not generalisable to the typical population but rather skewed by the underlying familial genetic likelihood. This could in turn be related to subclinical cases of microcephaly or pregnancy complications that haven't been accounted for. Nevertheless, bootstrap analysis showed that removing random individuals did not change the overall direction of the effect, confirming that the observed association is not the result of only a few outliers. In addition, broad pregnancy complications were controlled for in the models, by accounting for both growth restriction (via birth weight) and being born preterm (by adjusting age according to gestational age at birth). More research would be

needed to understand the interaction between brain growth and autism-related genetics in independent cohorts.

Even though no significant sex difference was observed in terms of autistic traits, an interaction was observed with sex, in their association with longitudinal brain growth, with males showing a more pronounced negative correlation. This is not in the direction predicted by the "brain overgrowth" theory, which would stipulate an added effect of brain growth and the male sex on autistic traits. By contrast, males with decreased brain growth between late pregnancy and early infancy, had the highest Q-CHAT scores in the second year of life. This sex-by-brain-growth interaction could be indicating an increased susceptibility of males, rather than an interaction with baseline sex differences in brain growth. These speculations will need to be confirmed in independent longitudinal cohorts that are large enough to allow for sex-stratified analyses.

Limitations of this study include the small sample size and the enrichment of the cohort with families at a higher likelihood for autism. The latter may be introducing genetic factors into the findings, that may not generalise in the general population. Pregnancy parameters and subclinical complications may also not have been adequately controlled for in the models that included birth weight and gestational age at birth.

In conclusion, Study 5 did not find evidence that brain overgrowth is associated with autistic traits in infancy. However, a subset of individuals that were high-scorers on autistic traits may be characterised by reduced perinatal brain growth, as well as low brain volume and surface area in early infancy (~2 months of age). This may be more apparent in children of families with an autistic relative (parent or sibling) as well as in males.

5.4.3 Study 6: Regional sex differences in infant neuroanatomy

This study follows up Study 5, with analyses of regional differences in brain structure, which characterise the interaction between sex differences in early infancy (2 months) and the emergence of autistic traits in late infancy (18 months) in both males and females.

The first analysis of baseline sex differences showed that these are more pronounced in 29 areas of the brain, after controlling for birth weight and adjusting significance thresholds for multiple testing using FDR. These correspond to half of the regions tested

and included most areas in the temporal lobe and prefrontal areas of the cortex, along with several striatal structures and the cerebellum. Similar data from large-scale population cohorts remain scarce at this age range, with most data on structural sex differences being reported in later childhood and adolescence (Ruigrok et al. 2014; Kaczkurkin, Raznahan, and Satterthwaite 2019).

In a recent longitudinal study of prenatal sexual differentiation of the brain (n=162 fetuses with 1 to 3 prenatal scans), only the temporal cortex showed significantly higher white matter volume in males, after controlling for intracranial volume and for multiple testing. (Studholme et al. 2020). In terms of more finely mapped regions, the authors reported a greater rate of growth for the temporal cortex, as well as baseline differences in parieto-occipital regions, including the cingulum, occipital cortex and the insular cortex. Different cross-sectional studies of the neonatal brain have also found sex differences in the temporal and occipital lobes, as well as in regions including the cingulate gyrus and insula (n=68 neonates and n=143 1-month olds respectively). But these were driven mainly by differences in total brain volume and they were either marginally or non-significant after correcting for this (Lehtola et al. 2019; Dean et al. 2018) These findings are consistent with the list of regions that were identified in this study. However, the analysis presented here was controlled for birth weight (rather than whole-brain measures), in order to control for sex differences in terms of overall size, but also to maintain important variance relating to the brain. Therefore, it is important to note that the regional sex differences reported in this study for the infant brain, may be the result of processes that affect the entire brain, rather than each region separately.

In addition to comparative studies of sex differences, a previous study by our group found an association between corpus callosum volume in childhood with amniotic testosterone levels measured during gestation (Chura et al. 2010). In this study, the volume of the corpus callosum was significantly larger in males but did not significantly correlate to infant autistic traits. This would further indicate that the link between autistic traits and sex differentiation processes includes series of genetic and hormonal parameters, besides testosterone. For example, estrogens, such as estradiol, have been linked to both autism and with the regulation of brain volume (Baron-Cohen et al. 2019; Shi et al. 2015). More research would be needed to study these complex endocrine interactions and their organisational effects on specific brain regions.

The brain regions that were significantly larger in males after controlling for birth weight, were all tested for association with autistic traits in later infancy. Almost half of them (13 out of 29) were negatively associated with autistic traits at nominal significance ($p<0.05$), in particular within temporal regions (Table 5.7). The volume of the middle temporal pole, the fusiform gyrus, the thalamus, and the midbrain remained significant after correction with multiple testing. As with total brain volume in Study 5.2, this effect was driven mostly by individuals with a family history of autism.

This is the first study to investigate the association between autistic traits and regional brain volume, as early as the 2nd month of life. The findings are consistent with the differences reported in other MRI studies in childhood and throughout life. Specifically, a study of correlations between brain structure and children's autistic traits in Generation R (ages 9 to 12) reported decreased surface area in the inferior temporal region, which includes the fusiform and part of the temporal pole (Alemany et al. 2021). In addition, a large cross-sectional study of autistic individuals (ages 2 to 64 years old) found decreased cortical thickness throughout the temporal lobe (Rooij et al. 2018). The thalamus and midbrain have also been identified in studies of sex differences in brain structure (Herting et al. 2012), as well as in autism (Lai et al. 2013).

Functionally, it is unclear how differences in regional brain volume may translate to behavioural or neuropsychiatric phenotypes. However, it is reasonable to assume that reduced volume in the third month of life represents a maladaptive trait, as the brain is rapidly expanding in terms of cortical thickness and surface area during this time (Lyall et al. 2015). In addition, evidence from case reports of lesions, neurodegenerative conditions and various experimental models (e.g. rodents or organoids) may provide additional functional insight. Both the fusiform and the temporal pole have been implicated in face recognition, with lesions in either of them leading to prosopoagnosia (Josephs et al. 2008; Barton et al. 2002). Based on these first clinical observations, many of the first functional studies of autism, focused on these regions, as part of the newly conceptualised theory-of-mind network (Castelli et al. 2000). The temporal poles in particular, first gained attention because of their proximity to the amygdala, which had been implicated in autism since the start of neurocognitive research into the condition (Baron-Cohen et al. 2000). Since then, a series of findings in targeted, region-of-interest analyses have revealed structural and functional differences in these regions, in the

brains of autistic individuals. Specifically regarding the temporal pole, underconnectivity was first reported in autistic adults, during a task of social salience, in which participants were meant to recognise social-like motives in animated, moving triangles during an MRI brain scan (Castelli et al. 2000). In autistic children, decreased volume was found in their right temporal pole (n=21, mean age=9.3 (2.2)), compared to undiagnosed age-matched controls (Boddaert et al. 2004). In addition, a compilation of clinical neuroradiology reports for the brain scans of n=77 autistic children, found an enrichment for myelination-related alterations in the temporal poles, among several other temporal lobe abnormalities (Boddaert et al. 2009). Myelination differences were also indicated in a study of autistic adolescents, who showed atypical patterns of age-related cortical thinning in the right temporal pole, as well as both fusiform gyri (Wallace et al. 2010). Similar patterns, as well as cortical atrophy and decreased connectivity of the temporal poles were seen in a more recent study of n=22 autistic adolescents in Brazil (Pereira et al. 2018).

Besides case-control differences in autism, the temporal pole has also been implicated in many important aspects of cognition, of particular significance to the infant brain. For example, activation of the region was shown to underpin the recognition of human speech and of familiar voices in particular, in fMRI and PET neuroimaging studies respectively (Crinion et al. 2003; Nakamura et al. 2001). The proximity of the temporal pole to the limbic system (e.g. the amygdala) and several sensory perception pathways (e.g. the visual stream along the temporal cortex) has also prompted neuropsychologists to propose that the temporal pole is the point of integration of emotion with sensory perception (Olson et al. 2007). In addition, there is substantial clinical evidence that implicates the region in semantic dementia, leading to speculation that the temporal poles play a very significant role in the learning and retrieval of semantic concepts throughout life, in addition to face recognition (Patterson et al. 2007).

The fusiform gyrus too has been linked to face recognition, sex differences, and autism (Lotze et al. 2019; Dalton et al. 2005; Josephs et al. 2008). With regards to the latter, a post-mortem cellular study detected a lower number of neurons in several layers of the fusiform, after controlling for cortical grey matter as a whole (n=7 cases, mean age= 12.1 (2.80 years old) (Van Kooten et al. 2008). In addition, many studies of brain activity tasks, have reported differences in activation of the fusiform during face-processing tasks in

childhood (Pierce and Redcay 2008), adolescence and adulthood (Pierce and Redcay 2008), replicating the findings of many earlier, smaller studies that first conceptualised autism as a deficit in mentalising/theory of mind (Critchley et al. 2000; Schultz et al. 2000). Interestingly, a more recent fMRI study that also included images relating to their autistic cohort's restricted interests, reported that the fusiform gyrus was "recruited" by the participants' non-social interests, rather than face processing. The authors speculated that atypical connectivity of the fusiform gyrus may link both of the core features of autism, by replacing a specialisation for social perception with network activity that is more specific to non-social subjects (Foss-Feig et al. 2016).

The findings regarding the temporal pole and fusiform are also of developmental significance, given this study's prospective design. A functional deficit in these regions in early infancy, could potentially lead to impaired social learning and a series of neurocognitive challenges in later life. This has been indicated by a series of behavioural studies that found a reduced ratio of social-vs-non-social attention in autism as early as 6 months of age (Maestro, 2002). Based on the brain regions identified here, it is interesting to speculate that an early postnatal deficit in face and/or human speech recognition may be sufficient to delay social learning, speech acquisition and lead to sensory overload in social situations in later life. These neurodevelopmental sequalae would need to be confirmed in larger longitudinal studies that monitor neurocognitive development and behaviour from birth to late childhood.

Finally, it is important to note that baseline sex differences remain relevant for early neurodevelopment. The temporal poles are amongst the regions most frequently identified in MRI studies as being larger in males, across all ages (Ruigrok et al. 2014). In this study, infant sex significantly interacted with regional volume in most of the identified regions in predicting later autistic traits, with the association between the two being more apparent in females, rather than males. This is consistent with previous findings in the activation of the fusiform gyrus, in a study of infants with tuberous sclerosis; a genetic condition that increases the likelihood for autism by ~ 50%. Similar to the study presented here, female infants with tuberous sclerosis showed stronger associations between fusiform underconnectivity and later autistic traits, than male infants with the condition, as early as 1 year of age (Scherrer et al. 2020). This interaction with infant sex could reflect reduced variance in males (a 'ceiling effect') or increased

susceptibility in females with high autistic traits and at high familial likelihood for autism. In terms of outcomes, sex differences in social-vs-non-social attention have also been described as early as 36 hours after birth (Connellan et al. 2000). Larger studies that would allow for sex-stratified analyses could investigate the interaction between early postnatal sex differences in regional brain growth and neurocognitive development in infancy.

In conclusion, Study 6 of regional differences according to sex and autistic traits, has yielded functionally relevant findings for the pathophysiology of autism. This is the first study to implicate a part of the temporal pole and the fusiform gyrus with an analysis that didn't involve a targeted, region-of-interest design. It is also the first study to link specific brain alterations to autistic traits in infants. Finally, it is the first longitudinal study that identifies a possible neurostructural biomarker for autistic traits, as early as the second month of life. These findings should be treated as tentative, until they are replicated in an independent cohort, in families with and without a history of autism, as well as separately in males and females. Nevertheless, they are consistent with prior literature and decades-long learned speculations on the neurocognitive underpinnings of autistic traits. If replicated, they could facilitate early postnatal diagnosis of infants at high familial likelihood, leading to targeted interventions and improved learning outcomes in later life.

The potential of integrating genetics in screening protocols is significant, since new sequencing and genotyping technologies could provide more molecular insight than protocols based on familial likelihood. However, the heritable components of autism may interact with prenatal physiology as well, as indicated in this study. In the next chapter, this notion is investigated further as the genetics of autism are studied together with the genetics of endocrine conditions and of specific prenatal factors that have been linked to autism.

Chapter 6
Genetics and sex-related factors

Summary of studies

Study 7: Rare variance: Enrichment of autism genes in male placentas

In this study, an enrichment analysis was conducted, based on a list of genes that corresponded to differentially expressed transcripts (FDR<0.1) between male and female placentas, in 1st trimester chorionic villi samples of viable pregnancies. These were compared to a list of high-confidence autism genes, based on syndromic forms of the condition or an overabundance of rare variants in sequencing studies. There was significant enrichment of X-linked of autism genes in male-biased placental gens, independently of gene length (n=5 genes, p<0.001). There was no overlap in autosomal genes. The overlapping genes on the X-chromosome are further analysed in terms of their function and regulation, as well as the different phenotypes that have been associated with them and autism.

Study 8: Common variance: Genetic correlations between autism & endocrine outcomes

In this study, summary statistics of GWAS were used to study the genetic correlation between neurodevelopment and sex steroid-related traits and conditions. The latter included genotype data for bioactive testosterone, estradiol and postnatal PlGF levels, as well as genotypes of PCOS, age of menarche and androgenic alopecia. Genetic correlation was calculated based on LD Score regression and the results were corrected for multiple testing with FDR. Common variance for autism did not correlate to the genetics of testosterone, estradiol or PlGF, but it was associated with the genotypes for early age of menarche (b=-0.109, FDR-q=0.004) and a relative protection from androgenic alopecia (b=-0.135, FDR-q=0.007).

6.1 Introduction

In Chapter 5, it was shown that familial likelihood for autism (based on diagnosed relatives) was associated with neuroanatomical and neurodevelopmental outcomes in early infancy, in a cohort as small as n=27. As was discussed in Chapter 1, most of the liability for autism can be attributed to genetic factors. Heritability estimates are mostly derived from twin-pair studies and genome-wide association studies and have been consistently high for both (Colvert et al. 2015; Huguet et al. 2016; Klei et al. 2012). However, discovering specific genes that capture a large percentage of the variance has proven challenging.

Exome sequencing studies have been more successful, uncovering many syndromic forms for the condition that are often attributed to *de novo* variants and including both copy number variants and single-gene mutations (Satterstrom et al. 2020). The Simons Foundation for Autism Research (SFARI) has collected information on all of these genes, compiling curated lists, based on the available evidence (Banerjee-Basu et al. 2010). These are scored according to the number of "hits" across all studies, the strength of the evidence for each and whether these are involved in syndromic or non-syndromic forms of the condition. Most of them relate to the regulation of the cell cycle in embryonic pathways and the function of neuron synapses.

Yet, together these identified genes account for less than 10% of the variance in the population, with the majority attributed to common variance (Gaugler et al. 2014). In order to capture this and discover specific genes, two related case-control genome-wide association studies (GWAS) have been conducted (Psychiatric Genomics Consortium 2017)(Grove et al. 2019). The largest included n=18,381 autistic people diagnosed in Denmark who were linked to a certified diagnosis via the National Psychiatric Register and were genotyped as part of iPSYCH (Grove et al. 2019). This study led to the discovery of five genome-wide significant loci. In their conclusions, the authors estimate that a considerably larger sample would be needed to uncover more genes and their related molecular pathways. As with all complex heritable traits, genetic variants of intermediate frequency will also need to be discovered via sequencing, in order to bridge the gap between polygenic and monogenic forms of the condition (Manolio et al. 2009).

However, it still remains unclear how this largely 'genetic' condition may affect males and females differently and lead to different prevalence rates for each sex. Two hypotheses have been put forward to resolve this (Werling and Geschwind 2015). First, that autistic males and females may be characterised by different genetic factors, which functionally converge on the neuronal or only on the behavioural level, leading to different liability distributions for each sex. Second, that both males and females are affected by the same genetic variants but underlying physiological "protective" factors in the latter interact with genetic liability and result in a sex-specific threshold for diagnosis, within the same liability-threshold model. In accordance with this, exome sequencing studies have consistently found that autistic females have a larger number of loss-of-function mutations, compared to males (Jacquemont et al. 2014; Kosmicki et al. 2017; Satterstrom et al. 2020). Hormonal factors (such as prenatal steroids) or differences in physiology (such as differing growth rates) may be mediating this 'female protective effect' (Baron-Cohen et al. 2019; Blanken et al. 2018).

On the molecular level, the interaction between autism genes and sex may be bidirectional. Autism-related genetic variants may be directly affecting sex differentiation mechanisms, including steroid hormone levels, leading to different effects for each genetic variant in males and females. Alternatively, steroid hormones may be involved in the regulation of the same autism-related genes, resulting in relative differences in how their gene products are expressed, translated, and "tolerated" in males and females.

With regards to the former, candidate gene studies have reported associations of autism and autistic traits with polymorphisms in hormone receptors (e.g. the estrogen receptor ESR2), as well steroid-regulating enzymes (e.g. aromatase) (Chakrabarti et al. 2009; Crider et al. 2014). But these findings have not been replicated when the whole genome was assessed in GWAS (Grove et al. 2019). With regards to the latter hypothesis of a mediatory role of sex steroids, a study of testosterone administration in developing neurons, found that the genes affected by the hormone (identified via ChIp-Seq) significantly overlapped with autism-related genes, including SFARI genes and genes that are differentially expressed in the autistic brain (identified by RNA-Seq post-mortem) (Quartier et al. 2018). But a wider comparison of all the genes that have steroid-responsive promoters, failed to find any enrichment. Instead, a moderate correlation of

autism-relate variance was reported with genes relating to sex differences in anthropometry (e.g. height) (Mitra et al. 2016). The same study reported sex heterogeneity with regard to X-chromosome-linked genetic variants associated with autism. Genetic variance on the X-chromosome could also be mediating a female protective effect, particularly on genes that escape inactivation or are characterised by haploinsufficiency when mutated. It is also important to note that the androgen receptor is encoded by a gene on the X chromosome. But in autism, studies have again produced mixed results regarding a significant enrichment for X-linked genes, or more specifically in regions that escape X-chromosome inactivation (Schutz et al. 2002; Gong et al. 2008; Nava et al. 2012; Chung et al. 2011).

So, there is little evidence that autism genes directly regulate steroids or are lie directly downstream of steroid-responsive elements. Nevertheless, the functional interaction between steroid hormones and autism genetics may be indirect and further downstream, at the level of molecular pathways and networks. In addition, male vulnerability may be mediated by factors that are not specific to autism and also extend to other neurodevelopmental conditions that also show sex differences in prevalence. Consistently, a study that analysed genetic variants associated with autism, together with ADHD, anxiety and many more comorbid traits, reported that their combined downstream targets and associated molecular pathways converged on networks that featured prominent roles for the estrogen receptor (ER-2), as well as aromatase (Leticia Diaz-Beltran et al. 2017). Moreover, the interaction between autism genes and sex differentiation processes, may be specific to early development, embryonic pathways, and particularly prenatal physiology. As previously mentioned, one of the few studies to report an overlap between testosterone targets and autism-related genes, focused their enrichment analysis on developing neurons (Quartier et al. 2018). However, steroid-synthesising tissues, such as the placenta, have not yet been analysed in a similar way, despite consistent reports of sex differences in gene expression, genetic mosaicism, and widespread escape from X-chromosome inactivation in females (Gonzalez et al. 2018; Gong et al. 2018; Gong et al. 2018; Coorens et al. 2021).

Alternatively, if autism genes and steroid-related molecular pathways interact beyond prenatal development, then this may also affect health outcomes throughout life and lead to higher prevalence of steroid-related symptoms and conditions in autistic people.

Although, epidemiological evidence is certainly consistent with this, particularly for conditions such as PCOS (Pohl et al. 2014; Cherskov et al. 2018; Simantov et al. 2021), the association between autism and steroid-related conditions has not been specifically shown at the genetic level.

In order to better inform the discussion on the interaction between sex and genetics in autism, according to these parameters, two brief exploratory analyses are presented in Studies 7 and 8. These respectively pertain to rare and common variants as well as prenatal and lifelong outcomes. In Study 7, genes that show sex differences in placental expression are checked for enrichment for high-confidence autism genes that have been consistently implicated to the condition by exome sequencing or in syndromic forms of the condition (SFARI categories 1 and S). In Study 8, autism-related common genetic variance (captured via GWAS) is studied in associated with the genetic variance relating to steroid hormone levels in adulthood (UK Biobank) and of the placental growth factor (PlGF), as well as to the genetics of complex steroid-related traits (publicly available data on age of menarche, PCOS and androgenic alopecia).

6.2 Methods & Results

6.2.1: Study 7 - Rare variance: Enrichment of autism genes in male placentas

Prenatal gene expression differences between the placentas of males and females have been reported in a study by Gonzales et al, conducted in Cedars-Sinai Medical Center of UCLA (n=17 females, n=22 males). This is the only study that has investigated these differences in humans and during term-time, at a time-point corresponding to 10.5 - 13.5 weeks gestation. This was achieved with RNA sequencing of the discarded chorionic villi samples, following prenatal genetic screening of the pregnancies, which were all carried to term. No additional information was reported for any clinical reasons that led to prescription of CVS sampling. The same placentas were then collected after birth and their gene expression was compared to measurements during gestation. Except birth weight, cohort covariates such as maternal age or crown rump length did not significantly differ between the sexes. Sex chromosomes were analysed separately to autosomes and found to contribute significantly to gene expression differences between the sexes. This was mainly driven by genes on the X chromosome and remained consistent throughout gestation and at birth. On the contrary, most of the autosomal genes with sex differences in the 1st trimester samples, did not show the same differences when placentas assessed at birth.

For the purposes of this enrichment analysis, a list of genes was created, that corresponded to the transcripts identified by Gonzales et al, as being significantly different between the sexes (both up- and down-regulated), at an FDR-corrected level of significance of $q<0.1$. This list comprised of n=135 genes, n=31 of which are on the X chr.

Regarding autism, a list of high-confidence genes was assembled, based on the curated database of SFARI. Only genes with the top confidence score of "1" (n=206), as well as "syndromic" genes were included (n=143). Based on SFARI policy, the former score is only awarded to genes with a minimum of three independent discoveries in sequencing studies and a likely loss-of-function effect (Satterstrom et al. 2020). The latter characterisation ('syndromic') refers to genes of identified syndromes that also substantially increase the likelihood for autism in those affected. The two categories are not mutually exclusive, with the final list that was checked for enrichment numbering n=286 different autism-related genes, of which n=34 are on the X chromosome.

Enrichment analysis was conducted separately for autosomes and the X chromosome, by comparing the number of genes overlapping between the two lists (autism and placenta), to the total number of coding genes in the genome. Logistic regression models were used, in order to control for gene length as a covariate in these comparisons. Information on identified coding genes, gene locations and lengths were obtained from Gene Ensembl.

There was no overlap between autosomal genes that were differentially expressed in male/female placentas, and high-confidence autism genes. The comparison that was specific on the X-chromosome, identified a total overlap of n=5 genes (Table 6.1). This was significant in a logistic regression model that controlled for gene length and compared this to the total number of genes on each list (n=34 for autism, n=31 for the placenta) and on the X-chromosome. The model showed that this enrichment was significant at $p<0.0001$, for an OR=11 (3.6 - 33.7). All five of the genes were differentially expressed in placentas at FDR-corrected $p<0.05$, with four being upregulated in females, compared to males (Table 6.1).

Table 6.1: SFARI Genes that show significant sex differences in placental expression.

Gene ID	***Gene Name***	***M/F***	***Autism / Total***	***Gene Function***	***SFARI Score***	***Conditions***
SMC1A	structural maintenance of chromosomes 1A	Female upregulated	2/8	Segregation of sister chromatids during division	S + 1	Cornelia de Lange - 2, DD/NDD, EPS Rett-like phenotypes
KDM5C	lysine demethylase 5C	Female upregulated	6/27	transcription and chromatin remodelling	1	EPS, Autism, EP, DD/NDD
ARHGEF 9	Cdc42-guanine nucleotide exchange factor (GEF) 9	Male upregulated	4/11	Rho-like GTPase, mTOR regulator	1	EPS, ID, Autism
HDAC8	Histone deacetylase 8	Female upregulated	1/8	represses transcription in large multiprotein complexes	S	Cornelia de Lange - 5
DDX3x	DEAD box helicase 3	Female upregulated	7/27	transcription regulation, mRNA assembly, splicing	1	DD/NDD, ADHD, EPS, ID, Autism, EP

6.2.2: Study 8 - Common variance: Genetic correlations between autism & endocrine outcomes

For the purposes of genetic correlation analyses, publicly available summary statistics of GWAS were collected, regarding autism, hormone levels and hormone-related traits. Datasets for these were obtained via the NHGRI-EBI Catalogue (Table 6.2).

Table 6.2: GWAS of hormone levels and hormone-related traits that were investigated in association with autism. Bio T: free fraction of circulating testosterone, based on testosterone and SHBG

	Bio T	***Bio T***	***Estradiol***	***PlGF***	***PCOS***	***Age of menarche***	***Alopecia***
Study	*Ruth SK et al, Nat Med, 2020*			*Folkersen L et al, Nat Metab, 2020*	*Day F et al, PLoS Gen, 2018*	*Day F et al, Nat Genet,2017*	*Hagenaars SP et al, PLoS Genet, 2017*
Cohorts	UK-Biobank			various, SCALLOP	various in UK, NL, USA	ReproGen, 23andMe, UKB	UK Biobank
N	178,782	188,507	206,927	21,758	24,267	329,345	52,874
Sex	males	females	males	both	Female cases, mixed controls	females	males
Age	40 - 69	40 - 69	40 - 69	>40 yo	27 - 60	NA	40 - 69
Loci	125	147	22	2	14	389	250

In terms of neurodevelopment, two outcomes were selected. First, clinically diagnosed autism, based on the latest GWAS (n=46,350 (18,381 cases))(Grove et al. 2019), that identified five loci at genome-wide level of significance and corresponded to both males and females with the condition, as identified and certified by the iPSYCH consortium based in Denmark, as well as the Psychiatric Genomics Consortium that had previously published the first autism GWAS (Psychiatric Genomics Consortium 2017). The second outcome that was investigated corresponded to the entirety of the iPSYCH cohort and included some of the same individuals with autism (n=6,939 overlap in cases), as well as anyone with ADHD, affective disorder, bipolar disorder, anorexia, or schizophrenia, who were diagnosed and genotyped in Denmark (total n=65,534 with controls) (Schork et al. 2019). Therefore, the second trait outcome included the first (26.2 % of iPSYCH had

autism) but also individuals with conditions that are often comorbid with autism. This second aggregate outcome was included in this chapter, in order to increase power in correlation analyses and investigate the specificity of potential findings.

Regarding hormone levels and steroid-related conditions, summary statistics of several GWAS were obtained online via publicly available resources (Table 6.2). These corresponded to several independent cohorts that did not overlap with the Danish iPSYCH cohort.

Bioavailable testosterone was calculated in the UK Biobank, by dividing total testosterone levels to the concentrations of SHBG for each individual (Ruth et al. 2020). This was calculated for both males and females and then linked to genotype data. In the same cohort, estradiol level measurements were available in both genders, but the GWAS for estradiol levels was restricted to males, as no heritable component corresponded to this trait was found in females ($h2<2$). This was probably due to the low sample number, as the cohort consisted of mainly post-menopausal women whose estradiol levels were below the level of detection (78% of the female cohort).

Placental growth factor (PlGF) levels were measured during adulthood, in circulating serum, as part of proteomic analyses that were aggregated into a meta-analysis of different studies (SCALLOP consortium) that investigated associations of serum biomarkers with health outcomes. In this study, circulating PlGF levels were significantly associated with the genetics of cardiovascular disease, an effect that the authors attributed to the angiogenesis role of the factor, which extends beyond pregnancy (Folkersen et al. 2020).

The common variance for PCOS included in this Chapter only included clinically diagnosed cases, using a variety of diagnostic criteria. In the corresponding GWAS, this was combined with cases from 23andMe (based on self-report) and resulted in many genome-wide significant loci that were linked to steroid hormone regulation and gonadotropin regulation, independently of the method of diagnostic ascertainment (F. Day et al. 2018). For the purposes of this study, self-report data on PCOS were excluded.

The GWAS on age of menarche, on the other hand, included self-report genotype data from 23andMe, meta-analysed together with data from the UK Biobank and the Reproductive Genetics Consortium (Day et al. 2017).

Finally, androgenic alopecia was ascertained in adult males in the UK Biobank, older than 40 years old. The trait was analysed categorically, based on the degrees of hair loss and age of onset. The X chromosome was analysed separately to the autosomes, given the established and disproportionate role of the androgen receptor in the liability of the trait, and was not included in the summary statistics (Hagenaars et al. 2017) or Study 8.

Genetic correlation of common variance was based on LD scores that were developed for each HapMap locus (Bulik-Sullivan et al. 2015). Variables of summary statistics were prepared according to this method's specifications. A breakdown of sample size for each SNP was included, where available. If SNP identifiers were missing, these were derived based on SNP location and the NCBI-SNP database. Multiple testing correction was conducted with application of a false discovery rate (FDR), based on the number of tests (n=7) for each neuropsychiatric outcome separately.

Of all the examined hormones and traits, only the common variance of age of menarche and alopecia correlated to the common variance for autism (Table 6.3 in bold). The former correlation extended to the wider iPSYCH dataset that included additional psychiatric conditions, while the latter was marginally non-significant, after controlling for multiple testing. Both of the correlations were negative in direction, with higher genetic burden for autism being associated with a lower age of menarche and reduced likelihood for hair loss.

Table 6.3: Pairwise genetic correlations based on LD scores of GWAS summary statistics. q is FDR-corrected value of statistical significance. 'Bio T': the levels of free testosterone, based on circulating total testosterone and SHBG.

	Bio T males	***Bio T females***	***Estradiol males***	***PLGF***	***PCOS***	***Age of menarche***	***Alopecia***
	h2: 0.13 (0.01)	h2: 0.16 (0.01)	h2: 0.02 (0.01)	h2: 0.12 (0.05)	h2: 0.13 (0.02)	h2:0.21 (0.01)	h2: 0.30 (0.05)
Autism	b=-0.01	b=-0.01	b=-0.06	b=0.06	b=-0.075	**b=-0.109**	**b=-0.135**
h2: 0.19	SD=0.04	SD=0.01	SD=0.08	SD=0.09	SD=0.084	**SD=0.031**	**SD=0.044**
(0.02)	z=-0.25	z=-0.20	z=-0.78	z=0.61	z=-0.893	**z=-3.482**	**z=-3.077**
	p=0.80	p=0.84	p=0.438	p=0.55	p=0.372	**p=0.0005**	**p=0.0021**
	q=0.84	q=0.84	q=0.767	q=0.77	q=0.767	**q=0.0035**	**q=0.0074**
iPSYCH combined	b=-0.07	b=0.08	b=-0.01	b=-0.08	b=-0.021	**b=-0.124**	b=-0.106
h2: 0.13	SD=0.04	SD=0.04	SD=0.07	SD=0.09	SD=0.09	**SD=0.036**	SD=0.044
(0.01)	z=-1.61	z=2.01	z=-0.16	z=-0.85	z=-0.23	**z=-3.452**	z=-2.402
	p=0.11	p=0.045	p=0.09	p=0.39	p=0.818	**p=0.0006**	p=0.0163
	q=0.154	q=0.105	q=0.154	q=0.455	q=0.818	**q=0.0042**	q=0.057

6.3 Discussion

6.3.1: Study 7 - Genetic Overlap on the X

This analysis compared the gene expression differences in the placenta, during mid-pregnancy, to a list of genes that have been associated with autism. Enrichment of autism genes in sex-biased placental gene expression was mixed. While there was no genetic overlap, when autosomes were considered, there was a significant enrichment, when the analysis was restricted to the X-chromosome. This was due to an overlap of five genes on the X, namely SMC1A, KDM5C, ARHGEF9, HDAC8 and DDX3x.

Sex differences in placental function and vulnerability have been known to clinicians for a while. But the molecular background to this has only been studied relatively recently, following the discovery of high-throughput genomic methods, such as microarrays and next-generation-sequencing. Consistently, across all human studies, placental gene expression differences between the sexes, can largely be attributed to differences in the dosage of genes, located on the X and Y chromosomes (Tsai et al. 2011; Gong et al. 2018). In the study by Gonzales et al, prenatal differences in the expression of genes on the X were consistent with measurements conducted at birth. On the contrary, differences in autosomes fluctuated according to gestational age (Gonzalez et al. 2018). Further analysis showed that the placental X-linked genes that differed by sex, were not restricted to regions that are known to escape X-chromosome inactivation, but rather extended across the length of the chromosome.

In a more recent, large-scale study of placental genomics (at birth), researchers were able to compare gene expression differences not only between the sexes, but also within the placentas of the same individuals. They also reported significant sex differences, stemming from the X chromosome. But intraindividual comparisons, paired with genotyping, also revealed that there was pronounced genetic mosaicism within the same placentas, which extended to transcriptional differences (Coorens et al. 2021). This complex genetic landscape could potentially be attributed to the tissue's clonal expansion and driven by rapid cycles of DNA replication and cell division. This could in turn lead to inefficiencies in chromosome methylation, X-chromosome inactivation and genetic instability, leading to accumulation of mutations confined within clonal cell populations, similarly to neoplastic growth. So, sex differences in placental genomics and ultimately

function could be attributed to the tissue's complex genetic landscape, gene dosage effects on the X-chromosome and this controlled state of genetic instability.

This phenomenon may also be affected by the effects of hormones and the person's own genetic background. Male placentas have been found to express higher levels of DHEAS; the main steroid precursor for both androgens and estrogens (Gong et al. 2019). In the study by Gonzales et al, unsupervised analysis of the identified variants via the IPA software, identified the estrogen receptor-1 and progesterone as potential upstream regulators of the identified sex differences (Gonzalez et al. 2018). It is also important to note that sex steroid hormones have pronounced effects on methylation, by directly acting on histone modification enzymes, as well as the genome itself (via their receptors)(Cleys et al. 2015). Therefore, sex differences in placental gene expression are probably not independent to the effects of sex steroid hormones.

Regarding autism, prenatal steroidogenesis, methylation differences in the placenta and genetic instability have all been associated with increased likelihood for the condition (Eichler and Zimmerman 2008). In this study, five genes were identified that may be contributing to the interactions between these factors.

All five of the genes take part in the regulation of genomic processes, such as transcription, gene replication and mitotic segregation. However, some have also been linked to steroid-related phenotypes. For example, KDM5C encodes a DNA-binding transcription regulator that is involved in chromatin remodelling. But a common genetic variant directly upstream of this gene (rs140498714) was identified in the largest GWAS of testosterone levels in older adults of the UK Biobank (Ruth et al.2020). Syndromes linked to the same locus, have also been associated with abnormalities in the formation of the male external genitalia. The main example is the 'Mental Retardation, X-Linked, Syndromic, Claes-Jensen Type (MRXSCJ), which, in addition to neurodevelopmental symptoms, also features short stature, cryptorchidism, micropenis and endocrine symptoms in later life, like amenorrhea (in females), alopecia (in males) and excessive body hair growth in neonates and children (Carmignac et al. 2020). In addition, in a mouse knockout model for Kdm5c, there was a trend for significant elevation in circulating testosterone levels ($p=0.07$) and a significant increase in the animals' aggressive behaviour. These changes were attributed to changes in the brain, where

dendrite differences, an increase in spines and upregulation of androgen targets, were noted in the amygdala and frontal cortex (Iwase et al. 2016).

Similarly, DD3X encodes a helicase involved in transcription and splicing, which has been linked to the main symptoms of Klinefelter syndrome. Individuals with this syndrome have increased prevalence for autism, but also frequently have defects in primary and secondary sex differentiation, with frequent eunochoid body proportions and primary hypogonadism (small gonads and decreased testosterone) (Hong and Reiss 2014; Bonomi et al. 2017). In a mouse model of the condition, DD3X was seen to escape X-chromosome inactivation, leading to a significant up-regulation of the transcript in the brain of the Klinefelter-type, compared to the brains of both male and female mice (Werler et al. 2011). It is interesting to speculate that loss-of-function mutations in autism may have the opposite effect and lead to sex steroid excess.

Contrary to the other genes, which were downregulated in male placentas, the transcript for ARHGEF9 was found to be upregulated in males. This gene encodes a guanine nucleotide exchange factor, that takes part in molecular cascades involving CDC42 and the mTOR pathway. In a clinical setting, ARHGEF9-related syndromes have been associated with autism, developmental delays and speech impairment, with epilepsy being a common symptom in most cases (Bhat et al. 2016; Alber et al. 2017). On the molecular level, the gene product was reported to contribute to the formation of GABAnergic and glycinergic synapses in the brain. Interestingly, common variance around the gene has also been linked to total testosterone levels (in men) (Ruth et al. 2020), as well as male-pattern alopecia (within an intron of the gene)(Hagenaars et al. 2017).

Finally, mutations in two of the genes that were identified in this analysis (DDX3X, SMC1A), can lead to Cornelia-de-Lange syndrome (CdLS). This rare genetic condition is characterised by mental and physical delays, microcephaly and a characteristic facial phenotype with short brows and synophrys (Kline et al. 2018). The children often exhibit autistic behaviours in response to overstimulation. This symptomatology is often worse in males, than females. Interestingly, children with the condition are often born with excess body hair, but this may be the result of dysfunctions in epithelial differentiation of the skin, rather than an example of hirsutism due to excess testosterone. Research into the endocrine correlates of the condition is limited, with unpublished conference report

describing significant differences in testosterone (Ascaso et al. 2019). On the molecular level, clinical sequencing in CdLS has revealed widespread defects in transcription and genetic instability (Yuan et al. 2015).

All of these examples confirm a molecular link between genetic pathways involved in neurodevelopment, genetic instability and in some cases, sex differences and the endocrine system. However, caution should be employed in the interpretation of the observed enrichment, as the result may be confounded by the 'X-factor'. Genes on the X have been found to be highly expressed in the brain, relative to other chromosomes (Nguyen and Disteche 2006). Consistently, there is also a relative overabundance of X-linked genes that are associated with mental disabilities (Zechner et al. 2001), and X-linked common variance in the UK Biobank has more recently been shown to be associated with neuroanatomical differences in many brain regions (Mallard et al. 2021). Therefore, in the context of Study 7, the SFARI-curated list of genes may not be specific to autism, but rather reflect a link between the human brain and the X-chromosome. In addition, there is potential for clinical ascertainment bias in the study by Gonzalez et al, as the CVS samples pertained to pregnancies that were recommended genetic screening. No clinical information is reported by the authors, other than relatively advanced maternal age (mean=39.4[SD=2.4]years old), so their genomic results may not be generalisable to prenatal placentas in the wider population.

In conclusion, this enrichment analysis offers a first indication that placental sex differences on the genetic level, may be overlapping with the genetic liability for autism. More research is needed to study placental gene expression profiles that are specific to autism, as well the role of the autosomes.

6.3.2: Study 8 - Genetic Correlation Analysis

The majority of the genetic liability for autism can be attributed to common variance (Gaugler et al. 2014). In this exploratory analysis, the genetics of autism were examined in association with the genetics of several steroid-related traits and conditions. It is important to note that these genetic correlations only capture the polygenic component of each trait, relating to common genetic variance They aren't tests of a link on the clinical level. In addition, each GWAS is affected by varying degrees of statistical power, as well

as the specific demographic characteristics of each cohort. Particularly regarding steroid hormone levels and PlGF levels, their assessments in the corresponding GWAS were all postnatal and in late adulthood, setting them apart from the other studies presented in this thesis that included prenatal measurements of the same hormones. This may be the reason that no genetic correlation was found between the genetic variance associated with testosterone, estradiol or PlGF levels with autism, despite previous clinical and epidemiological evidence. Alternatively, elevated steroidogenesis in autism may be due to genetic factors that are not captured well by the latest GWAS, but rather related to rare variants, the X-chromosome or to interactions between genes and the wider endocrine system.

For example, polycystic ovaries syndrome (PCOS) is a complex and heterogeneous condition that includes multiple hormonal systems and cannot (yet) be attributed to a single pathophysiological cause. In the context of autism, elevated rates of the condition have been reported in women with the condition (Cherskov et al. 2018). However, the evidence is certainly more conclusive for the effects of maternal PCOS on autism likelihood in the children, following replications in independent cohorts (Katsigianni et al. 2019). In this study, no genetic correlation was noted between the genotypes associated with PCOS and the ones for autism. This could be an indication that the link between the two conditions may be attributed to 'environmental' factors, such as increased androgen exposure in utero. The placentas of women with PCOS have been found to have lower levels of aromatase, potentially exposing the fetus to elevated levels of maternal androgens (Maliqueo et al. 2013). Consistently, new evidence in humans indicates that PCOS may be "transmitted" in a transgenerational way, whereby high levels of prenatal androgens in mothers with the condition, induce PCOS in their daughters via conditioning of the HPG axis, rather than via direct genetic effects (Risal et al. 2019). This could partly explain why the strong epidemiological link of PCOS with autism likelihood, does not appear to be mediated by genetic factors in this analysis or in epidemiological studies (Cesta et al. 2020).

On the contrary, this analysis showed that the genotypes associated with hair loss in men correlated negatively with autism. The effect size is considerable (b=-0.14 [0.04], p=0.007) and higher than the genetic correlation of the same trait (androgenic alopecia) with the genetics of bioavailable testosterone in men (b=0.10 [0.03], p=0.003). The

direction of effect is surprising, given previous findings of elevated circulating androgens in autism postnatally (Ruta et al. 2011). On the other hand, several recent studies in this thesis (Studies 1 and 2)(Baron-Cohen et al. 2019; Tsompanidis et al. 2021) and by other research groups (Bilder et al. 2019), have reported that autism is associated with higher levels of estrogens, which could, in turn, have a protective effect on androgenic alopecia postnatally. No previous clinical or epidemiological study has investigated whether androgenic alopecia is less frequent in autism, and most reports of symptoms relating to increased steroids have focused on women and their reproductive health (Pohl et al. 2014; Simantov et al. 2021). If this genetic correlation extends to the clinical level, then this could be seen as additional evidence that a potential 'steroidopathy' in autism affects males and females in different ways. A relative 'protection' from androgenic alopecia would also be consistent with the 'gender incoherence' theory, and similar findings of reduced masculinisation in secondary sex characteristics in autistic males (Bejerot et al. 2012). It is also important to note that androgenic alopecia is a complex, polygenic trait that likely involves several factors other than androgen levels/sensitivity, such as hair thickness, follicle vascularisation and epithelial aging. Several studies have reported a genetic link between the condition and neurodegenerative conditions, such as Parkinson's (Heilmann-Heimbach et al. 2017; Li et al. 2012). In this genetic correlation presented here, genotype data was restricted to the autosomes and did not include variance relating to the androgen receptor on the X-chromosome. Nevertheless, the result, albeit surprising, remains significant and likely captures a common latent factor in the maintenance of follicle health in men and the likelihood for autism.

Finally, the analysis showed that autism correlated negatively with age of menarche in women. This finding is novel since it uses the latest meta-analysis data on age of menarche. However, a similar trend had been reported in a previous GWAS for this trait but not discussed in their main text (Perry et al. 2014). This finding is consistent with previous epidemiological reports of a negative association between puberty onset and autistic traits (May et al. 2021), as well as higher likelihood for early onset in people diagnosed with autism (Herman-Giddens et al. 2012; Corbett et al. 2020). However, delayed puberty, in the context of hypogonadotrophic hypogonadism (e.g. in Klinefelter's), has also been associated with increased likelihood for concurrent autism

(OR=5.7 [2.6-12.6]), ADHD and other neurodevelopmental conditions (Ohlsson et al. 2019).

Puberty onset, as well as age of menarche are both complex traits, that feature an interplay between gonadal steroids, hypothalamic signalling, and pituitary rhythmicity. It's still unclear how autism affects the maturation of these components in childhood and puberty. Atypical rates of cortical thinning during adolescence have been reported for several brain regions in autistic individuals. In one study, the observed patterns of cortical thinning interacted with sex and were proposed to be mediated by an initial resistance to steroid-induced neuronal pruning, followed by an accelerated rate in later adolescence (Nunes et al. 2020). Faster pubertal development has also been linked with increasing symptom severity for other aspects of mental health. Males and females that mature earlier than their peers report more depressive symptoms (Mendle et al. 2010; Copeland et al. 2010) and are at increased odds of attempting suicide (Wichstrøm 2000), with the rate of pubertal development being a particularly strong predictor of depression in males (Mendle et al. 2010). A survey of over 35,000 Finnish teenagers showed that early onset of puberty was associated with internalising (e.g. depression, anxiety, psychosomatic problems, bulimia) and externalizing behaviour (substance-use, bullying, truancy) in females, but only the latter in males (Kaltiala-Heino et al. 2003). These reports are also consistent with the genetic correlation that was observed in this study, between early puberty onset and the genetics of several psychiatric conditions (Table 6.3). Therefore, this link may not be specific to autism and likely relate to both genetic and environmental factors that shape the HPA/G axis prenatally and throughout childhood.

In brief, the common genetic variance for autism did not correlate with the variance associated with steroid hormone levels in childhood. This may be because of low power in capturing the common genetic variance for autism, as well as the relatively low SNP-based heritability for postnatal hormone levels. However, the genetics of autism were found to correlate negatively to two complex, steroid-related traits; namely age of menarche and androgenic alopecia. The reason for this is unknown but likely involves a complex interaction between steroid hormones, the HPG axis and processes involved in cellular maturation. Additional research, both clinical and molecular, would be needed to add context to these correlations.

Chapter 7
General Discussion and Future Directions

Summary of chapter

In this chapter, the findings of all studies are critically assessed and discussed together with other relevant discoveries that were reported since work on this thesis commenced. All these lines of evidence support a prominent role of the prenatal steroid system and estrogens in particular, with regard to autistic traits and autism likelihood. However, prenatal steroidogenesis should be viewed as an interconnected system of several maternal, placental and fetal variables. Specifically, the estrogenic placenta may be the converging point where fetal sex and autism genetics interact to mediate liability. More research is needed into the molecular mechanisms of these interactions and on the role of endocrine conditions, such as PCOS and gestational diabetes. In addition, the proposed overlap between autism and "masculinisation" may need to be reassessed in light of the many endocrine variables that have now been associated with the condition besides testosterone. The question on the role of biological sex in autism remains clinically relevant but new epistemological approaches are needed that will also consider the condition's neurocognitive and behavioural heterogeneity.

7.1 Summary of the findings

This thesis aimed to investigate the ways in which biological sex interacts with the perinatal environment, resulting in associations with autistic traits or a diagnosis of autism in the children. The eight studies that are presented in this thesis, cover a range of sex-related factors, several perinatal time-points of exposure and neurodevelopmental outcomes from early infancy to late childhood. In addition, genetic factors are explored both in terms of familial likelihood and with the study of genetic variance (common and rare). The findings of each study are discussed in detail at the end of each chapter. In brief, they include the following

Study 1 - Sex steroids and autism (Chapter 2)

- Cohorts: Danish Psychiatric Registry (n= 98 male cases, n= 177 male controls)
- Time-point: First to second trimester (mean=14.9 weeks)
- Finding: Autistic males have higher levels of estradiol, estrone, estriol and progesterone in their amniotic fluid, compared to undiagnosed controls.

Study 2 - Sex steroids and traits (Chapter 3)

- Cohorts: the Cambridge Ultrasound Siblings and Parents study (n=122)
- Time-point: First trimester (mean=12.7 weeks)
- Findings: Free estradiol correlates the autistic traits of women during pregnancy, while total estradiol correlates to the autistic traits of the male, but not female infants. A clinical history of maternal hirsutism is also associated with higher autistic traits (in the mother) and higher circulating estradiol levels during pregnancy.

Study 3 – Sex and the placenta (Chapter 4)

- Cohorts: Generation R, the Netherlands (n=3469 cohort, n=65 with autism)
- Time-point: First (mean= 13.5 weeks) and second trimester (mean=20.6 weeks)
- Findings: Placental-derived markers of angiogenesis show pronounced sex differences in maternal plasma. Male-like patterns are associated with higher autistic traits in females and the likelihood of future autism diagnosis in males. Specifically, sex differences in PlGF concentrations mediate sex differences in SRS Scores.

Studies 4 - 6 – Sex and Infant Development (Chapter 5)

- Cohorts: the Cambridge Imaging and Longitudinal Development (CHILD) cohort, Cambridge, (n=41 prenatal & n=27 postnatal)
- Time-point: Third trimester and third month postnatally
- Findings:

- Study 4: Postnatal testosterone levels (during and after mini-puberty) are not associated with autistic traits or familial likelihood of diagnosis, based on first-degree relatives.
- Study 5: Low total brain volume and surface area, as well as a decreased perinatal rate of brain growth are associated with autistic traits in infancy. This is driven by individuals with a family history of autism and by males, rather than females.
- Study 6: Of all the sexually-dimorphic regions in early infancy (>2 months of age), the hypothalamus and areas involved in face recognition, are negatively associated with higher autistic traits in later infancy (>18 months of age). Significant interactions with family history of autism are noted.

Study 7 – Autism genes and gene expression differences in the placenta (Chapter 6)

- Cohort: Placental samples from pregnancies undergoing CNV prenatal testing (n=17 females, n=22 males)(Gonzalez et al. 2018)
- Finding: Genes on the X chromosome that are differentially expressed between males and females, are enriched for genes involved in X-linked autism and other neurodevelopmental conditions.

Study 8 – Genetic correlations between autism & endocrine outcomes (Chapter 6)

- Cohorts: Various GWAS, including UK Biobank and other cohorts of adults
- Finding: Common genetic variance that is associated with autism, correlates to the variance for early age of menarche in women and to the genetics of reduced hair-loss in men over the age of 40.

Many of these studies have also failed to replicate previous findings or identify evidence that would be consistent with previous theories on the interaction between sex differences and autism likelihood. Specifically, no association was found of testosterone

levels alone, prenatally or postnatally, with an autism diagnosis (Study 1) or with autistic traits in women and their infants (Study 2 or Study 4). In addition, no genetic correlation was found between genotypes associated with autism and the genetics of testosterone levels in adulthood. Furthermore, the association between brain volume and infant autistic traits was not consistent with the brain overgrowth theory, which predicts higher volumes and faster rates of brain growth in individuals with high autistic traits or autism (Study 5). Finally, sex differences in neurodevelopmental vulnerability persisted, even when accounting for differences in prenatal exposure to sex steroid hormones. This was shown particularly in Study 2, when the association of infant autistic traits with maternal estradiol was more significant in males, rather than females.

Both the positive and the null findings in these studies, suggest that sex steroid hormones, and androgens in particular, may not be directly responsible for increasing autism liability in males, but rather interact with maternal health, fetal sex and fetal genetics to affect neurodevelopment, in more complex ways. Given the considerable heterogeneity in the condition itself, as well as the behavioural differences between males and females, it may be improbable to ever attribute autism to a parsimonious, pathophysiological cause for both sexes. Nevertheless, the findings in this thesis suggest that estradiol and the placenta may play a central role, mediating the effects of many of these factors, as well as contributing to the sex differences in autism liability.

7.2 The role of the sex steroid system

The significance of prenatal estrogens, such as estradiol is now shown by two studies in this thesis, which found an association with both an autism diagnosis in males (Chapter 2) and autistic traits in males (Chapter 3). These findings have now been peer-reviewed and published (Baron-Cohen et al. 2019; Tsompanidis et al. 2021). In addition, a separate study by Bilder et al, also found significantly higher levels of estradiol in the maternal serum of pregnancies of children later diagnosed with autism (Bilder et al. 2019). These findings appear to contradict a similar study, in which significantly *lower* levels of estriol were reported in maternal serum (Windham et al. 2016). This may be due to differences in the timing of the samples, since serum samples in the latter corresponded to the second trimester (15 to 20 weeks), rather than the late first (Chapter 2: 14.9 weeks). In addition, estriol is mostly formed from DHEA, rather than the main activators of the androgen

receptor (testosterone and androstenedione), which act as precursors to estradiol and estriol respectively. When compared to other estrogens (Figure 2.3), fetal estriol levels also had the lower OR in terms of autism likelihood in the comparative study in amniotic fluid of Study 1.

Therefore, prenatal sex steroid excess in autism may be particularly skewed to steroidogenic pathways that are associated with androgens (the Δ4 and Δ5) and their aromatisation into estrogens prenatally by the placenta (Figure 1.1).

A single neurological biomarker has not yet been identified, on the molecular, cytoarchitectural or network level (section 1.3, Chapter 1). Studies have variably shown deficits in long-range connectivity, a skewed excitation/inhibition balance, excess number of spines and atypical patterns of cortical thickness throughout the brain (Schaer et al. 2013; Lee et al. 2017; Durand et al. 2012; Romero-Garcia et al. 2019). However, many reports also implicate the hypothalamus, which regulates endocrine homeostasis, facilitates social motivation and the output of the limbic system, among many other functions which are affected in autism (Caria, Ciringione, and de Falco 2020).

Sex steroid excess in autism could then be attributed to a developmental hypothalamic deficit, which could in turn lead to inefficient inhibitory feedback by circulating steroids and an overall increase in their levels at various points in life and prenatally (Pitteloud et al. 2008). Other variables in this complex system include the interaction of pregnancy complications and maternal stress with rate-limiting enzymes (e.g. aromatase or 11β-HSD)(Peña, Monk, and Champagne 2012), the integrity of barriers such as the trophoblast or the blood-brain-barrier (BBB) (which peripheral signals need to cross to mediate inhibitory feedback), as well as synaptic switching of GABBA from excitation to inhibition, which occurs perinatally in humans (Ganguly et al. 2001).

Interestingly, estradiol appears to regulate this developmental switching, as well as the formation of spines, dendrites and synapses in developing neurons (Nunez et al. 2008; Hoffman et al. 2016; Schwarz et al. 2008). In addition, estradiol interacts with the brain's immune system components (e.g., glia, cytokines) to regulate the rates of neuronal apoptosis and proliferation in the cortex (McCarthy and Wright 2017). More recently, in human iPSC-derived brain organoids, steroids were seen to directly regulate neuron proliferation, synaptogenesis and alter the ratios between excitatory and inhibitory

neurons (Kelava et al. 2020). Therefore, autism could be linked to a 'steroidopathy' that is secondary to an underlying dysfunction of the central nervous system and the "conditioning" effects of prenatal sex steroids on the developing gonad and brain.

In this thesis, direct effects of steroids on the brain could not be tested, but specific findings are consistent with these scenarios. First, a genetic correlation was observed between the common variance for autism and the genotypes associated with earlier age of menarche (Study 8). This link is mirrored in epidemiological data (Corbett et al. 2020), along with increased prevalence for a variety of steroid-related symptoms (Pohl et al. 2014; Simantov et al. 2021). In addition, in the same prospective Cambridge cohort, in which high amniotic testosterone was seen to correlate to high autistic traits (Auyeung et al. 2009), an interaction with puberty was identified. In brief, the same association persists for adolescent autistic traits but only in individuals with earlier puberty onset and a faster rate of physical maturation (Dooley et al. 2022). Moreover, in this thesis, one of the few brain regions that was associated with both sex differences as well as autistic traits, was the infant thalamus (Study 6). Given the small size of the infants' brains, this study's parcellation method could not draw boundaries between the infant thalamus and smaller regions around it, so the observed associations may also include the adjacent hypothalamus; the primary location of GnRh neurons that regulate sex steroid hormone levels, as well as puberty onset. It is interesting to speculate that lower brain volumes, in the region, as indicated in Study 6, may affect inhibitory feedback loops (Figure 1.1), leading to increased signalling to the pituitary and steroidogenic excess that includes both androgens and estrogens (Pitteloud et al. 2008). Alternatively, more severe deficits in the region could lead to the opposite outcome (primary hypogonadism), as is the case in Klinefelter's syndrome (Ohlsson et al. 2019). These two endocrine phenotypes may be associated with different kinds of autism (e.g., idiopathic vs syndromic) but would require clinical trials to establish with confidence (GnRh tests, aromatisation assays etc.). Future studies could shed further light into the links between sex steroids and neurodevelopment, by measuring the autistic traits of individuals with rare syndromes of gonadotropic resistance/dysregulation. These include complete/partial androgen insensitivity syndrome, aromatase excess syndrome, Kallmann syndrome and idiopathic cases of precocious puberty. Finally, additional epidemiological studies are needed in order to better describe and raise awareness among clinicians for the endocrine

comorbidities and steroid-related symptoms in autistic men and women (Simantov et al. 2021).

7.3 The role of the placenta and the prenatal environment

Three recent discoveries, together with findings in this thesis, raise the possibility that steroid excess in autism may not be only attributed to the autistic person's own genetics or neural substrates, but rather originate in the conditioning effects of the prenatal environment.

The first of these discoveries is with regard to PCOS. Maternal PCOS increases the odds of autism in the children by 35 to 59%. This has now been replicated in independent cohorts around the world (Kosidou et al. 2016; Cherskov et al. 2018; Berni et al. 2018), as well as in a meta-analysis (Katsigianni et al. 2019). In addition, this appears to be independent of genetic factors, as shown by the lack of a genetic correlation using GWAS data in Chapter 5 and other epidemiological studies that found no familial clustering in their associations (Cesta et al. 2020). The ultimate cause of PCOS is unclear, but animal models for the condition have long suggested that the condition may be related to androgen levels *in utero* and be induced by exogenous administration of androgens prenatally. This has only been recently shown in humans in a rare clinical study of transgenerational susceptibility (Risal et al. 2019). In brief, PCOS in the mothers induces PCOS in offspring by affecting their differentiating ovaries and affecting their HPG axis for life. In autism, higher rates of PCOS have been found in both autistic women as well as their mothers (Cherskov et al. 2018), raising the possibility of similar prenatal conditioning occurring in the context of autism.

Secondly, novel research on iPSC-derived brain organoids, as well as findings on iPSC-derived neurons, show that sex steroid hormones have significant developmental effects on the fundamental processes of human brain development, namely cortical expansion, and synapse formation (Shum et al. 2015; Mukherjee et al. 2017; Sellers et al. 2020; Kelava et al. 2020). Specifically, testosterone was shown to favour the differentiation and proliferation of excitatory over inhibitory neurons, providing a pathophysiological pathway that is consistent with findings in autism (Kelava et al. 2020).

Third, a series of recently published studies have identified a novel pathway of the so-called 'placenta-brain axis' in mice (Vacher et al. 2021). It was discovered that a placenta-derived steroid that is derived from progesterone (i.e., allopregnanolone) was able to reach the rodent brain and affect neurodevelopment and regulate social behaviour in postnatal life. Targeted knockout experiments, as well as rescuing paradigms, confirmed that allopregnanolone signalling in the developing cerebellum was needed to maintain social attention. Interestingly, this effect interacted with sex, since knockout of the steroid's downstream pathway affected male mice more than females.

Together with the findings presented in this thesis, on elevated prenatal estrogens (Studies 1 & 2), their link to maternal hirsutism (Study 2) and an overlap of autism with placental sex differences (Studies 3 and 7), these lines of evidence suggest that autism may be linked to lifelong hyperandrogenic traits due to the prenatal 'conditioning' effects of sex steroids and the placenta (Figure 7.1).

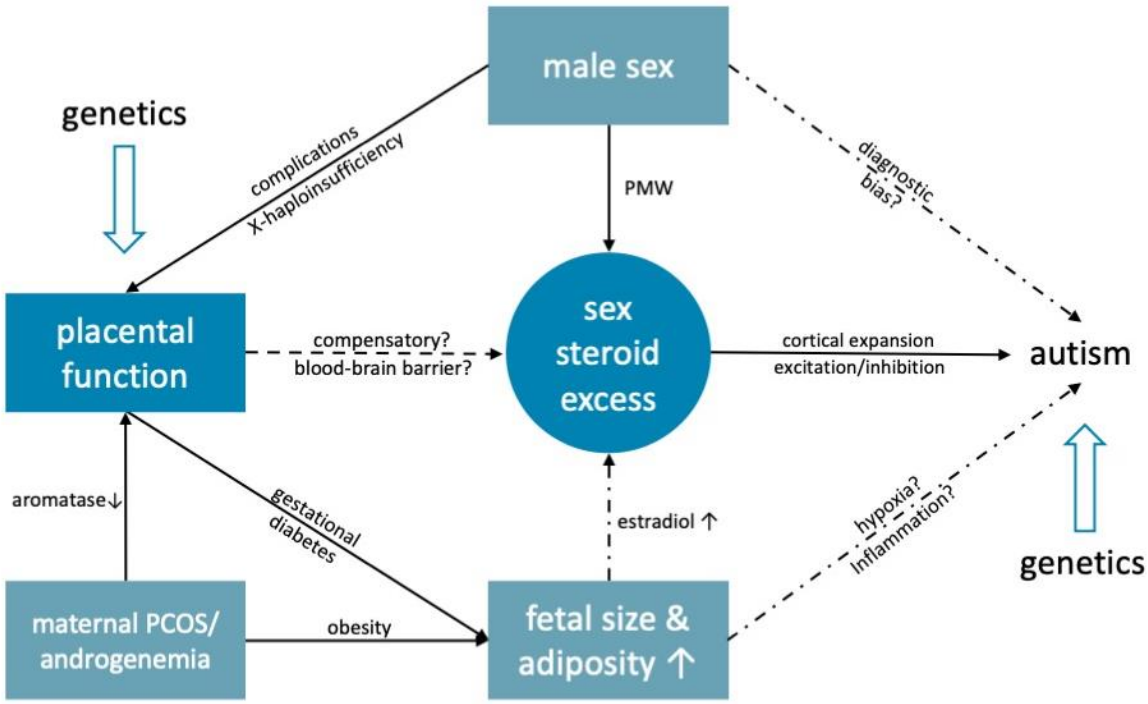


Figure 7.1: Proposed model of the prenatal interactions between fetal sex, the placenta and steroid system. Dotted lines indicate plausible but relatively unexplored pathophysiological links in the context of autism. PMW: prenatal masculinisation window

Both of these components (i.e., sex steroids and the placenta) could be mediating the effects of several other pregnancy complications, which have been shown to increase autism likelihood in other epidemiological studies. For example, pregnancy complications, such as gestational diabetes have been linked to autism (Wan et al. 2018) and often lead to an increase in fetal size and adiposity (Logan et al. 2017). In turn, this increase may be affecting endocrine pathways, , leading to compensatory increases in placental size (Desoye and Hauguel-De Mouzon 2007), as well as higher levels of estradiol via local aromatisation. These adaptative mechanisms may be clinically plausible but should be further investigated on the clinical and molecular level. Similarly, maternal PCOS has also been associated with decreased aromatase in the placenta (Maliqueo et al. 2013), as well as higher BMI in the offspring (Bahri Khomami et al. 2019). It is also interesting to speculate that higher levels of placental angiogenic factors, such as the PlGF (Chapter 4) could increase the permeability of the fetal as blood brain barrier to circulating steroids, as this has been shown for the maternal side in rodents (Schreurs et al. 2012). Importantly, this multifactorial system would be expected to interact with fetal sex, since males produce more steroids during the prenatal masculinisation window (PMW) and via their placentas (e.g. DHEAS), but are also more prone to placental complications (e.g. implantation failures or hypertension) and placental dysfunction (e.g. due to X-linked variants)(Figure 7.2) (Welsh et al 2014; Gong et al. 2018; Verburg et al. 2016).

All of these pathophysiological pathways are speculative and should be investigated in detailed clinical studies, as well as in molecular studies of placental function in autism. In addition, the role of genetics should be further investigated, since autism-associated genotypes appear to be independent to the genes associated with steroid levels in postnatal life (Study 8) (Mitra et al. 2016). Autism-associated genetics may rather interact with placental function, as shown in this thesis (Study 7) and another study of the placental methylome (Zhu et al. 2019). Moreover, hormonal pathways that are linked to adiposity and inflammation should be further analysed, as they interact with sex steroid pathways (e.g., in the context of PCOS) and may also affect neurodevelopment via other hormones and cytokines, independently of steroids. Finally, sex differences in prenatal factors (e.g. placental insufficiency) may not be specific to autism, but also

extend to other neurodevelopmental conditions and be mediated by other clinical outcomes such as iatrogenic preterm delivery (Santos et al. 2020).

7.4 The role of 'maleness'

The prenatal testosterone theory was first proposed as a way to explain the sex ratio in diagnoses, but also as a potential explanation of male-type shifts in the cognitive and behavioural traits of autistic people (i.e., the 'extreme male brain') (Baron-Cohen et al. 2005; Greenberg et al. 2018). As was discussed in Chapter 1, similar male-type shifts have been observed in the facial features, brain structures, biochemical profiles and connectivity patterns in autistic children and adults (Tan et al. 2020; Lai et al. 2013; Majewska et al. 2014; Floris et al. 2018). Atypical sex differentiation, with both male-type and female-type shifts in secondary sex characteristics, has also been noted, particularly after puberty (i.e., the 'gender incoherence') (Bejerot et al. 2012).

It is possible that prenatal or lifelong exposure to atypical levels of sex steroids could result in these different patterns of sex differentiation (Chapter 1, section 1.5). However, caution is needed in discerning association from causation. There is still little experimental, 'proof-of-concept' evidence, which can show if excess "masculinisation" is sufficient to cause autism in animals or humans. It remains to be seen how sex steroids directly affect the autistic brain or the regions mediating the condition's core traits (communication challenges and restricted interests). The data stemming from in vitro and animal model studies is certainly consistent with this (Lai et al. 2013), but more work is needed in humans to prove that male-type shifts extend beyond behavioural or structural patterns and to study how they interact with genes and hormones.

In addition, there are still remaining epistemological questions, on the nature of autism itself, as a neurocognitive and behavioural construct. Years of research have proved that autism can be consistently diagnosed, and that it's largely heritable (Gaugler et al. 2014). However, the condition's heterogeneity in behaviour, physiology and genetics is considerable. In addition, there has been less progress in identifying reliable biomarkers and understanding how autistic traits relate not only to biological sex, but also to social gender (Lai and Szatmari 2020). Since autism is comprised by two separate behavioural domains, namely social (e.g., challenges in communication) and non-social (e.g., restricted interests), it's possible that these interact differently with the underlying

biology, as well as with gender differences in cognition. Interestingly, two psychometric measures of these domains (empathising and systemising), were found to share little genetic correlation in the general population, despite their significant heritability (Warrier et al. 2019). So, the spectrum of autistic traits may be better conceptualised on two, rather than one axis, as described in earlier work by Baron-Cohen et al. on 'brain types' (Goldenfeld et al. 2005). However, employing this multi-dimensional framework in biomedical and clinical settings, will create new challenges, particularly with regard to diagnostic thresholds and their interactions with sex. In brief, more research is needed to understand how the underlying biology, as well as implicit gender biases, inform the variance in the two domains, but also how different diagnostic thresholds are set for each sex and for each domain.

7.5 Concluding Remarks

The effects of biological sex on neurodevelopment and autism likelihood remain as complex as the history of Psychiatry itself. Fortunately, autism researchers have acknowledged this and are committed in tackling any biases in the ways people's behaviours are analysed and classified (Baron-Cohen 2017). Undoubtedly autism as a condition, as well as the way it is diagnosed in males and females, will keep evolving. Nevertheless, the findings in this thesis show that perinatal differences between males and females are important and can predict autism-related outcomes in infancy and childhood. This does not mean that autism will or should ever be diagnosed before birth. Instead, this line of research may enable earlier and more reliable diagnoses postnatally, leading to awareness of co-occurring conditions, better provision of services, and improved learning outcomes for autistic people. Lastly, one can hope that this work not only contributes to the understanding of autism and infant neurodevelopment, but that it also may lead to an appreciation of the complexity, diversity, and unique evolution of our human brain.

Chapter 8

Addendum: Evolutionary perspectives on prenatal sex differences and autism

Preface:

This chapter is written as an addendum to the main thesis (Chapters 1 - 7), since it had not been part of the original research plan and it goes beyond the scope of the main research question on autism likelihood. After the completion of this thesis' studies, it became apparent that many of the findings, as well as recently published discoveries in the field, have evolutionary connotations that warrant further discussion. In particular, the proposed link between the placenta and the brain, may be better understood in the context of evolutionary pressures that have led to many human-specific adaptations.

8.1 The placenta and the endocrine regulation of the prenatal environment

The placenta may have escaped the focus of clinicians and developmental neuroscientists for many years. But evolutionary scientists have been reporting on the tissue's significance for decades. The shape, size and morphology of the placenta are considered very informative traits, in the context of the evolution, as they are often species-specific and quick to adapt to external pressures, in order to secure short-term and long-term viability (Power and Schulkin 2012). In the human placenta, comparative studies in mammals have identified the following important characteristics.

Compared to most other mammals, anthropoid primates are amongst the few species that have a haemochorial placenta (Martin 2003). This means that their invading trophoblast cells reach deep in the endometrium and fuse with the maternal blood vessels, bathing the placenta in the maternal circulation. This brings the fetus and mother's environments in direct contact that is only mediated by one cellular layer (the syncytiotrophoblast). The evolutionary significance of this morphology is unclear, as it has recently been shown to be an ancestral, rather than a derived state (Elliot and Crespi 2009). In humans, haemochorial placentas may be specifically allowing greater integration between maternal and fetal endocrinology, since the invading trophoblast cells have also acquired an active promoter sequence that allows the expression of aromatase, which is not present in other mammals with similar placentas (e.g. rodents) (Kamat et al. 1999).

Hormonally, the placenta of anthropoid primates is also characterised by a novel ability to synthesise chorionic gonadotropin (CG in humans - hCG) in the first half of gestation. The gene for this peptide is derived from a duplication and modification of the gene for the beta subunit of thee luteinising hormone (LH) (Hallast et al. 2008). CG regulates the first phases of placentation (adhesion, implantation, and differentiation), while the syncytiotrophoblast cells are in the initial stages of invading the uterine wall. At this time, the cytotrophoblast layer is covering the entirety of the amniotic sac and blocking access to the maternal circulation, maintaining a transient state of hypoxia in the fetal compartment that may favour cell differentiation, rather than rapid, aerobic cell proliferation. Following a peak in the late first trimester, GC kickstarts placental steroidogenesis and then declines in later pregnancy. The role of maintaining placentation and preventing uterine rejection is then 'passed on' to progesterone, which gradually increases until initiation of labour (Kallen 2004).

The prenatal metabolic pathways that lead to the formation of progesterone and other steroids have also gone changes in primates, compared other mammals. The most characteristic trait regards an inability by the placenta to metabolise progesterone locally into DHEA (the source of all sex steroids), in the absence of expression of the necessary enzyme (CYP17A1) (Lockwood 2004). Instead, this task falls on the maternal and fetal adrenals, which are fully integrated into placental steroidogenesis. The adrenals utilise placental progesterone and form DHEAS. This steroid is then fed back into the placenta (via haemochorial transfer), leading to the synthesis of androgens (androstenedione and testosterone), which are in turn rapidly aromatised to estrogens (estrone and estradiol)(Kallen 2004)(Figure 1.1). This process is enhanced by the gradual regression of the cytotrophoblast layer, which results in more direct contact between the maternal and fetal circulations after mid-pregnancy. In addition, the fetal adrenal zone that produces DHEAS is expanded and never fully regresses after labour (in contrast to other mammals).

The maternal and adrenal axes are further enhanced by another primate-specific adaption of placental signalling; the synthesis and release of placental CRH (Sirianni et al. 2005). This potent hormone recruits the HPA/G axes of the mother and infant, stimulating the release of ACTH by their pituitaries and leading to a constant upregulation of steroidogenesis, that is evident in the gradual increases of both progesterone and estrogens throughout pregnancy.

Based on these adaptations, the primate placenta can then be considered to have adapted into a 'steroidogenic engine' (Figure 7.1) that utilises the deep invasion of the trophoblast, in order to integrate the maternal and fetal endocrine capacities and bathe the developing fetus in estrogens.

Comparative studies of prenatal endocrinology among primate species are rare. Yet both direct and indirect evidence suggests that the processes of prenatal steroidogenesis may be even more enhanced in humans (Figure 8.1):

First, the human lineage has been found to have more duplication events for gonadotropin genes, leading to a greater number of functional hCG variants (Hallast et al. 2008).

Second, the human placenta is considered to be more invasive than in other primates, leading to higher prevalence of related complications. Consistently, humans are the only species of primate known to exhibit hyperemesis during pregnancy (i.e., 'morning sickness'); a condition that is now attributed to the cross-reactivity of very high levels of hCG with the thyroid (Haddow et al. 2008). This would indicate that humans also have higher levels of hCG, compared to other primates that have acquired the ability to synthesise this hormone.

Third, humans have been found to have a much higher peak-level of estrogens in maternal circulation, than chimps and gorillas (Czekala et al. 1983; Smith et al. 1999)(Figure 8.1). In fact, the human placenta is estimated to produce more estrogens over the course of a single pregnancy, than the entire amount produced over a woman's lifetime, outside of pregnancy. If this estrogenic excess is further confirmed in additional comparative studies, then it could potentially account for human susceptibility to estrogen-dependent conditions throughout life, such as endometriosis and breast cancer.

Composite figures redacted for copyright reasons. Copyright holders are as indicated in the citations in the legend.

Figure 8.1: Prenatal estrogenic excess in humans
***A:** Comparative graph for urinary excretion of estrogen (mainly E1 and E2) during pregnancy in humans, orangutans and gorillas. Reprinted from: Czekala et al. 1983*
***B**: Comparative graph for urinary excretion of E2 in chimpanzees (squares) and gorillas (rhombi), peaking at ~21,000 pmol/L = 5720.5 pg/ml. Reprinted from: Smith et al. 1999*
***C**: Graph of prenatal steroid hormones in maternal serum of humans (clinical average), showing rapid linear elevation in estradiol levels, peaking at ~17,000 pg/mL = 62407 pmol/l. Source: Wikimedia*

In brief, the regulation of the prenatal environment via placental steroid synthesis, has many primate-specific features. These may have gone further adaptation in humans that favours an excess in prenatal sex steroid synthesis. The adaptive molecular mechanisms of these adaptations may be varied and include multiple systems. For example, in many New World monkeys, George Chrousos was the first to describe a derived state of endocrine resistance to all steroid hormones (Chrousos 1999). This was shown to lead to high circulating levels of androgens, estrogens and corticosteroids, and was likely mediated by modifications in the way steroid-receptors bind to the genome, in tissue-specific ways.

The adaptive capabilities of the endocrine system and the placenta have long been known in Evolutionary Science (Martin 2003; Power and Schulkin 2012). Yet, a revaluation of the role of the placenta in evolution may be needed, as new discoveries have been made regarding the tissue's mediation of sex differences on the genomic level (Gong et al. 2018), lifelong conditioning for health-related outcomes (Calkins and Devaskar 2011) and, lastly, a link of the placenta to brain development and behaviour in sex-specific ways (Rosenfeld 2020; Vacher et al. 2021).

8.2 Sex differences in the placenta and evolution

Placental function is affected by the sex of the fetus (Verburg et al. 2016). Genetic differences start at conception and are mainly attributed to X-chromosome dosage effects of genes that escape inactivation (Gonzalez et al. 2018; Gong et al. 2018). This leads to differences in the levels of proteins involved in placental angiogenesis as early as the first trimester, with males having significantly lower levels, independently of placental weight (Brown et al. 2014). In Study 3, it was first shown that this pattern changes during the second trimester, as the placental growth-factor increased faster in males than in females, leading to significantly higher levels in the former. This phenomenon may be attributed to an interaction with the sex steroid system, as PLGF and DHEAS levels have been found to correlate in other experiments (Lowin et al. 2012). In addition, the timing coincides with the activation of the fetal testes and the prenatal androgen peak underlying the prenatal masculinisation window (Welsh, Suzuki, and Yamada 2014).

These genetic and proteomic observations are likely not independent to the clinical observations of sex differences in placental complications. Male fetuses are more likely to fail to implant in the first half of gestation, as well as exhibit placental hypertension, gestational diabetes and restrictions in intrauterine growth (Davis and Pfaff 2014; Murji et al. 2012). On a subclinical level, many of these sex differences have been attributed to altered 'strategies' regarding energy usage, particularly in the presence of maternal adversity, with males 'prioritising' nutrient uptake, compared to signs of adaptive changes in the placentas of females (Davis and Pfaff 2014).

Over the years, there has been extensive analysis of these clinical differences "in light of evolution". Maternal evolutionary pressures are qualitatively and quantitatively different to those of their male partners (Stanyon and Bigoni 2014). Mothers ought to balance the evolutionary benefits of procreation, with the substantial needs, in time and energy, for the gestation and breastfeeding of human children, who are in turn characterised by a more energy-demanding brain and a prolonged childhood, compared to other anthropoid primates. On the other hand, fathers benefit from high turn-over of conceptions (either with one or with more female partners) ensuring that each of their offspring survives to term. These differing evolutionary pressures are translated in the prenatal system via epigenetic regulation and parent-specific placental imprinting of several genes that regulate energy usage and growth (Lester and Marsit 2018; Wilkins and Haig 2003). Consistently, the susceptibility to preterm birth has been found to interact with maternal, rather than paternal genetic variance (Little 2009).

More recently, the placenta was found to feature extensive X-chromosome inactivation and genetic mosaicism due to a transient state of genetic instability (Gonzalez et al. 2018; Gong et al. 2018; Coorens et al. 2021). Given karyotypic differences between the sexes, these phenomena may also mediate sex differences and be attributed to the different pressures (maternal and fetal) outlined above. Interestingly, it has been proposed that X-linked dosage effects in the placenta may act as a means of indirect screening for aneuploidies, but also of the sex of the fetus, by the maternal decidua, leading to sex-specific regulation of implantation. In addition, the regulation of early hCG synthesis by the placenta, is affected by genetic instability, to such a degree that hCG levels are now used for screening of aneuploidies (Wright et al. 2010). But hCG levels are also affected by the sex of the fetus (particularly in the 1st trimester) (Steier et al. 1999; Adibi et al.

2015). Consistently, in this thesis (Chapter 3) an interaction of hCG levels was observed with sex, when predicting an infants' later autistic traits. In brief, the placenta functions as the 'arena' where maternal and paternal/fetal evolutionary interests collide. Placental sex differences in genetics and proteomics are likely affected by these pressures and may be mediating some of the adaptions that are unique to the human prenatal environment (Power and Schulkin 2012).

The different evolutionary pressures for males and females, further interact on the social level, shaping mating strategies (e.g. transient monogamy vs harem polygamy) and cultural/anthropological norms (e.g. marriage contracts) (Dunbar and Fleagle 1999; Stanyon and Bigoni 2014). A recent proposal by Eric Keverne et al, has also identified the placenta as the mediator of transgenerational conditioning of the maternal and fetal HPG/A axes, in response to some of those pressures (Keverne 2013)(Figure 8.2). According to this theory, maternal crosstalk with the placenta via hormones, can shape maternal behaviour during pregnancy, as well as the future mating behaviours of the offspring by the lifelong 'conditioning' of their hypothalamus. This intriguing hypothesis is consistent with findings in this thesis (Chapter 3) and with the evidence for transgenerational transmission of PCOS between mothers and daughters (Risal et al. 2019). However, the precise molecular details of such a mechanism remain unclear, as only one molecular pathway was identified by Keverne (Keverne 2015). It's also not clear if the 'conditioning' effects of placental hormones on the fetal brain are restricted to maternal nurturing and mating behaviours, or whether they also affect neurodevelopment and cognition for both males and females.

In light of recent findings on the placenta-brain axis (Vacher et al. 2021), as well as in this thesis (Chapters 2-4), it may be interesting to speculate that evolutionary pressures relating to sex differences and the placenta, may also have shaped the sex differentiation of human physiology and of the human brain.

Composite figures redacted for copyright reasons. Copyright holders are as indicated in the citations in the figurer legends.

Figure 8.2: Keverne E. - Transgenerational conditioning of maternal behaviour via the placenta.

A: *Transgenerational model as proposed by Eric Keverne, whereby maternal and placental hormones interact to affect the development of their counterparts in the offspring. Maternal imprinting proposed as evolutionary mechanism that mediates the evolutionary interests of the matriline.*

B*: Neuroendocrine conditioning of maternal behaviour. Speculative examples include nurturing and reproductive behaviour in females, extending beyond their own estrous and offspring. Links of steroids and the placenta to the brain (e.g., downregulation of GnRH) still need to be proven in proof-of-concept experiments.*

8.3 Sex differentiation in human evolution

Sexual selection has been a powerful force in the evolution of human physiology and anatomy. Since Darwin, evolutionary scientists often assumed that male competition in humans was enhancing sex differences and their secondary sexual characteristics, as is the case in many other species (e.g., the peacock). However, recent comparative studies in non-human primates, as well as extinct hominin species, have revealed the opposite may be true in modern humans (Stewart-Williams and Thomas 2013; Stanyon and Bigoni 2014). Data on body weight, muscle mass, canine tooth length and other features, all indicate that male-typical sexual dimorphism in humans is reduced, compared to most of their primate relatives (Figure 8.3). On the other hand, human females have evolved specific post-pubertal adaptations, compared to their primate relatives, that favour consecutive reproductive cycles and mating outside of estrous. These adaptations have been attributed to the evolutionary effects of both polygyny and polyandry in human societies with increasing group sizes, leading to a 'mutual mate choice' model of mating, as well as a gradual shift from harem-like monogamy (e.g. in gorillas) to various degrees of pair-bonding between males and females (Johnstone et al. 1996).

However, much less is known in terms of the molecular and physiological adaptations that underpin these shifts in sexual dimorphism. Interestingly, many of the features of anatomically modern humans, could be attributed to the signalling of sex steroids and estrogens in particular. These include the following:

1. Secondary sex characteristics in females. Contrary to other primates that 'signal' estrous with transient changes in physiology, human females have developed permanent changes in their anatomy (e.g., enlarged breasts), signalling post-pubertal maturity, independently to the monthly cycles of ovulation. Human breast development is estrogen-dependent, as shown in the preponderance of tumours that respond to hormone treatment, conditions that feature gynecomastia (e.g. aromatase excess), as well as GWAS studies of the trait (Eriksson et al. 2012) .
2. Lack of hirsutism. Humans have evolved skin with a drastically reduced number of terminal hairs (i.e., fur), with the exception of the scalp. This trait may not be directly attributed to the pressures of sexual selection, but rather to the need for thermoregulation. Nevertheless, the degree of hirsutism interacts evidently with sex

in humans, as well as the relative levels of androgens and estrogens in individuals of both sexes. For example, exogenous estrogen administration leads to a reduction in hirsutism, even in post-pubertal, chromosomally male humans.

3. Elevated digit ratios. A comparative study of the 2D:4D digit ratio in anthropoid primates, as well as other hominin species, has revealed that humans have higher ratios, to all with the exception of the gibbon (Nelson and Shultz 2010). This trend was strongly predicted by mating strategies, since both gibbons and humans form pair-bonds and are more monogamous, compared to other primate societies and what the fossil records suggest for early hominins (Stanyon and Bigoni 2014). An elevation of the digit ratio is also consistent with previously mentioned data on prenatal estrogenic excess in humans (Figure 8.1) and may be linked to human adaptations in placental steroidogenesis leading to steroid excess (e.g., high hCG).
4. Feminised facial features and neoteny. Similarly, to digit ratios, certain anatomical features on the face (e.g., brow ridge thickness, facial length) appear to have undergone gradual changes in hominins, that match a "juvenile" as well as more "female" phenotype. The evidence tying these traits to steroid hormone levels is more tentative (Whitehouse et al. 2015). Yet, the trend towards "craniofacial feminisation" did not appear to stop with *Homo Sapiens* speciation but continue to be evident in skeletal remains as recently as the Middle Pleistocene (Cieri et al. 2014). These findings are mirrored in the digit ratios of the same remains and, together with other studies, were interpreted by the authors as evidence of a "self-domestication" process in humans, whereby neoteny and a reduction in secondary sex characteristics correlate with increases in group sizes.
5. Increased pelvic width in females. Even though many of the sexually dimorphic features in males are relatively reduced (compared to other primates), pelvic size is an exception to this and a rare example of an enhanced sex difference in human anatomy. The female pelvis undergoes considerable remodelling during puberty, increasing in width, compared to males. The opposite happens after menopause. The regulation of these patterns appear to be independent to the general sexual dimorphism for body size (e.g. femur length)(Kurki 2011). Evidence from human pelvic growth trajectories, rare genetic syndromes, as well as studies in other primate species and rodents, have confirmed that pelvic growth is dependent on estrogens, rather than growth hormones (Huseynov et al. 2016). The authors of the largest

comparative study among different primate species concluded that "the conserved mammalian endocrine system strongly constrains the evolution of the pattern of pelvic differences but enables rapid evolutionary change of the magnitude of sexual dimorphism."(Fischer et al. 2021) Therefore, humans appear to have acquired adaptations in terms of "degree", rather than having developed an independent mechanism that increases pelvic sex differences. This is also consistent with previously mentioned findings on estrogenic excess in humans (Figure 8.1). Different kinds of evolutionary pressures act on the human pelvis, which has adapted to allow the birth of fetuses with larger heads, as well as to provide support to the pelvic floor during bipedalism.

This link between estrogens and the adaptation of the human pelvis also relates to the development of the brain itself, since increases in pelvic width were necessary for giving birth to neonates with larger brains. Analysis of estrogen-responsive elements in the human genome, have also implicated estrogenic signalling to the evolution of brain size in humans (Shi et al. 2015). Assays using exogenous estradiol showed suppression of many microcephaly-associated genes. The same effect was noted when the sequences were added to assays of macaque and chimpanzee cell lines, prompting the authors to suggest that human advantages may be attributed to estrogenic excess and/or prolonging the developmental time-window for the effects of estrogens; a hypothesis that is consistent with previous theories on 'neoteny' (Somel et al. 2009).

For this reason and in light of the findings in this thesis, it is interesting to speculate that the steroid-related and placenta-related physiological adaptations mentioned above, are also linked to neurodevelopment and the evolution of human-specific behaviours.

Composite figures redacted for copyright reasons. Copyright holders are as indicated in the citations in the figure legend.

Figure 8.3: Reduction in sex differences in humans.

A: Percentage of difference of male to female body size, in a series of fossil hominins, arrayed left-to-right by projected age of specimens and species. Reprinted from: Stanyon and Bigoni 2014

B: Distribution of digit ratios, showing elevation in humans, compared to their closest Old-World relatives. The more distant gibbons (Hylobates) have even higher digit ratios. This pattern is associated with higher degrees of pair-bonding (PB: pair-bonding /NPB: non-pair-bonded).

Reprinted from: Nelson and Shultz, 2010

8.4 Neurodevelopment and autistic traits in light of evolution

Few human traits are as evidently derived, as the anatomical and functional properties of the human brain. Since the days of Darwin, the "faculties" of "man" have been extensively analysed, with much speculation on the evolutionary pressures that have shaped them through the years (Darwin 1875). The relative significance of each aspect of cognition may never be objectively evaluated and understood "in light of Evolution". It is certain though that: 1. Humans have the highest encephalisation quotient (deviation from predicted brain mass vs total body mass) than all other animals. 2. Humans form social groups that are larger than other primates, as well as extinct members of the genus *Homo*.

When these two parameters are examined together in anthropoid primates and hominins, a correlation becomes apparent. Larger brains (and particular the size of the neocortex) are associated with larger group sizes (Dunbar 2009). This observation prompted the proposition of the 'social brain' theory of human brain evolution by Robin Dunbar et al, which attributes cortical expansion, to the requirements of increasingly complex social systems in ever growing hunter-gatherer groups and ultimately human societies. In recent years, many lines of evidence (e.g. on cognition and use of language) are in accordance with the 'social brain' theory (Dunbar 2016). This hypothesis has also offered a novel perspective, since social cognition and communication have been, for the first time, recognised as significant, human-specific traits, alongside other behaviours, such as ingenuity in toolmaking, representational painting/sculpting and ritualistic burials.

Based on these hypotheses, it has become apparent that autism, as well as autistic traits in the wider population, posit an evolutionary dilemma. Autism is a neurodevelopmental condition with a large heritable component (Gaugler et al. 2014), so it's reasonable to assume that a part of its aetiology interacts with human evolution. Behaviourally, autism is diagnosed based on a phenotype that includes both social challenges (reduced social intent, attention, communication skills), as well as atypical non-social behaviours (repetitive habits, restricted interests). In particularly, traits relating to the social domain have been specifically linked to a reduction in cognitive empathy, which is the ability to anticipate, describe and understand other people's emotional states and motivations (Baron-Cohen et al. 2013).

According to the 'social brain' theory, autism would appear to be a condition that is not selected for by the prevailing forces of human evolution. This unfortunate notion would be consistent with other traits that often co-occur with the condition, such as language delay, intellectual disability, and challenges with establishing relationships and creating families. However, autism and its related traits also give rise to aspects of cognition that society has long considered to be specific to humans, including analytical and detailed thinking, mechanical ingenuity, and the capacity to understand complex systems of cause and effect. In addition, autistic people's attention to detail is often accompanied by creativity and "talents" that are considered specific to humans (e.g. resourcefulness, drawing/painting, fantastical world-building etc.). These links between autism and human evolution have been emphasised recently in a book by Simon Baron-Cohen, in which autistic individuals were proposed to be the "pattern seekers" that helped early human societies become more efficient hunters, as well as the inventors of systemised agriculture (Baron-Cohen 2020).

Since autism liability is largely genetic, it may be easier to understand the condition "in the light of evolution", by studying selection signatures in the genes that have been associated with the condition. So far, studies of both common and rare variance have produced mixed results. Rare variants that lead to autism, often correspond to conserved genes in mammals, that regulate the cell cycle, differentiation and take part in the formation of neuronal synapses (Banerjee-Basu and Packer 2010; Pembroke, Hartl, and Geschwind 2021). Some of these high-confidence autism genes have even been found to regulate the social behaviour of bees, prompting the study's authors to describe this observation as evidence for "deep evolutionary conservation" in autism genetics (Shpigler et al. 2017). In addition, an analytical study of the properties of autism genes, reported that these are "evolutionary ancient", with larger transcripts, a high density of noncoding elements and, lastly, an "intolerance" to variants compared to the rest of the genome (Casanova et al. 2019). However, rare variants in many of these genes often lead to intellectual disability (Banerjee-Basu and Packer 2010), and for this reason, may not be linked specifically to subclinical autistic traits, which still capture the majority of variance in the general population. On the contrary, common genetic variance for autism and related traits, correlates to a large degree positively with IQ, educational attainment, and was found to correspond to genes that form human-specific functional networks

(Polimanti and Gelernter 2017). Moreover, human-accelerated regions (HARs) of the genome, which diverged recently in humans, show an enrichment for both autism-linked genes and genes relating to neural function (Doan et al. 2016). Therefore, based on its associated genetics, it remains unclear if autism represents a disruption of evolutionary conserved neuronal physiology, or a human-specific pattern of derived cognitive traits.

Finally, it is important to note the polygenic variance, associated with autism, is as heterogeneous as the condition itself (Warrier et al. 2019). In order to understand autism in terms of evolution, we may then need to consider the condition on the neurocognitive and social level, as well as the way sex and sex-related differences interact with these variables and with human-specific evolutionary pressures.

8.5 A theory on the evolution of the social brain via prenatal steroids

According to the social brain theory, group sizes in hominins increased together with the size of their cortex. This trend may have continued in *Homo Sapiens* (Cieri et al. 2014) and arguably offered our species the comparative advantages that enabled mass emigration, natural resource management, and the formation of human societies around the world.

The social brain theory is providing a link between two traits that can be robustly inferred from the paleoanthropological record, namely group size and brain size. However, the theory does not specify any of the physiological or behavioural adaptations that were required in order to enable an increase in both of the traits. As outlined above, several other theories have been put forward to account for these, based on studies of human-specific traits. For example, an elevation in digit ratios and a concurrent reduction in anatomical sex differences (Figure 8.3) has been proposed to be associated with a change in mating strategies (from male competition and polygamous harems to pair-bonding), which itself can be attributed to the effects of large group sizes (polygyny and polyandry). In addition, placental hormones, and genetic imprinting, have been proposed to 'condition' maternal and offspring endocrinology, in order to enable maternal nurturing and female reproductive behaviours that extend beyond the immediate postnatal period and estrous (Figure 8.2). These proposals are consistent with apparent

increases in the total estrogenic capacities of the human placenta, compared to other primates (Figure 8.1).

In this thesis, an association was discovered between maternal estrogen levels and autism/autistic traits in males (Studies 1 and 2). In addition, the sex of the child was shown to interact with this effect (Study 2) and be partly mediated by placental sex differences in angiogenesis (Study 3). There was no evidence that this effect extended postnatally, in the absence of the placenta (Study 4). On the contrary, autistic traits in infancy were associated with sex differences in neuroanatomy (e.g., reduced brain size, reduced volume in face-recognition cortical areas) that began prenatally (Studies 5 and 6). A genetic predisposition for autism was linked to this (based on familial likelihood - Studies 5,6), but also showed overlap with placental sex differences (based on X-linked rare variants -Study 7) and with outcomes in later life relating to steroid signalling, such as early age of menarche and protection from androgenic alopecia (based on GWAS data - Study 8). In brief, prenatal estrogens, steroid-related traits and the placenta were associated with sex differences in neurodevelopmental outcomes, relating to autism.

Based on these findings and the points outlined above, it may become possible to infer a more precise physiological framework for early neurodevelopment, in the wider context of the social brain theory of evolution (Figure 8.4). To this end, I propose that placental steroidogenesis and estrogens in particular:

1. have evolved to reach higher levels prenatally, compared to other hominids.
2. contribute to the rapid cortical expansion of the human neocortex.
3. directly affect brain regions relating to social development, including face recognition, communicative intent, and empathy.
4. contribute to a reduction in physiological sex differences, particularly in terms of male differentiation and male-type competition.
5. contribute to human-specific physiological features, such as infant neoteny and female secondary characteristics that obscure estrous.
6. have evolved due to matrilineal pressures to decrease male aggression, and infanticide, as well as to facilitate pair-bonding and to prolong the period of child-rearing.

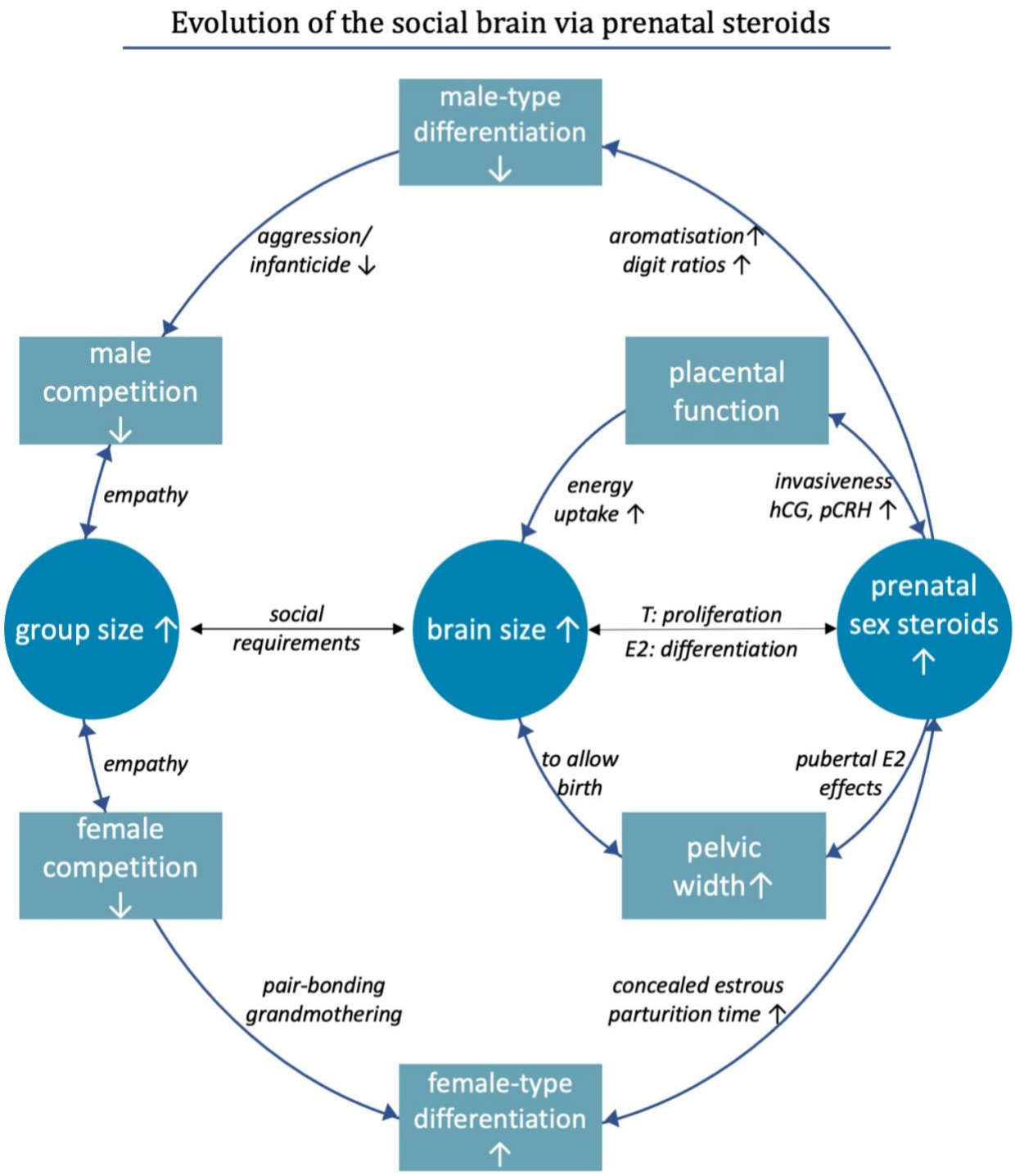


Figure 8.4: Evolution of the social brain via prenatal steroids. Circular diagram denotes co-evolution of interconnected systems, rather than a linear sequence of adaptations. Outer circle is based on the requirements stemming from the social brain theory and particularly the balancing between male and female competition in increasing group sizes. Inner circle refers to the placenta-brain link and recent data in human models of cortical expansion (Kelava et al. 2021) and pelvic growth (Huseynov 2016).

Hypothesis: A reduction in male aggression and an increase in human empathy, pair-bonding and cooperation was mediated by the matriline and by adaptions that favoured placental estrogenic excess.

T: testosterone, E2: estradiol, hCG: human choriogonadotropin, pCRH: placental corticotropin

Linking human speciation to the functions of estrogens and the placenta may also partly explain other aspects of human physiology. For example, recent adaptations in the system, including sex steroid excess, may be reducing the number of ovarian oocytes that are formed prenatally, or accelerating the rate of follicular atresia throughout life. These traits could then hasten menopause and induce the so-called "grandmothering" effect in human societies, which has arguably contributed significantly to their advancement (Kim et al. 2012). In addition, placental effects may have led to transgenerational, cumulative adaptations, leading to each successive generation carrying the hormonal conditioning of the previous ones. This is consistent with findings on PCOS transmission (Risal et al. 2019), the proposal by Eric Keverne on specific molecular pathways (Keverne 2015), as well as recent discoveries on imprinting and the epigenetic effects of sex steroids (Stanyon and Bigoni 2014). Therefore, the placenta could be leading to rapid adaptations that are not entirely dependent on genetic variance, shortening the evolutionary time that is required for the adaptation of the human HPG/A axis and related behaviours. If the proposed placenta-brain link is true, this feature could also explain the rapid cortical expansion in the human lineage, as well as indications that this is still continuing in *Homo Sapiens* (Cieri et al. 2014). Consistently, anecdotal reports in contemporary human populations have also noted an increase in placental weights over time (Pinar et al. 1996). Compared to their primate relatives, humans also feature higher rates of placenta-related pregnancy complications (e.g., hyperemesis, preeclampsia etc) and postnatal conditions (e.g., endometriosis), indicating that a derived state of steroidogenic excess may be leading to human-specific maladaptive traits. All of these speculations will need to be rigorously tested in animal models and large human cohorts.

8.6: Autism as the result of excess adaptation

This notion of a maladaptive trait due to prenatal adaptations, could also be extended to our understanding of autism. As mentioned before, it is not clear whether autism or autistic traits are selected for by the prevailing pressures that led to human speciation. Given the condition's heterogeneity, on the genetic and behavioural level, it may be prudent to avoid "just-so" narratives and instead analyse the emergence of autism in the wider context of neurodevelopment.

The findings in this thesis and by others, have provided considerable evidence that prenatal sex steroid excess is associated with both autism and autistic traits in the children. The molecular impact of steroids on the developing brain (beyond sex differentiation) is unclear, but these appear to regulate the proliferation, differentiation, and migration of radial glial cells, in recent experiments involving human stem cell-derived neurons and organoids (Kelava et al. 2020; Sellers et al. 2015). These neural precursors establish a scaffolding for the organisation of cortical columns, during the first stages of cortical expansion. This link to the development of the human neocortex is consistent with evidence that was outlined previously, on how placental steroidogenesis may have been a driving force for many human-specific adaptations (sections 8.1-2).

It is then interesting to speculate that an excess in sex steroids may have been selected for in the human lineage, but also to occasionally lead to maladaptive phenotypes in cases when these adaptations go over and above physiological tolerance. For example, under specific prenatal circumstances (e.g., maternal PCOS, inefficient aromatisation, androgen increases in the PMW), steroid excess could be having an adverse impact on this complex, derived process (e.g., reduced inhibitory connectivity between cortical layers). Initial data from testosterone administration on brain organoids are consistent with this hypothesis, but more work is needed to explore the interplay between the different stages of cortical expansion, the rates of excitation/inhibition and a role of auto/paracrine aromatisation in the human brain (Kelava et al. 2020).

In an evolutionary context, the brain regions that are more affected by sex steroid excess, may coincide with the regions that have evolved more rapidly in humans. According to the social brain theory, these include cortical regions that enhance communication, empathy, as well as subcortical regions (e.g., amygdala) that regulate reactivity in social situations. Interestingly, in the context of autism, these domains have been found to be both hypo- and hyperactive. Most autistic people report challenges with social communication and the conscious understanding of their own and others' thinking and motivations. But many also show high levels of affective empathy, and a social awareness that becomes so overwhelming that it results in avoidant behaviours from childhood (e.g. avoiding eye contact) (Baron-Cohen et al. 2000). In brief, there is considerable heterogeneity in the social cognition of autistic individuals, which could be related to an imbalance in the components that regulate interpersonal communication in humans (e.g.,

social reactivity, empathy and communicative intent), rather than a simple deficit in all aspects of social development.

It is then interesting to speculate that autism could be a maladaptive neurodevelopmental phenotype, which is partly attributed to a derived propensity for prenatal sex steroid excess and rapid expansion of the 'social brain' in humans. Male susceptibility, compared to females, could then be explained by both physiological differences (e.g., androgen excess in males due to the activation of the fetal testes), as well as in terms of Evolution, since endocrine adaptations may have been 'designed' to meet the evolutionary needs of the matriline, rather than those of the 'male brain' (Figure 8.2).

8.7 Addendum Conclusion

In the 'Descent of Man', Charles Darwin was the first to analyse human evolution in terms of sexual selection. In that book, he argued that the human brain may have evolved due to male competition and the attractiveness of "man's intellect" to the opposite gender. He concluded that "man with all his noble qualities...still bears in his bodily frame the indelible stamp of his lowly origin" (Darwin 1875). Humans certainly carry the physiological characteristics of our close relatives. But the role of sex in human evolution may have been misunderstood in this regard. Although Darwin's monumental legacy cannot be disputed, it is now increasingly recognised that his analyses had been affected by the biases of his time and the widespread belief in male pre-eminence over women. For better or for worse, there cannot be any certainty in the study of Evolution. Nevertheless, the findings in this thesis and recent discoveries on prenatal steroids, on the placenta and on human cognition, all raise an intriguing possibility; that the "noble qualities" of our species may not be attributable to the attractive intellect of "man", but rather to the maternal conditioning and social capacities of women.

Bibliography

Aaronson, Ian A., Murat A. Cakmak, and Lyndon L. Key. 1997. "Defects of the Testosterone Biosynthetic Pathway in Boys with Hypospadias." *Journal of Urology* 157 (5). https://doi.org/10.1016/S0022-5347(01)64893-6.

Abel, Kathryn M., Christina Dalman, Anna C. Svensson, Ezra Susser, Henrik Dal, Selma Idring, Roger T. Webb, Dheeraj Rai, and Cecilia Magnusson. 2013. "Deviance in Fetal Growth and Risk of Autism Spectrum Disorder." *American Journal of Psychiatry*. https://doi.org/10.1176/appi.ajp.2012.12040543.

Adibi, J. J., M. K. Lee, S. Saha, W. J. Boscardin, A. Apfel, and R. J. Currier. 2015. "Fetal Sex Differences in Human Chorionic Gonadotropin Fluctuate by Maternal Race, Age, Weight and by Gestational Age." *Journal of Developmental Origins of Health and Disease*. https://doi.org/10.1017/S2040174415001336.

Al-Haddad, Benjamin J.S., Bo Jacobsson, Shilpi Chabra, Dominika Modzelewska, Erin M. Olson, Raphael Bernier, Daniel A. Enquobahrie, et al. 2019. "Long-Term Risk of Neuropsychiatric Disease after Exposure to Infection in Utero." *JAMA Psychiatry*. https://doi.org/10.1001/jamapsychiatry.2019.0029.

Alber, Michael, Vera M. Kalscheuer, Elysa Marco, Elliott Sherr, Gaetan Lesca, Marianne Till, Gyri Gradek, et al. 2017. "ARHGEF9 Disease." *Neurology: Genetics* 3 (3). https://doi.org/10.1212/NXG.0000000000000148.

Alemany, Silvia, Elisabet Blok, Philip R. Jansen, Ryan L. Muetzel, and Tonya White. 2021. "Brain Morphology, Autistic Traits, and Polygenic Risk for Autism: A Population-Based Neuroimaging Study." *Autism Research*, July. https://doi.org/10.1002/AUR.2576.

Allison, C., F E Mathew, L Ruta, G Pasco, R Soufer, C Brayne, T Charman, and S. Baron-Cohen. 2021. "The Quantitative Checklist for Autism in Toddlers (Q–CHAT). A Population Screening Study with Follow-up: The Case for Multiple Time–Point Screening for Autism." *BMJ Open Paediatrics*.

Allison, C, S Baron-Cohen, S Wheelwright, T Charman, J Richler, G Pasco, and C Brayne. 2008. "The Q-CHAT (Quantitative CHecklist for Autism in Toddlers): A Normally Distributed Quantitative Measure of Autistic Traits at 18-24 Months of Age: Preliminary Report." *J Autism Dev Disord* 38 (8): 1414–25. https://doi.org/10.1007/s10803-007-0509-7.

American Psychiatric Association. 2013. "DSM-5 Diagnostic Classification." In *Diagnostic and Statistical Manual of Mental Disorders*. https://doi.org/10.1176/appi.books.9780890425596.x00diagnosticclassification.

Amiri, M, S Lamballais, E Geenjaar, LME Blanken, H El Marroun, H Tiemeier, and T White. 2020. "Environment-Wide Association Study (E n WAS) of Prenatal and Perinatal Factors Associated With Autistic Traits: A Population-Based Study." *Autism Research : Official Journal of the International Society for Autism Research* 13 (9): 1582–1600. https://doi.org/10.1002/AUR.2372.

Anderson, G M, A Jacobs-Stannard, K Chawarska, F R Volkmar, and H J Kliman. 2007.

"Placental Trophoblast Inclusions in Autism Spectrum Disorder." *Biol Psychiatry* 61 (4): 487–91. https://doi.org/10.1016/j.biopsych.2006.03.068.

Andescavage, Nickie N., Adre Du Plessis, Robert McCarter, Ahmed Serag, Iordanis Evangelou, Gilbert Vezina, Richard Robertson, and Catherine Limperopoulos. 2017. "Complex Trajectories of Brain Development in the Healthy Human Fetus." *Cerebral Cortex* 27 (11): 5274–83. https://doi.org/10.1093/cercor/bhw306.

Ascaso, A, M Romero de Ávila, M Llorente, L Trujillano, F Ramos, J Pie, and G Bueno. 2019. "Endocrine Evaluation of 29 Cornelia de Lange Syndrome Patients (CdLS)." In *ESPE*, PP1-229. Vienna, Austria.

Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium. 2017. "Meta-Analysis of GWAS of over 16,000 Individuals with Autism Spectrum Disorder Highlights a Novel Locus at 10q24.32 and a Significant Overlap with Schizophrenia." *Molecular Autism* 8: 21. https://doi.org/10.1186/s13229-017-0137-9.

Auyeung, B, K Taylor, G Hackett, and S Baron-Cohen. 2010. "Foetal Testosterone and Autistic Traits in 18 to 24-Month-Old Children." *Mol Autism* 1 (1): 11. https://doi.org/10.1186/2040-2392-1-11.

Auyeung, Bonnie, Jag Ahluwalia, Lynn Thomson, Kevin Taylor, Gerald Hackett, Kieran J O'Donnell, and Simon Baron-Cohen. 2012. "Prenatal versus Postnatal Sex Steroid Hormone Effects on Autistic Traits in Children at 18 to 24 Months of Age." *Molecular Autism 2012 3:1* 3 (1): 1–5. https://doi.org/10.1186/2040-2392-3-17.

Auyeung, Bonnie, Simon Baron-Cohen, Emma Ashwin, Rebecca Knickmeyer, Kevin Taylor, and Gerald Hackett. 2009. "Fetal Testosterone and Autistic Traits." *British Journal of Psychology*. https://doi.org/10.1348/000712608X311731.

Auyeung, Bonnie, Simon Baron-Cohen, Emma Chapman, Rebecca Knickmeyer, Kevin Taylor, and Gerald Hackett. 2006. "Foetal Testosterone and the Child Systemizing Quotient." *European Journal of Endocrinology, Supplement*. https://doi.org/10.1530/eje.1.02260.

Avants, Brian B., Nick Tustison, and Gang Song. 2009. "Advanced Normalization Tools (ANTS)." *Insight Journal*. https://doi.org/http://hdl.handle.net/10380/3113.

Aydin, Ezra, Rosemary Holt, Daren Chaplin, Rebecca Hawkes, Carrie Allison, Gerald Hackett, Topun Austin, et al. 2019a. "Fetal Anogenital Distance Using Ultrasound." *Prenatal Diagnosis* 39 (7): 527–35. https://doi.org/10.1002/pd.5459.

Bahri Khomami, Mahnaz, Anju E. Joham, Jacqueline A. Boyle, Terhi Piltonen, Chavy Arora, Michael Silagy, Marie L. Misso, Helena J. Teede, and Lisa J. Moran. 2019. "The Role of Maternal Obesity in Infant Outcomes in Polycystic Ovary Syndrome—A Systematic Review, Meta-Analysis, and Meta-Regression." *Obesity Reviews*. https://doi.org/10.1111/obr.12832.

Bale, Tracy L. 2016. "The Placenta and Neurodevelopment: Sex Differences in Prenatal Vulnerability." *Dialogues in Clinical Neuroscience*. https://doi.org/10.31887/dcns.2016.18.4/tbale.

Banerjee-Basu, Sharmila, and Alan Packer. 2010. "SFARI Gene: An Evolving Database for

the Autism Research Community." *DMM Disease Models and Mechanisms*. https://doi.org/10.1242/dmm.005439.

Baron-Cohen, S, S Cassidy, B Auyeung, C Allison, M Achoukhi, S Robertson, A Pohl, and M C Lai. 2014. "Attenuation of Typical Sex Differences in 800 Adults with Autism vs. 3,900 Controls." *PLoS One* 9 (7): e102251. https://doi.org/10.1371/journal.pone.0102251.

Baron-Cohen, S, M V Lombardo, B Auyeung, E Ashwin, B Chakrabarti, and R Knickmeyer. 2011. "Why Are Autism Spectrum Conditions More Prevalent in Males?" *PLoS Biol* 9 (6): e1001081. https://doi.org/10.1371/journal.pbio.1001081.

Baron-Cohen, S, J Richler, D Bisarya, N Gurunathan, and S Wheelwright. 2003. "The Systemizing Quotient: An Investigation of Adults with Asperger Syndrome or High-Functioning Autism, and Normal Sex Differences." *Philos Trans R Soc Lond B Biol Sci* 358 (1430): 361–74. https://doi.org/10.1098/rstb.2002.1206.

Baron-Cohen, S, H A Ring, E T Bullmore, S Wheelwright, C Ashwin, and S C Williams. 2000. "The Amygdala Theory of Autism." *Neuroscience and Biobehavioral Reviews* 24 (3): 355–64. http://www.ncbi.nlm.nih.gov/pubmed/10781695.

Baron-Cohen, S, S Wheelwright, R Skinner, J Martin, and E Clubley. 2001. "The Autism-Spectrum Quotient (AQ): Evidence from Asperger Syndrome/High-Functioning Autism, Males and Females, Scientists and Mathematicians." *J Autism Dev Disord* 31 (1): 5–17. https://www.ncbi.nlm.nih.gov/pubmed/11439754.

Baron-Cohen, Simon., Svetlana. Lutchmaya, and Rebecca. Knickmeyer. 2004. *Prenatal Testosterone in Mind : Amniotic Fluid Studies*. MIT Press.

Baron-Cohen, Simon. 2002. "The Extreme Male Brain Theory of Autism." *Trends in Cognitive Sciences*. https://doi.org/10.1016/S1364-6613(02)01904-6.

———. 2017. "Editorial Perspective: Neurodiversity – a Revolutionary Concept for Autism and Psychiatry." *Journal of Child Psychology and Psychiatry and Allied Disciplines*. https://doi.org/10.1111/jcpp.12703.

Baron-Cohen, Simon, Bonnie Auyeung, B Nørgaard-Pedersen, D M Hougaard, Morsi W Abdallah, L Melgaard, A S Cohen, Bhismadev Chakrabarti, Liliana Ruta, and Michael V Lombardo. 2015. "Elevated Fetal Steroidogenic Activity in Autism." *Molecular Psychiatry* 20 (June): 369–376. https://doi.org/10.1038/mp.2014.48.

Baron-Cohen, Simon, Rebecca C. Knickmeyer, and Matthew K. Belmonte. 2005. "Sex Differences in the Brain: Implications for Explaining Autism." *Science*. https://doi.org/10.1126/science.1115455.

Baron-Cohen, Simon, Helen Tager-Flusberg, and Michael V. Lomardo. 2013. *Understanding Other Minds: Perspectives from Developmental Social Neuroscience*. *Oxford University Press*.

Baron-Cohen, Simon, Alexandros Tsompanidis, Bonnie Auyeung, Bent Nørgaard-Pedersen, David M. Hougaard, Morsi Abdallah, Arieh Cohen, and Alexa Pohl. 2019. "Foetal Oestrogens and Autism." *Molecular Psychiatry*. https://doi.org/10.1038/s41380-019-0454-9.

Baron-Cohen, Simon, Sally Wheelwright, Richard Skinner, Joanne Martin, and Emma

Clubley. 2001. “The Autism-Spectrum Quotient (AQ): Evidence from Asperger Syndrome/High-Functioning Autism, Males and Females, Scientists and Mathematicians.” *Journal of Autism and Developmental Disorders*. https://doi.org/10.1023/A:1005653411471.

Baron-Cohen, Simon. 2020. *The Pattern Seekers*. Allen Lane - Penguin Books Ltd.

Barrett, E S, K M Hoeger, S Sathyanarayana, D H Abbott, J B Redmon, R H N Nguyen, and S H Swan. 2018. “Anogenital Distance in Newborn Daughters of Women with Polycystic Ovary Syndrome Indicates Fetal Testosterone Exposure.” *J Dev Orig Health Dis*, 1–8. https://doi.org/10.1017/S2040174417001118.

Barton, Jason J.S., Daniel Z. Press, Julian P. Keenan, and Margaret O’Connor. 2002. “Lesions of the Fusiform Face Area Impair Perception of Facial Configuration in Prosopagnosia.” *Neurology* 58 (1): 71–78. https://doi.org/10.1212/WNL.58.1.71.

Becker, Esther B.E., and Catherine J. Stoodley. 2013. “Autism Spectrum Disorder and the Cerebellum.” In *International Review of Neurobiology*. Vol. 113. https://doi.org/10.1016/B978-0-12-418700-9.00001-0.

Becker, Marianne, Klaus Oehler, Carl Joachim Partsch, Ulrike Ulmen, Renate Schmutzler, Henning Cammann, and Volker Hesse. 2015. “Hormonal ‘minipuberty’ Influences the Somatic Development of Boys but Not of Girls up to the Age of 6 Years.” *Clinical Endocrinology* 83 (5): 694–701. https://doi.org/10.1111/CEN.12827.

Beek, C van de, J H Thijssen, P T Cohen-Kettenis, S H van Goozen, and J K Buitelaar. 2004. “Relationships between Sex Hormones Assessed in Amniotic Fluid, and Maternal and Umbilical Cord Serum: What Is the Best Source of Information to Investigate the Effects of Fetal Hormonal Exposure?” *Horm Behav* 46 (5): 663–69. https://doi.org/10.1016/j.yhbeh.2004.06.010.

Bejerot, S, J M Eriksson, S Bonde, K Carlstrom, M B Humble, and E Eriksson. 2012. “The Extreme Male Brain Revisited: Gender Coherence in Adults with Autism Spectrum Disorder.” *Br J Psychiatry* 201: 116–23. https://doi.org/10.1192/bjp.bp.111.097899.

Bejerot, Susanne, and Jonna M. Eriksson. 2014. “Sexuality and Gender Role in Autism Spectrum Disorder: A Case Control Study.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0087961.

Benavides, Amanda, Andrew Metzger, Alexander Tereshchenko, Amy Conrad, Edward F. Bell, John Spencer, Shannon Ross-Sheehy, Michael Georgieff, Vince Magnotta, and Peg Nopoulos. 2018. “Sex-Specific Alterations in Preterm Brain.” *Pediatric Research 2018 85:1* 85 (1): 55–62. https://doi.org/10.1038/s41390-018-0187-5.

Bergen, N. E., M. I. Bouwland-Both, R. P.M. Steegers-Theunissen, A. Hofman, H. Russcher, J. Lindemans, V. W.V. Jaddoe, and E. A.P. Steegers. 2015. “Early Pregnancy Maternal and Fetal Angiogenic Factors and Fetal and Childhood Growth: The Generation R Study.” *Human Reproduction* 30 (6): 1302–13. https://doi.org/10.1093/humrep/dev070.

Berni, T R, C L Morgan, E R Berni, and D A Rees. 2018. “Polycystic Ovary Syndrome Is Associated With Adverse Mental Health and Neurodevelopmental Outcomes.” *J Clin Endocrinol Metab* 103 (6): 2116–25. https://doi.org/10.1210/jc.2017-02667.

Beversdorf, D. Q., S. E. Manning, A. Hillier, S. L. Anderson, R. E. Nordgren, S. E. Walters, H. N. Nagaraja, W. C. Cooley, S. E. Gaelic, and M. L. Bauman. 2005. "Timing of Prenatal Stressors and Autism." *Journal of Autism and Developmental Disorders*. https://doi.org/10.1007/s10803-005-5037-8.

Bhat, Gifty, Danielle LaGrave, Alison Millson, John Herriges, Allen N. Lamb, and Reuben Matalon. 2016. "Xq11.1-11.2 Deletion Involving ARHGEF9 in a Girl with Autism Spectrum Disorder." *European Journal of Medical Genetics* 59 (9). https://doi.org/10.1016/j.ejmg.2016.05.014.

Bilder, Deborah A, M Sean Esplin, Hilary Coon, Paul Burghardt, Erin A S Clark, Alison Fraser, Ken R Smith, et al. 2019. "Early Second Trimester Maternal Serum Steroid-Related Biomarkers Associated with Autism Spectrum Disorder." *Journal of Autism and Developmental Disorders*, August. https://doi.org/10.1007/s10803-019-04162-2.

Blanken, L M E, A Dass, G Alvares, J van der Ende, N K Schoemaker, H El Marroun, M Hickey, et al. 2018. "A Prospective Study of Fetal Head Growth, Autistic Traits and Autism Spectrum Disorder." *Autism Res* 11 (4): 602–12. https://doi.org/10.1002/aur.1921.

Blutstein, Tamara, Peter J. Baab, H. Ronald Zielke, and Jessica A. Mong. 2009. "Hormonal Modulation of Amino Acid Neurotransmitter Metabolism in the Arcuate Nucleus of the Adult Female Rat: A Novel Action of Estradiol." *Endocrinology*. https://doi.org/10.1210/en.2008-1701.

Boddaert, N., N. Chabane, H. Gervais, C. D. Good, M. Bourgeois, M. H. Plumet, C. Barthélémy, et al. 2004. "Superior Temporal Sulcus Anatomical Abnormalities in Childhood Autism: A Voxel-Based Morphometry MRI Study." *NeuroImage* 23 (1): 364–69. https://doi.org/10.1016/J.NEUROIMAGE.2004.06.016.

Boddaert, Nathalie, Mônica Zilbovicius, Anne Philipe, Laurence Robel, Marie Bourgeois, Catherine Barthélemy, David Seidenwurm, et al. 2009. "MRI Findings in 77 Children with Non-Syndromic Autistic Disorder." *PLOS ONE* 4 (2): e4415. https://doi.org/10.1371/JOURNAL.PONE.0004415.

Bölte, Sven, Fritz Poustka, and John N. Constantino. 2008. "Assessing Autistic Traits: Cross-Cultural Validation of the Social Responsiveness Scale (SRS)." *Autism Research*. https://doi.org/10.1002/aur.49.

Bonnet-Brilhault, Fréderique, Toky A. Rajerison, Christian Paillet, Marie Guimard-Brunault, Agathe Saby, Laura Ponson, Gabriele Tripi, Joëlle Malvy, and Sylvie Roux. 2018. "Autism Is a Prenatal Disorder: Evidence from Late Gestation Brain Overgrowth." *Autism Research* 11 (12): 1635–42. https://doi.org/10.1002/aur.2036.

Bonomi, Marco, Vincenzo Rochira, Daniela Pasquali, Giancarlo Balercia, E. A. Jannini, Alberto Ferlin, Aldo Calogero, et al. 2017. "Klinefelter Syndrome (KS): Genetics, Clinical Phenotype and Hypogonadism." *Journal of Endocrinological Investigation*. https://doi.org/10.1007/s40618-016-0541-6.

Breedlove, S. Marc. 2010. "Minireview: Organizational Hypothesis: Instances of the Fingerpost." *Endocrinology* 151 (9): 4116. https://doi.org/10.1210/EN.2010-0041.

Broere-Brown, Zoe A., Esme Baan, Sarah Schalekamp-Timmermans, Bero O. Verburg, Vincent W.V. Jaddoe, and Eric A.P. Steegers. 2016. "Sex-Specific Differences in Fetal and Infant Growth Patterns: A Prospective Population-Based Cohort Study." *Biology of Sex Differences*. https://doi.org/10.1186/s13293-016-0119-1.

Brown, M. A., M. D. Lindheimer, M. De Swiet, A. Van Assche, and J. M. Moutquin. 2001. "The Classification and Diagnosis of the Hypertensive Disorders of Pregnancy: Statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP)." *Hypertension in Pregnancy*. https://doi.org/10.1081/PRG-100104165.

Brown, Z A, S Schalekamp-Timmermans, H W Tiemeier, A Hofman, V W V Jaddoe, and E A P Steegers. 2014. "Fetal Sex Specific Differences in Human Placentation: A Prospective Cohort Study." *Placenta* 35: 359–64. https://doi.org/10.1016/j.placenta.2014.03.014.

Bulik-Sullivan, Brendan, Po Ru Loh, Hilary K. Finucane, Stephan Ripke, Jian Yang, Nick Patterson, Mark J. Daly, et al. 2015. "LD Score Regression Distinguishes Confounding from Polygenicity in Genome-Wide Association Studies." *Nature Genetics* 47 (3). https://doi.org/10.1038/ng.3211.

Calkins, Kara, and Sherin U. Devaskar. 2011. "Fetal Origins of Adult Disease." *Current Problems in Pediatric and Adolescent Health Care* 41 (6). https://doi.org/10.1016/j.cppeds.2011.01.001.

Canfield, R. E., J. F. O'Connor, S. Birken, A. Krichevsky, and A. J. Wilcox. 1987. "Development of an Assay for a Biomarker of Pregnancy and Early Fetal Loss." *Environmental Health Perspectives*. https://doi.org/10.1289/ehp.877457.

Caria, Andrea, Luciana Ciringione, and Simona de Falco. 2020. "Morphofunctional Alterations of the Hypothalamus and Social Behavior in Autism Spectrum Disorders." *Brain Sciences*. https://doi.org/10.3390/brainsci10070435.

Carmignac, Virginie, Sophie Nambot, Daphné Lehalle, Patrick Callier, Stephanie Moortgat, Valérie Benoit, Jamal Ghoumid, et al. 2020. "Further Delineation of the Female Phenotype with KDM5C Disease Causing Variants: 19 New Individuals and Review of the Literature." *Clinical Genetics* 98 (1). https://doi.org/10.1111/cge.13755.

Casanova, Emily L., Andrew E. Switala, Srini Dandamudi, Allison R. Hickman, Joshua Vandenbrink, Julia L. Sharp, Frank Alex Feltus, and Manuel F. Casanova. 2019. "Autism Risk Genes Are Evolutionarily Ancient and Maintain a Unique Feature Landscape That Echoes Their Function." *Autism Research* 12 (6). https://doi.org/10.1002/aur.2112.

Castelli, Fulvia, Francesca Happé, Uta Frith, and Chris Frith. 2000. "Movement and Mind: A Functional Imaging Study of Perception and Interpretation of Complex Intentional Movement Patterns." *NeuroImage* 12 (3): 314–25. https://doi.org/10.1006/nimg.2000.0612.

Cesta, C E, M Mansson, C Palm, P Lichtenstein, A N Iliadou, and M Landen. 2016. "Polycystic Ovary Syndrome and Psychiatric Disorders: Co-Morbidity and Heritability in a Nationwide Swedish Cohort." *Psychoneuroendocrinology* 73: 196–

203. https://doi.org/10.1016/j.psyneuen.2016.08.005.

Cesta, Carolyn E., Anna S. Öberg, Abraham Ibrahimson, Ikram Yusuf, Henrik Larsson, Catarina Almqvist, Brian M. D'Onofrio, et al. 2020. "Maternal Polycystic Ovary Syndrome and Risk of Neuropsychiatric Disorders in Offspring: Prenatal Androgen Exposure or Genetic Confounding?" *Psychological Medicine*. https://doi.org/10.1017/S0033291719000424.

Chakrabarti, Bhismadev, F Dudbridge, L Kent, Sally Wheelwright, G Hill-Cawthorne, Carrie Allison, S Banerjee-Basu, and Simon Baron-Cohen. 2009. "Genes Related to Sex Steroids, Neural Growth, and Social-Emotional Behavior Are Associated with Autistic Traits, Empathy, and Asperger Syndrome." *Autism Research* 2 (3): 157–77. https://doi.org/10.1002/aur.80.

Chapman, E, S Baron-Cohen, B Auyeung, R Knickmeyer, K Taylor, and G Hackett. 2006. "Fetal Testosterone and Empathy: Evidence from the Empathy Quotient (EQ) and the 'Reading the Mind in the Eyes' Test." *Soc Neurosci* 1 (2): 135–48. https://doi.org/10.1080/17470910600992239.

Chen, Xinxia, Linghua Kong, Terhi T. Piltonen, Mika Gissler, and Catharina Lavebratt. 2020. "Association of Polycystic Ovary Syndrome or Anovulatory Infertility with Offspring Psychiatric and Mild Neurodevelopmental Disorders: A Finnish Population-Based Cohort Study." *Human Reproduction*. https://doi.org/10.1093/humrep/deaa192.

Chen, Z, O Wang, M Nie, K Elison, D Zhou, M Li, Y Jiang, et al. 2015. "Aromatase Deficiency in a Chinese Adult Man Caused by Novel Compound Heterozygous CYP19A1 Mutations: Effects of Estrogen Replacement Therapy on the Bone, Lipid, Liver and Glucose Metabolism." *Mol Cell Endocrinol* 399: 32–42. https://doi.org/10.1016/j.mce.2014.09.016.

Cherskov, A, A Pohl, C Allison, H Zhang, R A Payne, and S Baron-Cohen. 2018. "Polycystic Ovary Syndrome and Autism: A Test of the Prenatal Sex Steroid Theory." *Transl Psychiatry* 8 (1): 136. https://doi.org/10.1038/s41398-018-0186-7.

Chowdhury, Vishwajit S., Kazutoshi Yamamoto, Takayoshi Ubuka, George E. Bentley, Atsuhiko Hattori, and Kazuyoshi Tsutsui. 2010. "Melatonin Stimulates the Release of Gonadotropin-Inhibitory Hormone by the Avian Hypothalamus." *Endocrinology*. https://doi.org/10.1210/en.2009-0908.

Chrousos, G. P. 1999. "Editorial: A New 'new' Syndrome in the New World: Is Multiple Postreceptor Steroid Hormone Resistance Due to a Coregulator Defect?" *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jc.84.12.4450.

Chung, Ren Hua, Deqiong Ma, Kai Wang, Dale J. Hedges, James M. Jaworski, John R. Gilbert, Michael L. Cuccaro, et al. 2011. "An X Chromosome-Wide Association Study in Autism Families Identifies TBL1X as a Novel Autism Spectrum Disorder Candidate Gene in Males." *Molecular Autism* 2 (1). https://doi.org/10.1186/2040-2392-2-18.

Chura, Lindsay R., Michael V. Lombardo, Emma Ashwin, Bonnie Auyeung, Bhismadev Chakrabarti, Edward T. Bullmore, and Simon Baron-Cohen. 2010. "Organizational Effects of Fetal Testosterone on Human Corpus Callosum Size and Asymmetry."

Psychoneuroendocrinology 35 (1): 122–32. https://doi.org/10.1016/j.psyneuen.2009.09.009.

Cieri, Robert L., Steven E. Churchill, Robert G. Franciscus, Jingzhi Tan, and Brian Hare. 2014. "Craniofacial Feminization, Social Tolerance, and the Origins of Behavioral Modernity." *Current Anthropology* 55 (4). https://doi.org/10.1086/677209.

Cleys, Ellane R., Jennifer L. Halleran, Vanessa A. Enriquez, Juliano C. Da Silveira, Rachel C. West, Quinton A. Winger, Russell V. Anthony, Jason E. Bruemmer, Colin M. Clay, and Gerrit J. Bouma. 2015. "Androgen Receptor and Histone Lysine Demethylases in Ovine Placenta E0117472." *PLoS ONE* 10 (2). https://doi.org/10.1371/journal.pone.0117472.

Colvert, Emma, Beata Tick, Fiona McEwen, Catherine Stewart, Sarah R. Curran, Emma Woodhouse, Nicola Gillan, et al. 2015. "Heritability of Autism Spectrum Disorder in a UK Population-Based Twin Sample." *JAMA Psychiatry* 72 (5). https://doi.org/10.1001/jamapsychiatry.2014.3028.

Connellan, Jennifer, Simon Baron-Cohen, Sally Wheelwright, Anna Batki, and Jag Ahluwalia. 2000. "Sex Differences in Human Neonatal Social Perception." *Infant Behavior and Development*. https://doi.org/10.1016/S0163-6383(00)00032-1.

Coorens, Tim H.H., Thomas R.W. Oliver, Rashesh Sanghvi, Ulla Sovio, Emma Cook, Roser Vento-Tormo, Muzlifah Haniffa, et al. 2021. "Inherent Mosaicism and Extensive Mutation of Human Placentas." *Nature* 592 (7852). https://doi.org/10.1038/s41586-021-03345-1.

Copeland, William, Lilly Shanahan, Shari Miller, E. Jane Costello, Adrian Angold, and Barbara Maughan. 2010. "Outcomes of Early Pubertal Timing in Young Women: A Prospective Population-Based Study." *American Journal of Psychiatry* 167 (10). https://doi.org/10.1176/appi.ajp.2010.09081190.

Corbett, Blythe A., Simon Vandekar, Rachael A. Muscatello, and Yasas Tanguturi. 2020. "Pubertal Timing During Early Adolescence: Advanced Pubertal Onset in Females with Autism Spectrum Disorder." *Autism Research* 13 (12). https://doi.org/10.1002/aur.2406.

Courchesne, Eric, Ruth Carper, and Natacha Akshoomoff. 2003. "Evidence of Brain Overgrowth in the First Year of Life in Autism." *JAMA* 290 (3): 337. https://doi.org/10.1001/jama.290.3.337.

Courchesne, Eric, Peter R. Mouton, Michael E. Calhoun, Katerina Semendeferi, Clelia Ahrens-Barbeau, Melodie J. Hallet, Cynthia Carter Barnes, and Karen Pierce. 2011. "Neuron Number and Size in Prefrontal Cortex of Children with Autism." *JAMA - Journal of the American Medical Association* 306 (18). https://doi.org/10.1001/jama.2011.1638.

Courchesne, Eric, Karen Pierce, Cynthia M. Schumann, Elizabeth Redcay, Joseph A. Buckwalter, Daniel P. Kennedy, and John Morgan. 2007. "Mapping Early Brain Development in Autism." *Neuron*. https://doi.org/10.1016/j.neuron.2007.10.016.

Couteur, A. L. Le, I. Gottesman, P. Bolton, E. Simonoff, E. Yuzda, M. Rutter, and A. Bailey. 1995. "Autism as a Strongly Genetic Disorder Evidence from a British Twin Study." *Psychological Medicine* 25 (1): 63–77.

https://doi.org/10.1017/S0033291700028099.

Crider, Amanda, Roshni Thakkar, Anthony O Ahmed, and Anilkumar Pillai. 2014. "Dysregulation of Estrogen Receptor Beta (ERβ), Aromatase (CYP19A1), and ER Co-Activators in the Middle Frontal Gyrus of Autism Spectrum Disorder Subjects." *Molecular Autism* 5 (1): 46. https://doi.org/10.1186/2040-2392-5-46.

Crinion, Jennifer T., Matthew A. Lambon-Ralph, Elizabeth A. Warburton, David Howard, and Richard J. S. Wise. 2003. "Temporal Lobe Regions Engaged during Normal Speech Comprehension." *Brain* 126 (5): 1193–1201. https://doi.org/10.1093/BRAIN/AWG104.

Critchley, Hugo D., Eileen M. Daly, Edward T. Bullmore, Steven C.R. Williams, Therese Van Amelsvoort, Dene M. Robertson, Andrea Rowe, et al. 2000. "The Functional Neuroanatomy of Social Behaviour: Changes in Cerebral Blood Flow When People with Autistic Disorder Process Facial Expressions." *Brain* 123 (11): 2203–12. https://doi.org/10.1093/brain/123.11.2203.

Croen, L A, O Zerbo, Y Qian, M L Massolo, S Rich, S Sidney, and C Kripke. 2015. "The Health Status of Adults on the Autism Spectrum." *Autism* 19 (7): 814–23. https://doi.org/10.1177/1362361315577517.

Cruz, Cynthia Dela, and Oduvaldo C.M. Pereira. 2012. "Prenatal Testosterone Supplementation Alters Puberty Onset, Aggressive Behavior, and Partner Preference in Adult Male Rats." *Journal of Physiological Sciences* 62 (2). https://doi.org/10.1007/s12576-011-0190-7.

Curran, E A, G W O'Keeffe, A M Looney, G Moloney, S V Hegarty, D M Murray, A S Khashan, and L C Kenny. 2018. "Exposure to Hypertensive Disorders of Pregnancy Increases the Risk of Autism Spectrum Disorder in Affected Offspring." *Mol Neurobiol* 55 (7): 5557–64. https://doi.org/10.1007/s12035-017-0794-x.

Czekala, N. M., K. Benirschke, H. McClure, and B. L. Lasley. 1983. "Urinary Estrogen Excretion during Pregnancy in the Gorilla (Gorilla Gorilla), Orangutan (Pongo Pygmaeus) and the Human (Homo Sapiens)." *Biology of Reproduction* 28 (2): 289–94. https://doi.org/10.1095/biolreprod28.2.289.

Dachew, Berihun Assefa, Abdullah Mamun, Joemer Calderon Maravilla, and Rosa Alati. 2018. "Pre-Eclampsia and the Risk of Autism-Spectrum Disorder in Offspring: Meta-Analysis." *British Journal of Psychiatry*. https://doi.org/10.1192/bjp.2017.27.

Dalton, Kim M, Brendon M Nacewicz, Tom Johnstone, Hillary S Schaefer, Morton Ann Gernsbacher, H H Goldsmith, Andrew L Alexander, and Richard J Davidson. 2005. "Gaze Fixation and the Neural Circuitry of Face Processing in Autism." *Nature Neuroscience* 8 (4): 519–26. https://doi.org/10.1038/nn1421.

Darwin, Charles. 2008. *The Descent of Man, and Selection in Relation to Sex*. *The Descent of Man, and Selection in Relation to Sex*. Vol. 1–2. https://doi.org/10.1038/011305a0.

Davis, Elysia Poggi, and Donald Pfaff. 2014. "Sexually Dimorphic Responses to Early Adversity: Implications for Affective Problems and Autism Spectrum Disorder." *Psychoneuroendocrinology*. https://doi.org/10.1016/j.psyneuen.2014.06.014.

Day, Felix, Tugce Karaderi, Michelle R. Jones, Cindy Meun, Chunyan He, Alex Drong, Peter Kraft, et al. 2018. "Large-Scale Genome-Wide Meta-Analysis of Polycystic Ovary Syndrome Suggests Shared Genetic Architecture for Different Diagnosis Criteria." *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1007813.

Day, Felix R., Deborah J. Thompson, Hannes Helgason, Daniel I. Chasman, Hilary Finucane, Patrick Sulem, Katherine S. Ruth, et al. 2017. "Genomic Analyses Identify Hundreds of Variants Associated with Age at Menarche and Support a Role for Puberty Timing in Cancer Risk." *Nature Genetics* 49 (6). https://doi.org/10.1038/ng.3841.

Dean, Douglas C., E. M. Planalp, W. Wooten, C. K. Schmidt, S. R. Kecskemeti, C. Frye, N. L. Schmidt, H. H. Goldsmith, A. L. Alexander, and R. J. Davidson. 2018. "Investigation of Brain Structure in the 1-Month Infant." *Brain Structure and Function* 223 (4): 1953–70. https://doi.org/10.1007/s00429-017-1600-2.

Desoye, Gernot, and Sylvie Hauguel-De Mouzon. 2007. "The Human Placenta in Gestational Diabetes Mellitus: The Insulin and Cytokine Network." *Diabetes Care* 30 (SUPPL. 2). https://doi.org/10.2337/dc07-s203.

Dewerchin, Mieke, and Peter Carmeliet. 2012. "PlGF: A Multitasking Cytokine with Disease-Restricted Activity." *Cold Spring Harbor Perspectives in Medicine*. https://doi.org/10.1101/cshperspect.a011056.

Diaz-Beltran, Leticia, Francisco J. Esteban, Maya Varma, Alp Ortuzk, Maude David, and Dennis P. Wall. 2017. "Cross-Disorder Comparative Analysis of Comorbid Conditions Reveals Novel Autism Candidate Genes." *BMC Genomics*. https://doi.org/10.1186/s12864-017-3667-9.

Doan, Ryan N., Byoung Il Bae, Beatriz Cubelos, Cindy Chang, Amer A. Hossain, Samira Al-Saad, Nahit M. Mukaddes, et al. 2016. "Mutations in Human Accelerated Regions Disrupt Cognition and Social Behavior." *Cell* 167 (2). https://doi.org/10.1016/j.cell.2016.08.071.

Dooley, Niamh, Amber Ruigrok, Rosemary Holt, Carrie Allison, Alexandros Tsompanidis, Jack Waldman, Bonnie Auyeung, Michael V. Lombardo, and Simon Baron-Cohen. 2022. "Is There an Association between Prenatal Testosterone and Autistic Traits in Adolescents?" *Psychoneuroendocrinology* 136. https://doi.org/10.1016/j.psyneuen.2021.105623.

Dunbar, R. I.M. 2009. "The Social Brain Hypothesis and Its Implications for Social Evolution." *Annals of Human Biology* 36 (5). https://doi.org/10.1080/03014460902960289.

Dunbar, Robin, and John G. Fleagle. 1999. "Primate Adaptation and Evolution." *The Journal of the Royal Anthropological Institute* 5 (4). https://doi.org/10.2307/2661176.

Dunbar, Robin I. M. 2016. "The Social Brain Hypothesis and Human Evolution." In *Oxford Research Encyclopedia of Psychology*. https://doi.org/10.1093/acrefore/9780190236557.013.44.

Durand, C M, J Perroy, F Loll, D Perrais, L Fagni, T Bourgeron, M Montcouquiol, and N Sans. 2012. "SHANK3 Mutations Identified in Autism Lead to Modification of

Dendritic Spine Morphology via an Actin-Dependent Mechanism." *Mol Psychiatry* 17 (1): 71–84. https://doi.org/10.1038/mp.2011.57.

Eichler, Evan E., and Andrew W. Zimmerman. 2008. "A Hot Spot of Genetic Instability in Autism." *New England Journal of Medicine* 358 (7). https://doi.org/10.1056/nejme0708756.

Elliot, M. G., and B. J. Crespi. 2009. "Phylogenetic Evidence for Early Hemochorial Placentation in Eutheria." *Placenta* 30 (11). https://doi.org/10.1016/j.placenta.2009.08.004.

Epperson, C N, D R Kim, and T L Bale. 2014. "Estradiol Modulation of Monoamine Metabolism: One Possible Mechanism Underlying Sex Differences in Risk for Depression and Dementia." *JAMA Psychiatry* 71 (8): 869–70. https://doi.org/10.1001/jamapsychiatry.2014.729.

Eriksson, Nicholas, Geoffrey M. Benton, Chuong B. Do, Amy K. Kiefer, Joanna L. Mountain, David A. Hinds, Uta Francke, and Joyce Y. Tung. 2012. "Genetic Variants Associated with Breast Size Also Influence Breast Cancer Risk." *BMC Medical Genetics* 13. https://doi.org/10.1186/1471-2350-13-53.

Escobar, J C, S S Patel, V E Beshay, T Suzuki, and B R Carr. 2011. "The Human Placenta Expresses CYP17 and Generates Androgens de Novo." *J Clin Endocrinol Metab* 96 (5): 1385–92. https://doi.org/10.1210/jc.2010-2504.

Etchell, Andrew, Aditi Adhikari, Lauren S. Weinberg, Ai Leen Choo, Emily O. Garnett, Ho Ming Chow, and Soo Eun Chang. 2018. "A Systematic Literature Review of Sex Differences in Childhood Language and Brain Development." *Neuropsychologia*. Neuropsychologia. https://doi.org/10.1016/j.neuropsychologia.2018.04.011.

Faul, Franz, Edgar Erdfelder, Axel Buchner, and Albert-Georg Lang. 2009. "Statistical Power Analyses Using G*Power 3.1: Tests for Correlation and Regression Analyses." *Behavior Research Methods* 41 (4): 1149–60. https://doi.org/10.3758/BRM.41.4.1149.

Fingher, Noa, Ilan Dinstein, Michal Ben-Shachar, Shlomi Haar, Anders M. Dale, Lisa Eyler, Karen Pierce, and Eric Courchesne. 2017. "Toddlers Later Diagnosed with Autism Exhibit Multiple Structural Abnormalities in Temporal Corpus Callosum Fibers." *Cortex* 97 (December): 291–305. https://doi.org/10.1016/j.cortex.2016.12.024.

Fischer, Barbara, Nicole D.S. Grunstra, Eva Zaffarini, and Philipp Mitteroecker. 2021. "Sex Differences in the Pelvis Did Not Evolve de Novo in Modern Humans." *Nature Ecology and Evolution* 5 (5). https://doi.org/10.1038/s41559-021-01425-z.

Floris, D L, M C Lai, T Nath, M P Milham, and A Di Martino. 2018. "Network-Specific Sex Differentiation of Intrinsic Brain Function in Males with Autism." *Mol Autism* 9: 17. https://doi.org/10.1186/s13229-018-0192-x.

Folkersen, Lasse, Stefan Gustafsson, Qin Wang, Daniel Hvidberg Hansen, Åsa K. Hedman, Andrew Schork, Karen Page, et al. 2020. "Genomic and Drug Target Evaluation of 90 Cardiovascular Proteins in 30,931 Individuals." *Nature Metabolism* 2 (10). https://doi.org/10.1038/s42255-020-00287-2.

Fombonne, Eric. 2009. “Epidemiology of Pervasive Developmental Disorders.” *Pediatric Research* 65 (6): 591–98. https://doi.org/10.1203/PDR.0b013e31819e7203.

Fombonne, Eric, Bernadette Rogé, Jacques Claverie, Stéphanie Courty, and Jeanne Frémolle. 1999. “Microcephaly and Macrocephaly in Autism.” *Journal of Autism and Developmental Disorders* 29 (2): 113–19. https://doi.org/10.1023/A:1023036509476.

Foss-Feig, Jennifer H., Rankin W. McGugin, Isabel Gauthier, Lisa E. Mash, Pamela Ventola, and Carissa J. Cascio. 2016. “A Functional Neuroimaging Study of Fusiform Response to Restricted Interests in Children and Adolescents with Autism Spectrum Disorder.” *Journal of Neurodevelopmental Disorders* 8 (1): 1–12. https://doi.org/10.1186/s11689-016-9149-6.

Ganguly, K, A F Schinder, S T Wong, and M Poo. 2001. “GABA Itself Promotes the Developmental Switch of Neuronal GABAergic Responses from Excitation to Inhibition.” *Cell* 105 (4): 521–32. https://www.ncbi.nlm.nih.gov/pubmed/11371348.

Gaugler, Trent, Lambertus Klei, Stephan J. Sanders, Corneliu A. Bodea, Arthur P. Goldberg, Ann B. Lee, Milind Mahajan, et al. 2014. “Most Genetic Risk for Autism Resides with Common Variation.” *Nature Genetics*. https://doi.org/10.1038/ng.3039.

Geschwind, Norman, and Albert M. Galaburda. 1985. “Cerebral Lateralization: Biological Mechanisms, Associations, and Pathology: I. A Hypothesis and a Program for Research.” *Archives of Neurology* 42 (5): 428–59. https://doi.org/10.1001/archneur.1985.04060050026008.

Gholipour, Ali, Caitlin K. Rollins, Clemente Velasco-Annis, Abdelhakim Ouaalam, Alireza Akhondi-Asl, Onur Afacan, Cynthia M. Ortinau, et al. 2017. “A Normative Spatiotemporal MRI Atlas of the Fetal Brain for Automatic Segmentation and Analysis of Early Brain Growth.” *Scientific Reports 2017 7:1* 7 (1): 1–13. https://doi.org/10.1038/s41598-017-00525-w.

Gilmore, John H., Weili Lin, Marcel W. Prastawa, Christopher B. Looney, Y. Sampath K. Vetsa, Rebecca C. Knickmeyer, Dianne D. Evans, et al. 2007. “Regional Gray Matter Growth, Sexual Dimorphism, and Cerebral Asymmetry in the Neonatal Brain.” *Journal of Neuroscience* 27 (6): 1255–60. https://doi.org/10.1523/JNEUROSCI.3339-06.2007.

Goldenfeld, Nigel, Simon Baron-Cohen, and Sally Wheelwright. 2005. “Empathizing and Systemizing in Males, Females, and Autism.” *Clinical Neuropsychiatry: Journal of Treatment Evaluation*.

Gong, S, M D Johnson, J Dopierala, F Gaccioli, U Sovio, M Constancia, G C Smith, and D S Charnock-Jones. 2018. “Genome-Wide Oxidative Bisulfite Sequencing Identifies Sex-Specific Methylation Differences in the Human Placenta.” *Epigenetics* 13 (3): 228–39. https://doi.org/10.1080/15592294.2018.1429857.

Gong, S, U Sovio, I L Aye, F Gaccioli, J Dopierala, M D Johnson, A M Wood, et al. 2018. “Placental Polyamine Metabolism Differs by Fetal Sex, Fetal Growth Restriction, and Preeclampsia.” *JCI Insight* 3 (13). https://doi.org/10.1172/jci.insight.120723.

Gong, Xiaohong, Elena Bacchelli, Francesca Blasi, Claudio Toma, Catalina Betancur, Pauline Chaste, Richard Delorme, et al. 2008. "Analysis of X Chromosome Inactivation in Autism Spectrum Disorders." *American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics* 147 (6). https://doi.org/10.1002/ajmg.b.30688.

González, A., C. Martínez-Campa, M. D. Mediavilla, C. Alonso-González, E. J. Sánchez-Barceló, and S. Cos. 2007. "Inhibitory Effects of Pharmacological Doses of Melatonin on Aromatase Activity and Expression in Rat Glioma Cells." *British Journal of Cancer*. https://doi.org/10.1038/sj.bjc.6603935.

González, Miriam, Alfredo Cabrera-Socorro, Carlos G Pérez-García, James D Fraser, Francisco J López, Rafael Alonso, and Gundela Meyer. 2007. "Distribution Patterns of Estrogen Receptor Alpha and Beta in the Human Cortex and Hippocampus during Development and Adulthood." *The Journal of Comparative Neurology* 503 (6): 790–802. https://doi.org/10.1002/cne.21419.

Gonzalez, Tania L., Tianyanxin Sun, Alexander F. Koeppel, Bora Lee, Erica T. Wang, Charles R. Farber, Stephen S. Rich, et al. 2018. "Sex Differences in the Late First Trimester Human Placenta Transcriptome." *Biology of Sex Differences* 9 (1). https://doi.org/10.1186/S13293-018-0165-Y.

Goodwin, Thomas M., Martin Montoro, Jorge H. Mestman, A. Eugene Pekary, and Jerome M. Hershman. 1992. "The Role of Chorionic Gonadotropin in Transient Hyperthyroidism of Hyperemesis Gravidarum." *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jcem.75.5.1430095.

Gottfredsdóttir, Helga, Kristín Björnsdóttir, and Jane Sandall. 2009. "How Do Prospective Parents Who Decline Prenatal Screening Account for Their Decision? A Qualitative Study." *Social Science and Medicine* 69 (2). https://doi.org/10.1016/j.socscimed.2009.05.004.

Greenberg, David M, Varun Warrier, Carrie Allison, and Simon Baron-Cohen. 2018. "Testing the Empathizing-Systemizing Theory of Sex Differences and the Extreme Male Brain Theory of Autism in Half a Million People." *Proceedings of the National Academy of Sciences of the United States of America*, November, 201811032. https://doi.org/10.1073/pnas.1811032115.

Grove, Jakob, Stephan Ripke, Thomas D. Als, Manuel Mattheisen, Raymond K. Walters, Hyejung Won, Jonatan Pallesen, et al. 2019. "Identification of Common Genetic Risk Variants for Autism Spectrum Disorder." *Nature Genetics* 51 (3): 431–44. https://doi.org/10.1038/s41588-019-0344-8.

Haddow, James E., Monica R. McClain, Geralyn Lambert-Messerlian, Glenn E. Palomaki, Jacob A. Canick, Jane Cleary-Goldman, Fergal D. Malone, et al. 2008. "Variability in Thyroid-Stimulating Hormone Suppression by Human Chronic Gonadotropin during Early Pregnancy." *Journal of Clinical Endocrinology and Metabolism* 93 (9). https://doi.org/10.1210/jc.2008-0568.

Hagenaars, Saskia P., W. David Hill, Sarah E. Harris, Stuart J. Ritchie, Gail Davies, David C. Liewald, Catharine R. Gale, David J. Porteous, Ian J. Deary, and Riccardo E. Marioni. 2017. "Genetic Prediction of Male Pattern Baldness." *PLoS Genetics* 13 (2). https://doi.org/10.1371/journal.pgen.1006594.

Hallast, Pille, Janna Saarela, Aarno Palotie, and Maris Laan. 2008. "High Divergence in Primate-Specific Duplicated Regions: Human and Chimpanzee Chorionic Gonadotropin Beta Genes." *BMC Evolutionary Biology* 8 (1). https://doi.org/10.1186/1471-2148-8-195.

Hamra, Ghassan B., Kristen Lyall, Gayle C. Windham, Antonia M. Calafat, Andreas Sjödin, Heather Volk, and Lisa A. Croen. 2019. "Prenatal Exposure to Endocrine-Disrupting Chemicals in Relation to Autism Spectrum Disorder and Intellectual Disability." *Epidemiology*. https://doi.org/10.1097/EDE.0000000000000983.

Harris, Paul A., Robert Taylor, Brenda L. Minor, Veida Elliott, Michelle Fernandez, Lindsay O'Neal, Laura McLeod, et al. 2019. "The REDCap Consortium: Building an International Community of Software Platform Partners." *Journal of Biomedical Informatics*. https://doi.org/10.1016/j.jbi.2019.103208.

Harris, Paul A., Robert Taylor, Robert Thielke, Jonathon Payne, Nathaniel Gonzalez, and Jose G. Conde. 2009. "A Metadata-Driven Methodology and Workflow Process for Providing Translational Research Informatics Support, J Biomed Inform." *J Biomed Inform*.

Hazlett, H C, H Gu, B C Munsell, S H Kim, M Styner, J J Wolff, J T Elison, et al. 2017. "Early Brain Development in Infants at High Risk for Autism Spectrum Disorder." *Nature* 542 (7641): 348–51. https://doi.org/10.1038/nature21369.

Hazlett, Heather Cody, Michele D Poe, Guido Gerig, Martin Styner, Chad Chappell, Rachel Gimpel Smith, Clement Vachet, and Joseph Piven. 2011. "Early Brain Overgrowth in Autism Associated with an Increase in Cortical Surface Area before Age 2 Years." *Archives of General Psychiatry* 68 (5): 467–76. https://doi.org/10.1001/archgenpsychiatry.2011.39.

Hecht, Patrick M., Melissa Hudson, Susan L. Connors, Michael R. Tilley, Xudong Liu, and David Q. Beversdorf. 2016. "Maternal Serotonin Transporter Genotype Affects Risk for ASD with Exposure to Prenatal Stress." *Autism Research*. https://doi.org/10.1002/aur.1629.

Heilmann-Heimbach, Stefanie, Christine Herold, Lara M. Hochfeld, Axel M. Hillmer, Dale R. Nyholt, Julian Hecker, Asif Javed, et al. 2017. "Meta-Analysis Identifies Novel Risk Loci and Yields Systematic Insights into the Biology of Male-Pattern Baldness." *Nature Communications* 8. https://doi.org/10.1038/ncomms14694.

Hensch, T K. 2005. "Critical Period Plasticity in Local Cortical Circuits." *Nat Rev Neurosci* 6 (11): 877–88. https://doi.org/10.1038/nrn1787.

Herman-Giddens, Marcia E., Jennifer Steffes, Donna Harris, Eric Slora, Michael Hussey, Steven A. Dowshen, Richard Wasserman, Janet R. Serwint, Lynn Smitherman, and Edward O. Reiter. 2012. "Secondary Sexual Characteristics in Boys: Data from the Pediatric Research in Office Settings Network." *Pediatrics* 130 (5). https://doi.org/10.1542/peds.2011-3291.

Herting, Megan M, Emily C Maxwell, Christy Irvine, and Bonnie J Nagel. 2012. "The Impact of Sex, Puberty, and Hormones on White Matter Microstructure in Adolescents." *Cerebral Cortex* 22 (9): 1979–92. https://doi.org/10.1093/cercor/bhr246.

Hines, M, M Constantinescu, and D Spencer. 2015. "Early Androgen Exposure and Human Gender Development." *Biol Sex Differ* 6: 3. https://doi.org/10.1186/s13293-015-0022-1.

Hirvikoski, Tatja, Ellenor Mittendorfer-Rutz, Marcus Boman, Henrik Larsson, Paul Lichtenstein, and Sven Bölte. 2016. "Premature Mortality in Autism Spectrum Disorder." *British Journal of Psychiatry* 208 (3): 232–38. https://doi.org/10.1192/bjp.bp.114.160192.

Hoffman, J. F., C. L. Wright, and M. M. McCarthy. 2016a. "A Critical Period in Purkinje Cell Development Is Mediated by Local Estradiol Synthesis, Disrupted by Inflammation, and Has Enduring Consequences Only for Males." *Journal of Neuroscience* 36 (39): 10039–49. https://doi.org/10.1523/JNEUROSCI.1262-16.2016.

Hogeveen, Kevin N., Patrice Cousin, Michel Pugeat, Didier Dewailly, Benoît Soudan, and Geoffrey L. Hammond. 2002. "Human Sex Hormone-Binding Globulin Variants Associated with Hyperandrogenism and Ovarian Dysfunction." *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI0214060.

Holingue, Calliope, Carol Newill, Li Ching Lee, Pankaj J. Pasricha, and M. Daniele Fallin. 2018. "Gastrointestinal Symptoms in Autism Spectrum Disorder: A Review of the Literature on Ascertainment and Prevalence." *Autism Research*. https://doi.org/10.1002/aur.1854.

Holland, Dominic, Linda Chang, Thomas M. Ernst, Megan Curran, Steven D. Buchthal, Daniel Alicata, Jon Skranes, et al. 2014. "Structural Growth Trajectories and Rates of Change in the First 3 Months of Infant Brain Development." *JAMA Neurology* 71 (10): 1266–74. https://doi.org/10.1001/JAMANEUROL.2014.1638.

Hong, David S., and Allan L. Reiss. 2014. "Cognitive and Neurological Aspects of Sex Chromosome Aneuploidies." *The Lancet Neurology*. https://doi.org/10.1016/S1474-4422(13)70302-8.

Hotchkiss, A K, C S Lambright, J S Ostby, L Parks-Saldutti, J G Vandenbergh, and L E Gray Jr. 2007. "Prenatal Testosterone Exposure Permanently Masculinizes Anogenital Distance, Nipple Development, and Reproductive Tract Morphology in Female Sprague-Dawley Rats." *Toxicol Sci* 96 (2): 335–45. https://doi.org/10.1093/toxsci/kfm002.

Howlin, Patricia, and Anna Asgharian. 1999. "The Diagnosis of Autism and Asperger Syndrome: Findings from a Survey of 770 Families." *Developmental Medicine and Child Neurology*. https://doi.org/10.1017/S0012162299001656.

Hu, V W, A Nguyen, K S Kim, M E Steinberg, T Sarachana, M A Scully, S J Soldin, T Luu, and N H Lee. 2009. "Gene Expression Profiling of Lymphoblasts from Autistic and Nonaffected Sib Pairs: Altered Pathways in Neuronal Development and Steroid Biosynthesis." *PLoS One* 4 (6): e5775. https://doi.org/10.1371/journal.pone.0005775.

Hughes, Ieuan A., John D. Davies, Trevor I. Bunch, Vickie Pasterski, Kiki Mastroyannopoulou, and Jane Macdougall. 2012. "Androgen Insensitivity Syndrome." In *The Lancet*. Vol. 380. https://doi.org/10.1016/S0140-6736(12)60071-3.

Huguet, Guillaume, Marion Benabou, and Thomas Bourgeron. 2016. "The Genetics of Autism Spectrum Disorders." *Research and Perspectives in Endocrine Interactions*. https://doi.org/10.1007/978-3-319-27069-2_11.

Hull, Laura, Meng Chuan Lai, Simon Baron-Cohen, Carrie Allison, Paula Smith, K. V. Petrides, and William Mandy. 2020. "Gender Differences in Self-Reported Camouflaging in Autistic and Non-Autistic Adults." *Autism*. https://doi.org/10.1177/1362361319864804.

Huseynov, Alik, Christoph P.E. Zollikofer, Walter Coudyzer, Dominic Gascho, Christian Kellenberger, Ricarda Hinzpeter, and Marcia S.Ponce De León. 2016. "Developmental Evidence for Obstetric Adaptation of the Human Female Pelvis." *Proceedings of the National Academy of Sciences of the United States of America* 113 (19). https://doi.org/10.1073/pnas.1517085113.

Ikeda, Takayuki, Li Sun, Naoki Tsuruoka, Yasuhito Ishigaki, Yasuo Yoshitomi, Yoshino Yoshitake, and Hideto Yonekura. 2011. "Hypoxia Down-Regulates SFlt-1 (SVEGFR-1) Expression in Human Microvascular Endothelial Cells by a Mechanism Involving MRNA Alternative Processing." *Biochemical Journal*. https://doi.org/10.1042/BJ20101490.

Iwase, Shigeki, Emily Brookes, Saurabh Agarwal, Aimee I. Badeaux, Hikaru Ito, Christina N. Vallianatos, Giulio Srubek Tomassy, et al. 2016. "A Mouse Model of X-Linked Intellectual Disability Associated with Impaired Removal of Histone Methylation." *Cell Reports* 14 (5). https://doi.org/10.1016/j.celrep.2015.12.091.

Jacquemont, Sébastien, Bradley P. Coe, Micha Hersch, Michael H. Duyzend, Niklas Krumm, Sven Bergmann, Jacques S. Beckmann, Jill A. Rosenfeld, and Evan E. Eichler. 2014. "A Higher Mutational Burden in Females Supports a 'Female Protective Model' in Neurodevelopmental Disorders." *American Journal of Human Genetics* 94 (3): 415–25. https://doi.org/10.1016/j.ajhg.2014.02.001.

Jamnadass, E S, J A Keelan, L P Hollier, M Hickey, M T Maybery, and A J Whitehouse. 2015. "The Perinatal Androgen to Estrogen Ratio and Autistic-like Traits in the General Population: A Longitudinal Pregnancy Cohort Study." *J Neurodev Disord* 7 (1): 17. https://doi.org/10.1186/s11689-015-9114-9.

Johnstone, Rufus A., John D. Reynolds, and James C. Deutsch. 1996. "Mutual Mate Choice and Sex Differences in Choosiness." *Evolution* 50 (4). https://doi.org/10.1111/j.1558-5646.1996.tb03912.x.

Josephs, K. A., J. L. Whitwell, P. Vemuri, M. L. Senjem, B. F. Boeve, D. S. Knopman, G. E. Smith, R. J. Ivnik, R. C. Petersen, and C. R. Jack. 2008. "The Anatomic Correlate of Prosopagnosia in Semantic Dementia." *Neurology* 71 (20): 1628–33. https://doi.org/10.1212/01.WNL.0000334756.18558.92.

Kaat, Aaron J., Amy M. Shui, Sheila S. Ghods, Cristan A. Farmer, Amy N. Esler, Audrey Thurm, Stelios Georgiades, et al. 2021. "Sex Differences in Scores on Standardized Measures of Autism Symptoms: A Multisite Integrative Data Analysis." *Journal of Child Psychology and Psychiatry and Allied Disciplines* 62 (1). https://doi.org/10.1111/jcpp.13242.

Kaczkurkin, Antonia N., Armin Raznahan, and Theodore D. Satterthwaite. 2019. "Sex

Differences in the Developing Brain: Insights from Multimodal Neuroimaging." *Neuropsychopharmacology*. Neuropsychopharmacology. https://doi.org/10.1038/s41386-018-0111-z.

Kallen, Caleb B. 2004. "Steroid Hormone Synthesis in Pregnancy." *Obstetrics and Gynecology Clinics of North America*. https://doi.org/10.1016/j.ogc.2004.08.009.

Kaltiala-Heino, Riittakerttu, Mauri Marttunen, Päivi Rantanen, and Matti Rimpelä. 2003. "Early Puberty Is Associated with Mental Health Problems in Middle Adolescence." *Social Science and Medicine* 57 (6). https://doi.org/10.1016/S0277-9536(02)00480-X.

Kaludjerovic, Jovana, and Wendy E Ward. 2012. "The Interplay between Estrogen and Fetal Adrenal Cortex." *Journal of Nutrition and Metabolism* 2012: 837901. https://doi.org/10.1155/2012/837901.

Kamat, Amrita, Katherine H. Graves, Margaret E. Smith, James A. Richardson, and Carole R. Mendelson. 1999. "A 500-Bp Region, ≈40 Kb Upstream of the Human CYP19 (Aromatase) Gene, Mediates Placenta-Specific Expression in Transgenic Mice." *Proceedings of the National Academy of Sciences of the United States of America* 96 (8). https://doi.org/10.1073/pnas.96.8.4575.

Katsigianni, Maria, Vasilios Karageorgiou, Irene Lambrinoudaki, and Charalampos Siristatidis. 2019. "Maternal Polycystic Ovarian Syndrome in Autism Spectrum Disorder: A Systematic Review and Meta-Analysis." *Molecular Psychiatry*. https://doi.org/10.1038/s41380-019-0398-0.

Kelava, Iva, Ilaria Chiaradia, Laura Pellegrini, Alex T Kalinka, and Madeline A Lancaster. 2022. "Androgens Increase Excitatory Neurogenic Potential in Human Brain Organoids." *Nature* 602 (7895): 112–16. https://doi.org/10.1038/s41586-021-04330-4.

Kern, J K, D A Geier, J B Adams, M R Troutman, G A Davis, P G King, and M R Geier. 2013. "Handgrip Strength in Autism Spectrum Disorder Compared with Controls." *J Strength Cond Res* 27 (8): 2277–81. https://doi.org/10.1519/JSC.0b013e31827de068.

Keverne, E. B. 2013. "Importance of the Matriline for Genomic Imprinting, Brain Development and Behaviour." *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2011.0327.

Keverne, Eric B. 2015. "Genomic Imprinting, Action, and Interaction of Maternal and Fetal Genomes." *Proceedings of the National Academy of Sciences of the United States of America* 112 (22). https://doi.org/10.1073/pnas.1411253111.

Kim, Peter S., James E. Coxworth, and Kristen Hawkes. 2012. "Increased Longevity Evolves from Grandmothering." *Proceedings of the Royal Society B: Biological Sciences* 279 (1749). https://doi.org/10.1098/rspb.2012.1751.

Kinney, Dennis K., Andrea M. Miller, David J. Crowley, Emerald Huang, and Erika Gerber. 2008. "Autism Prevalence Following Prenatal Exposure to Hurricanes and Tropical Storms in Louisiana." *Journal of Autism and Developmental Disorders*. https://doi.org/10.1007/s10803-007-0414-0.

Klei, Lambertus, Stephan J. Sanders, Michael T. Murtha, Vanessa Hus, Jennifer K. Lowe, A. Jeremy Willsey, Daniel Moreno-De-Luca, et al. 2012. "Common Genetic Variants, Acting Additively, Are a Major Source of Risk for Autism." *Molecular Autism* 3 (1). https://doi.org/10.1186/2040-2392-3-9.

Kline, Antonie D., Joanna F. Moss, Angelo Selicorni, Anne Marie Bisgaard, Matthew A. Deardorff, Peter M. Gillett, Stacey L. Ishman, et al. 2018. "Diagnosis and Management of Cornelia de Lange Syndrome: First International Consensus Statement." *Nature Reviews Genetics*. https://doi.org/10.1038/s41576-018-0031-0.

Knickmeyer, R, S Baron-Cohen, B A Fane, S Wheelwright, G A Mathews, G S Conway, C G Brook, and M Hines. 2006. "Androgens and Autistic Traits: A Study of Individuals with Congenital Adrenal Hyperplasia." *Horm Behav* 50 (1): 148–53. https://doi.org/10.1016/j.yhbeh.2006.02.006.

Knickmeyer, R, S Baron-Cohen, P Raggatt, and K Taylor. 2005. "Foetal Testosterone, Social Relationships, and Restricted Interests in Children." *J Child Psychol Psychiatry* 46 (2): 198–210. https://doi.org/10.1111/j.1469-7610.2004.00349.x.

Knickmeyer, Rebecca, Simon Baron-Cohen, Peter Raggatt, Kevin Taylor, and Gerald Hackett. 2006. "Fetal Testosterone and Empathy." *Hormones and Behavior* 49 (3): 282–92. https://doi.org/10.1016/j.yhbeh.2005.08.010.

Konkle, Anne T M, and Margaret M McCarthy. 2011. "Developmental Time Course of Estradiol, Testosterone, and Dihydrotestosterone Levels in Discrete Regions of Male and Female Rat Brain." *Endocrinology* 152 (1): 223–35. https://doi.org/10.1210/en.2010-0607.

Kooijman, M N, C J Kruithof, C M van Duijn, L Duijts, O H Franco, IJzendoorn M H van, J C de Jongste, et al. 2016. "The Generation R Study: Design and Cohort Update 2017." *Eur J Epidemiol* 31 (12): 1243–64. https://doi.org/10.1007/s10654-016-0224-9.

Kooten, Imke A.J. Van, Saskia J.M.C. Palmen, Patricia Von Cappeln, Harry W.M. Steinbusch, Hubert Korr, Helmut Heinsen, Patrick R. Hof, Herman Van Engeland, and Christoph Schmitz. 2008. "Neurons in the Fusiform Gyrus Are Fewer and Smaller in Autism." *Brain* 131 (4): 987–99. https://doi.org/10.1093/brain/awn033.

Kosidou, K, C Dalman, L Widman, S Arver, B K Lee, C Magnusson, and R M Gardner. 2016. "Maternal Polycystic Ovary Syndrome and the Risk of Autism Spectrum Disorders in the Offspring: A Population-Based Nationwide Study in Sweden." *Mol Psychiatry* 21 (10): 1441–48. https://doi.org/10.1038/mp.2015.183.

Kosmicki, Jack A., Kaitlin E. Samocha, Daniel P. Howrigan, Stephan J. Sanders, Kamil Slowikowski, Monkol Lek, Konrad J. Karczewski, et al. 2017. "Refining the Role of de Novo Protein-Truncating Variants in Neurodevelopmental Disorders by Using Population Reference Samples." *Nature Genetics*. https://doi.org/10.1038/ng.3789.

Kung, K T, M Constantinescu, W V Browne, R M Noorderhaven, and M Hines. 2016. "No Relationship between Early Postnatal Testosterone Concentrations and Autistic Traits in 18 to 30-Month-Old Children." *Mol Autism* 7: 15. https://doi.org/10.1186/s13229-016-0078-8.

Kung, K T, D Spencer, V Pasterski, S Neufeld, V Glover, T G O'Connor, P C Hindmarsh, I A Hughes, C L Acerini, and M Hines. 2016. "No Relationship between Prenatal Androgen Exposure and Autistic Traits: Convergent Evidence from Studies of Children with Congenital Adrenal Hyperplasia and of Amniotic Testosterone Concentrations in Typically Developing Children." *J Child Psychol Psychiatry* 57 (12): 1455–62. https://doi.org/10.1111/jcpp.12602.

Kung, Karson T.F., Wendy V. Browne, Mihaela Constantinescu, Rebecca M. Noorderhaven, and Melissa Hines. 2016. "Early Postnatal Testosterone Predicts Sex-Related Differences in Early Expressive Vocabulary." *Psychoneuroendocrinology* 68 (June): 111–16. https://doi.org/10.1016/j.psyneuen.2016.03.001.

Kung, Karson T.F., Ajay Thankamony, Ken K.L. Ong, Carlo L. Acerini, David B. Dunger, Ieuan A. Hughes, and Melissa Hines. 2021. "No Relationship between Prenatal or Early Postnatal Androgen Exposure and Autistic Traits: Evidence Using Anogenital Distance and Penile Length Measurements at Birth and 3 Months of Age." *Journal of Child Psychology and Psychiatry and Allied Disciplines* 62 (7). https://doi.org/10.1111/jcpp.13335.

Kurki, Helen K. 2011. "Pelvic Dimorphism in Relation to Body Size and Body Size Dimorphism in Humans." *Journal of Human Evolution* 61 (6). https://doi.org/10.1016/j.jhevol.2011.07.006.

Kyriakopoulou, Vanessa, Deniz Vatansever, Alice Davidson, Prachi Patkee, Samia Elkommos, Andrew Chew, Miriam Martinez-Biarge, et al. 2017. "Normative Biometry of the Fetal Brain Using Magnetic Resonance Imaging." *Brain Structure & Function* 222 (5): 2295. https://doi.org/10.1007/S00429-016-1342-6.

Lai, M C, M V Lombardo, J Suckling, A N Ruigrok, B Chakrabarti, C Ecker, S C Deoni, et al. 2013. "Biological Sex Affects the Neurobiology of Autism." *Brain* 136 (Pt 9): 2799–2815. https://doi.org/10.1093/brain/awt216.

Lai, Meng-Chuan, and Peter Szatmari. 2020. "Sex and Gender Impacts on the Behavioural Presentation and Recognition of Autism." *Current Opinion in Psychiatry* 33 (2): 117–23. https://doi.org/10.1097/YCO.0000000000000575.

Lai, Meng Chuan, Evdokia Anagnostou, Max Wiznitzer, Carrie Allison, and Simon Baron-Cohen. 2020a. "Evidence-Based Support for Autistic People across the Lifespan: Maximising Potential, Minimising Barriers, and Optimising the Person–Environment Fit." *The Lancet Neurology*. https://doi.org/10.1016/S1474-4422(20)30034-X.

Lai, Meng Chuan, Caroline Kassee, Richard Besney, Sarah Bonato, Laura Hull, William Mandy, Peter Szatmari, and Stephanie H. Ameis. 2019. "Prevalence of Co-Occurring Mental Health Diagnoses in the Autism Population: A Systematic Review and Meta-Analysis." *The Lancet Psychiatry* 6 (10). https://doi.org/10.1016/S2215-0366(19)30289-5.

Lamminmäki, Annamarja, Melissa Hines, Tanja Kuiri-Hänninen, Leena Kilpeläinen, Leo Dunkel, and Ulla Sankilampi. 2012. "Testosterone Measured in Infancy Predicts Subsequent Sex-Typed Behavior in Boys and in Girls." *Hormones and Behavior* 61 (4): 611–16. https://doi.org/10.1016/J.YHBEH.2012.02.013.

Lauritsen, Marlene B., Meta Jørgensen, Kreesten M. Madsen, Sanne Lemcke, Susanne Toft, Jakob Grove, Diana E. Schendel, and Poul Thorsen. 2010. "Validity of Childhood Autism in the Danish Psychiatric Central Register: Findings from a Cohort Sample Born 1990-1999." *Journal of Autism and Developmental Disorders* 40 (2): 139–48. https://doi.org/10.1007/s10803-009-0818-0.

Lee BK Karlsson H, DeVilbiss A, Newschaffer DRC, McGrath J, Eyles D, Magnusson C, Dalman C. 2018. "Prenatal Nutrition and Risk of Child ASD: Findings and Updates from the Stockholm Youth Cohort." *International Meeting for Autism Research (INSAR)*. de Doelen ICC, Rotterdam, Netherlands.

Lee, Eunee, Jiseok Lee, and Eunjoon Kim. 2017. "Excitation/Inhibition Imbalance in Animal Models of Autism Spectrum Disorders." *Biological Psychiatry*. https://doi.org/10.1016/j.biopsych.2016.05.011.

Lee, Joshua K., Derek S. Andrews, Sally Ozonoff, Marjorie Solomon, Sally Rogers, David G. Amaral, and Christine Wu Nordahl. 2021. "Longitudinal Evaluation of Cerebral Growth Across Childhood in Boys and Girls With Autism Spectrum Disorder." *Biological Psychiatry* 90 (5): 286–94. https://doi.org/10.1016/J.BIOPSYCH.2020.10.014.

Lehtola, S. J., J. J. Tuulari, L. Karlsson, R. Parkkola, H. Merisaari, J. Saunavaara, T. Lähdesmäki, N. M. Scheinin, and H. Karlsson. 2019. "Associations of Age and Sex with Brain Volumes and Asymmetry in 2–5-Week-Old Infants." *Brain Structure & Function* 224 (1): 501. https://doi.org/10.1007/S00429-018-1787-X.

Lester, Barry M., and Carmen J. Marsit. 2018. "Epigenetic Mechanisms in the Placenta Related to Infant Neurodevelopment." *Epigenomics*. https://doi.org/10.2217/epi-2016-0171.

Li, J, M Shi, Z Ma, S Zhao, G Euskirchen, J Ziskin, A Urban, J Hallmayer, and M Snyder. 2014. "Integrated Systems Analysis Reveals a Molecular Network Underlying Autism Spectrum Disorders." *Mol Syst Biol* 10: 774. https://doi.org/10.15252/msb.20145487.

Li, Rui, Felix F. Brockschmidt, Amy K. Kiefer, Hreinn Stefansson, Dale R. Nyholt, Kijoung Song, Sita H. Vermeulen, et al. 2012. "Six Novel Susceptibility Loci for Early-Onset Androgenetic Alopecia and Their Unexpected Association with Common Diseases." *PLoS Genetics* 8 (5). https://doi.org/10.1371/journal.pgen.1002746.

Lisse, Thomas S., Martin Hewison, and John S. Adams. 2011. "Hormone Response Element Binding Proteins: Novel Regulators of Vitamin D and Estrogen Signaling." *Steroids*. https://doi.org/10.1016/j.steroids.2011.01.002.

Little, Julian. 2009. "Invited Commentary: Maternal Effects in Preterm Birth-Effects of Maternal Genotype, Mitochondrial Dna, Imprinting, or Environment?" *American Journal of Epidemiology*. https://doi.org/10.1093/aje/kwp326.

Lockwood, Charles J. 2004. "The Initiation of Parturition at Term." *Obstetrics and Gynecology Clinics of North America*. https://doi.org/10.1016/j.ogc.2004.08.012.

Logan, Karen M., Chris Gale, Matthew J. Hyde, Shalini Santhakumaran, and Neena Modi. 2017. "Diabetes in Pregnancy and Infant Adiposity: Systematic Review and Meta-Analysis." *Archives of Disease in Childhood: Fetal and Neonatal Edition* 102 (1).

https://doi.org/10.1136/archdischild-2015-309750.

Long, Manhai, Mandana Ghisari, Lisbeth Kjeldsen, Maria Wielsøe, Bent Nørgaard-Pedersen, Erik Lykke Mortensen, Morsi W. Abdallah, and Eva C. Bonefeld-Jørgensen. 2019. "Autism Spectrum Disorders, Endocrine Disrupting Compounds, and Heavy Metals in Amniotic Fluid: A Case-Control Study." *Molecular Autism*. https://doi.org/10.1186/s13229-018-0253-1.

Loomes, R, L Hull, and W P L Mandy. 2017. "What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis." *J Am Acad Child Adolesc Psychiatry* 56 (6): 466–74. https://doi.org/10.1016/j.jaac.2017.03.013.

Lotze, Martin, Martin Domin, Florian H. Gerlach, Christian Gaser, Eileen Lueders, Carsten O. Schmidt, and Nicola Neumann. 2019. "Novel Findings from 2,838 Adult Brains on Sex Differences in Gray Matter Brain Volume." *Scientific Reports 2019 9:1* 9 (1): 1–7. https://doi.org/10.1038/s41598-018-38239-2.

Lowin, Torsten, Claudia Weidler, Zsuzsa Jenei-Lanzl, Silvia Capellino, Christoph G.O. Baerwald, Frank Buttgereit, and Rainer H. Straub. 2012. "Relationship between Placenta Growth Factor 1 and Vascularization, Dehydroepiandrosterone Sulfate to Dehydroepiandrosterone Conversion, or Aromatase Expression in Patients with Rheumatoid Arthritis and Patients with Osteoarthritis." *Arthritis and Rheumatism*. https://doi.org/10.1002/art.34338.

Lu, Shi-Fang, Qianxing Mo, Shan Hu, Carrie Garippa, and Neal G. Simon. 2003. "Dehydroepiandrosterone Upregulates Neural Androgen Receptor Level and Transcriptional Activity." *Journal of Neurobiology* 57 (2): 163–71. https://doi.org/10.1002/neu.10260.

Lutchmaya, S, S Baron-Cohen, and P Raggatt. 2002. "Foetal Testosterone and Eye Contact in 12-Month-Old Human Infants." *Infant Behavior & Development* 25 (3): 327–35. https://doi.org/Pii S0163-6383(02)00094-2Doi 10.1016/S0163-6383(02)00094-2.

Lutchmaya, Svetlana, Simon Baron-Cohen, and Peter Raggatt. 2001. "Foetal Testosterone and Vocabulary Size in 18- and 24-Month-Old Infants." *Infant Behavior and Development*. https://doi.org/10.1016/S0163-6383(02)00087-5.

Lyall, Amanda E., Feng Shi, Xiujuan Geng, Sandra Woolson, Gang Li, Li Wang, Robert M. Hamer, Dinggang Shen, and John H. Gilmore. 2015. "Dynamic Development of Regional Cortical Thickness and Surface Area in Early Childhood." *Cerebral Cortex* 25 (8). https://doi.org/10.1093/cercor/bhu027.

Lyall, K, D L Pauls, S L Santangelo, D Spiegelman, and A Ascherio. 2011. "Maternal Early Life Factors Associated with Hormone Levels and the Risk of Having a Child with an Autism Spectrum Disorder in the Nurses Health Study II." *J Autism Dev Disord* 41 (5): 618–27. https://doi.org/10.1007/s10803-010-1079-7.

Lyall, Kristen, Xuejuan Ning, Judy L Aschner, Lyndsay A Avalos, Deborah H Bennett, Deborah A Bilder, Nicole R Bush, et al. 2022. "Cardiometabolic Pregnancy Complications in Association with Autism-Related Traits as Measured by the Social Responsiveness Scale in ECHO." *American Journal of Epidemiology*, March, kwac061. https://doi.org/10.1093/aje/kwac061.

Lyall, Kristen, David L Pauls, Donna Spiegelman, Alberto Ascherio, and Susan L Santangelo. 2012. "Pregnancy Complications and Obstetric Suboptimality in Association with Autism Spectrum Disorders in Children of the Nurses' Health Study II." *Autism Research* 5 (1): 21–30. https://doi.org/10.1002/aur.228.

Mackay, D F, G C Smith, S A Cooper, R Wood, A King, D N Clark, and J P Pell. 2016. "Month of Conception and Learning Disabilities: A Record-Linkage Study of 801,592 Children." *Am J Epidemiol* 184 (7): 485–93. https://doi.org/10.1093/aje/kww096.

MacLusky, N J, and F Naftolin. 1981. "Sexual Differentiation of the Central Nervous System." *Science* 211 (4488): 1294–1302.

Madigan, Sheri, Dillon Browne, Nicole Racine, Camille Mori, and Suzanne Tough. 2019. "Association between Screen Time and Children's Performance on a Developmental Screening Test." *JAMA Pediatrics* 173 (3). https://doi.org/10.1001/jamapediatrics.2018.5056.

Maher, Gillian M., Gerard W. O'Keeffe, Christina Dalman, Patricia M. Kearney, Fergus P. McCarthy, Louise C. Kenny, and Ali S. Khashan. 2020. "Association between Preeclampsia and Autism Spectrum Disorder: A Population-Based Study." *Journal of Child Psychology and Psychiatry and Allied Disciplines*. https://doi.org/10.1111/jcpp.13127.

Maher, Gillian M., Gerard W. O'Keeffe, Patricia M. Kearney, Louise C. Kenny, Timothy G. Dinan, Molly Mattsson, and Ali S. Khashan. 2018. "Association of Hypertensive Disorders of Pregnancy With Risk of Neurodevelopmental Disorders in Offspring." *JAMA Psychiatry* 75 (8): 809. https://doi.org/10.1001/jamapsychiatry.2018.0854.

Majewska, Maria Dorota, Martin Hill, Ewa Urbanowicz, Paulina Rok-Bujko, Przemysław Bieńkowski, Irena Namysłowska, and Paweł Mierzejewski. 2014. "Marked Elevation of Adrenal Steroids, Especially Androgens, in Saliva of Prepubertal Autistic Children." *European Child and Adolescent Psychiatry*. https://doi.org/10.1007/s00787-013-0472-0.

Makieva, S, P T Saunders, and J E Norman. 2014. "Androgens in Pregnancy: Roles in Parturition." *Hum Reprod Update* 20 (4): 542–59. https://doi.org/10.1093/humupd/dmu008.

Maliqueo, M., G. Cruz, C. Espina, I. Contreras, M. García, B. Echiburú, and N. Crisosto. 2017. "Obesity during Pregnancy Affects Sex Steroid Concentrations Depending on Fetal Gender." *International Journal of Obesity*. https://doi.org/10.1038/ijo.2017.159.

Maliqueo, Manuel, Hernán E. Lara, Fernando Sánchez, Bárbara Echiburú, Nicolás Crisosto, and Teresa Sir-Petermann. 2013. "Placental Steroidogenesis in Pregnant Women with Polycystic Ovary Syndrome." *European Journal of Obstetrics and Gynecology and Reproductive Biology*. https://doi.org/10.1016/j.ejogrb.2012.10.015.

Mallard, Travis T., Siyuan Liu, Jakob Seidlitz, Zhiwei Ma, Dustin Moraczewski, Adam Thomas, and Armin Raznahan. 2021. "X-Chromosome Influences on Neuroanatomical Variation in Humans." *Nature Neuroscience* 24 (9).

https://doi.org/10.1038/s41593-021-00890-w.

Manolio, Teri A., Francis S. Collins, Nancy J. Cox, David B. Goldstein, Lucia A. Hindorff, David J. Hunter, Mark I. McCarthy, et al. 2009. "Finding the Missing Heritability of Complex Diseases." *Nature*. https://doi.org/10.1038/nature08494.

Martin, Robert D. 2003. "Human Reproduction: A Comparative Background for Medical Hypotheses." In *Journal of Reproductive Immunology*. Vol. 59. https://doi.org/10.1016/S0165-0378(03)00042-1.

Matagne, V., G. Rasier, M. C. Lebrethon, A. Gérard, and J. P. Bourguignon. 2004. "Estradiol Stimulation of Pulsatile Gonadotropin-Releasing Hormone Secretion in Vitro: Correlation with Perinatal Exposure to Sex Steroids and Induction of Sexual Precocity in Vivo." *Endocrinology* 145 (6). https://doi.org/10.1210/en.2003-1259.

May, Tamara, Karen Lee Jing Yi, Kate L. Loveland, Beverley Vollenhoven, and Katrina Williams. 2021. "Overlap of Autism and Conditions Associated with Atypical Sex Hormone Levels or Response: A Systematic Review and Meta-Analysis." *Research in Autism Spectrum Disorders*. https://doi.org/10.1016/j.rasd.2020.101693.

McCarthy, M. M., L. C. Kaufman, P. J. Brooks, D. W. Pfaff, and S. Schwartz-Giblin. 1995. "Estrogen Modulation of MRNA Levels for the Two Forms of Glutamic Acid Decarboxylase (GAD) in Female Rat Brain." *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.903600412.

McCarthy, Margaret M. 2009. "The Two Faces of Estradiol: Effects on the Developing Brain." *Neuroscientist*. https://doi.org/10.1177/1073858409340924.

McCarthy, Margaret M., and Christopher L. Wright. 2017. "Convergence of Sex Differences and the Neuroimmune System in Autism Spectrum Disorder." *Biological Psychiatry*. https://doi.org/10.1016/j.biopsych.2016.10.004.

McCarthy, Margaret M. 2008. "Estradiol and the Developing Brain." *Physiological Reviews* 88: 91–134. https://doi.org/10.1152/physrev.00010.2007.

McKenna, Brooke G., Yongchao Huang, Kévin Vervier, Dabney Hofammann, Mary Cafferata, Seima Al-Momani, Florencia Lowenthal, et al. 2021. "Genetic and Morphological Estimates of Androgen Exposure Predict Social Deficits in Multiple Neurodevelopmental Disorder Cohorts." *Molecular Autism* 12 (1): 43. https://doi.org/10.1186/s13229-021-00450-w.

McLaughlin, Kelsey, John W. Snelgrove, Melanie C. Audette, Atif Syed, Sebastian R. Hobson, Rory C. Windrim, Nir Melamed, Sergio Carmona, and John C. Kingdom. 2021. "PlGF (Placental Growth Factor) Testing in Clinical Practice: Evidence from a Canadian Tertiary Maternity Referral Center." *Hypertension* 77 (6): 2057–65. https://doi.org/10.1161/HYPERTENSIONAHA.121.17047.

Mendle, Jane, K. Paige Harden, Jeanne Brooks-Gunn, and Julia A. Graber. 2010. "Development's Tortoise and Hare: Pubertal Timing, Pubertal Tempo, and Depressive Symptoms in Boys and Girls." *Developmental Psychology* 46 (5). https://doi.org/10.1037/a0020205.

Mitra, Ileena, Kathryn Tsang, Christine Ladd-Acosta, Lisa A. Croen, Kimberly A. Aldinger, Robert L. Hendren, Michela Traglia, et al. 2016. "Pleiotropic Mechanisms Indicated

for Sex Differences in Autism." Edited by Jonathan Flint. *PLoS Genetics* 12 (11): e1006425. https://doi.org/10.1371/journal.pgen.1006425.

Mong, J. A., J. L. Nuñez, and M. M. McCarthy. 2002. "GABA Mediates Steroid-Induced Astrocyte Differentiation in the Neonatal Rat Hypothalamus." *Journal of Neuroendocrinology*. https://doi.org/10.1046/j.1365-2826.2002.00737.x.

Moore, Gaea Schwaebe, Anna Weber Kneitel, Cheryl K Walker, William M Gilbert, and Guibo Xing. 2012. "Autism Risk in Small- and Large-for-Gestational-Age Infants." *American Journal of Obstetrics and Gynecology* 206 (4): 314.e1-9. https://doi.org/10.1016/j.ajog.2012.01.044.

Mottron, Laurent, and Danilo Bzdok. 2020. "Autism Spectrum Heterogeneity: Fact or Artifact?" *Molecular Psychiatry*. https://doi.org/10.1038/s41380-020-0748-y.

Mukherjee, J, R A Cardarelli, Y Cantaut-Belarif, T Z Deeb, D P Srivastava, S K Tyagarajan, M N Pangalos, et al. 2017. "Estradiol Modulates the Efficacy of Synaptic Inhibition by Decreasing the Dwell Time of GABAA Receptors at Inhibitory Synapses." *Proc Natl Acad Sci U S A* 114 (44): 11763–68. https://doi.org/10.1073/pnas.1705075114.

Muller, C L, A M J Anacker, and J Veenstra-VanderWeele. 2016. "The Serotonin System in Autism Spectrum Disorder: From Biomarker to Animal Models." *Neuroscience* 321: 24–41. https://doi.org/10.1016/j.neuroscience.2015.11.010.

Murji, A, L K Proctor, A D Paterson, D Chitayat, R Weksberg, and J Kingdom. 2012. "Male Sex Bias in Placental Dysfunction." *Am J Med Genet A* 158A (4): 779–83. https://doi.org/10.1002/ajmg.a.35250.

Murphy, Vanessa E., Roger Smith, Warwick B. Giles, and Vicki L. Clifton. 2006. "Endocrine Regulation of Human Fetal Growth: The Role of the Mother, Placenta, and Fetus." *Endocrine Reviews*. https://doi.org/10.1210/er.2005-0011.

Nakamura, Katsuki, Ryuta Kawashima, Motoaki Sugiura, Takashi Kato, Akinori Nakamura, Kentaro Hatano, Sumiharu Nagumo, et al. 2001. "Neural Substrates for Recognition of Familiar Voices: A PET Study." *Neuropsychologia* 39 (10): 1047–54. https://doi.org/10.1016/S0028-3932(01)00037-9.

National Institute for Health and Care Excellence. 2012. "Autism Spectrum Disorder in Adults: Diagnosis and Management | Guidance and Guidelines | NICE." *Nice Clinical Guideline*, no. June 2012.

Nava, C., F. Lamari, D. Héron, C. Mignot, A. Rastetter, B. Keren, D. Cohen, et al. 2012. "Analysis of the Chromosome X Exome in Patients with Autism Spectrum Disorders Identified Novel Candidate Genes, Including TMLHE." *Translational Psychiatry* 2. https://doi.org/10.1038/tp.2012.102.

Nebel, Rebecca A., Jill Kirschen, Jinlu Cai, Young Jae Woo, Koshi Cherian, and Brett S. Abrahams. 2015. "Reciprocal Relationship between Head Size, an Autism Endophenotype, and Gene Dosage at 19p13.12 Points to AKAP8 and AKAP8L." *PLOS ONE* 10 (6): e0129270. https://doi.org/10.1371/JOURNAL.PONE.0129270.

Nelson, Emma, and Susanne Shultz. 2010. "Finger Length Ratios (2D:4D) in Anthropoids Implicate Reduced Prenatal Androgens in Social Bonding." *American Journal of*

Physical Anthropology 141 (3). https://doi.org/10.1002/ajpa.21157.

Neumeyer, A M, A Gates, C Ferrone, H Lee, and M Misra. 2013. "Bone Density in Peripubertal Boys with Autism Spectrum Disorders." *J Autism Dev Disord* 43 (7): 1623–29. https://doi.org/10.1007/s10803-012-1709-3.

Nguyen, Kim Di, and Christine M. Disteche. 2006. "Dosage Compensation of the Active X Chromosome in Mammals." *Nature Genetics* 38 (1): 47–53. https://doi.org/10.1038/ng1705.

Noriega, Nigel C., Dominique H. Eghlidi, Vasilios T. Garyfallou, Steven G. Kohama, Sharon G. Kryger, and Henryk F. Urbanski. 2010. "Influence of 17β-Estradiol and Progesterone on GABAergic Gene Expression in the Arcuate Nucleus, Amygdala and Hippocampus of the Rhesus Macaque." *Brain Research*. https://doi.org/10.1016/j.brainres.2009.10.011.

Nugent, Bridget M., and Tracy L. Bale. 2015. "The Omniscient Placenta: Metabolic and Epigenetic Regulation of Fetal Programming." *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2015.09.001.

Nunes, Adonay S., Vasily A. Vakorin, Nataliia Kozhemiako, Nicholas Peatfield, Urs Ribary, and Sam M. Doesburg. 2020. "Atypical Age-Related Changes in Cortical Thickness in Autism Spectrum Disorder." *Scientific Reports*. https://doi.org/10.1038/s41598-020-67507-3.

Nunez, Joseph L., Graham W. Aberdeen, Eugene D. Albrecht, and Margaret M McCarthy. 2008. "Impact of Estradiol on Gamma-Aminobutyric Acid- and Glutamate-Mediated Calcium Responses of Fetal Baboon (Papio Anubis) Hippocampal and Cortical Neurons." *Endocrinology* 149 (12): 6433–43. https://doi.org/10.1210/en.2007-1720.

O'Neill, Joseph, Ravi Bansal, Suzanne Goh, Martina Rodie, Siddhant Sawardekar, and Bradley S. Peterson. 2019. "Parsing the Heterogeneity of Brain Metabolic Disturbances in Autism Spectrum Disorder." *Biological Psychiatry*. https://doi.org/10.1016/j.biopsych.2019.06.010.

Ohlsson Gotby, Vide, Olle Söder, Louise Frisén, Eva Serlachius, Sven Bölte, Catarina Almqvist, Henrik Larsson, Paul Lichtenstein, and Kristiina Tammimies. 2019. "Hypogonadotrophic Hypogonadism, Delayed Puberty and Risk for Neurodevelopmental Disorders." *Journal of Neuroendocrinology* 31 (11). https://doi.org/10.1111/jne.12803.

Olson, Ingrid R., Alan Plotzker, and Youssef Ezzyat. 2007. "The Enigmatic Temporal Pole: A Review of Findings on Social and Emotional Processing." *Brain*. Brain. https://doi.org/10.1093/brain/awm052.

Orzack, Steven Hecht, J. William Stubblefield, Viatcheslav R. Akmaev, Pere Colls, Santiago Munné, Thomas Scholl, David Steinsaltz, and James E. Zuckerman. 2015. "The Human Sex Ratio from Conception to Birth." *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1416546112.

Pagalan, Lief, Celeste Bickford, Whitney Weikum, Bruce Lanphear, Michael Brauer, Nancy Lanphear, Gillian E. Hanley, Tim F. Oberlander, and Meghan Winters. 2019.

"Association of Prenatal Exposure to Air Pollution with Autism Spectrum Disorder." *JAMA Pediatrics*. https://doi.org/10.1001/jamapediatrics.2018.3101.

Panizzon, Matthew S., Christine Fennema-Notestine, Lisa T. Eyler, Terry L. Jernigan, Elizabeth Prom-Wormley, Michael Neale, Kristen Jacobson, et al. 2009. "Distinct Genetic Influences on Cortical Surface Area and Cortical Thickness." *Cerebral Cortex* 19 (11). https://doi.org/10.1093/cercor/bhp026.

Park, B Y, B K Lee, I Burstyn, L P Tabb, J A Keelan, A J O Whitehouse, L A Croen, et al. 2017. "Umbilical Cord Blood Androgen Levels and ASD-Related Phenotypes at 12 and 36 Months in an Enriched Risk Cohort Study." *Mol Autism* 8: 3. https://doi.org/10.1186/s13229-017-0118-z.

Park, B Y, D P Misra, J Moye, R K Miller, L Croen, M D Fallin, C Walker, C J Newschaffer, C M Salafia, and Consortium National Children's Study. 2018. "Placental Gross Shape Differences in a High Autism Risk Cohort and the General Population." *PLoS One* 13 (8): e0191276. https://doi.org/10.1371/journal.pone.0191276.

Parner, Erik T., Diana E. Schendel, and Poul Thorsen. 2008. "Autism Prevalence Trends over Time in Denmark: Changes in Prevalence and Age at Diagnosis." *Archives of Pediatrics and Adolescent Medicine* 162 (12). https://doi.org/10.1001/archpedi.162.12.1150.

Pasquali, Renato, Laura Zanotti, Flaminia Fanelli, Marco Mezzullo, Alessia Fazzini, Antonio Maria Morselli Labate, Andrea Repaci, Danilo Ribichini, and Alessandra Gambineri. 2016. "Defining Hyperandrogenism in Women with Polycystic Ovary Syndrome: A Challenging Perspective." *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jc.2015-4009.

Patterson, Karalyn, Peter J. Nestor, and Timothy T. Rogers. 2007. "Where Do You Know What You Know? The Representation of Semantic Knowledge in the Human Brain." *Nature Reviews Neuroscience 2007 8:12* 8 (12): 976–87. https://doi.org/10.1038/nrn2277.

Pembroke, William G., Christopher L. Hartl, and Daniel H. Geschwind. 2021. "Evolutionary Conservation and Divergence of the Human Brain Transcriptome." *Genome Biology* 22 (1). https://doi.org/10.1186/s13059-020-02257-z.

Peña, Catherine Jensen, Catherine Monk, and Frances A. Champagne. 2012. "Epigenetic Effects of Prenatal Stress on 11β-Hydroxysteroid Dehydrogenase-2 in the Placenta and Fetal Brain." *PLoS ONE* 7 (6). https://doi.org/10.1371/journal.pone.0039791.

Pereira, Alessandra M., Brunno M. Campos, Ana C. Coan, Luiz F. Pegoraro, Thiago J. R. de Rezende, Ignacio Obeso, Paulo Dalgalarrondo, Jaderson C. da Costa, Jean-Claude Dreher, and Fernando Cendes. 2018. "Differences in Cortical Structure and Functional MRI Connectivity in High Functioning Autism." *Frontiers in Neurology* 0 (JUL): 539. https://doi.org/10.3389/FNEUR.2018.00539.

Perrot-Sinal, T. S., A. P. Auger, and M. M. McCarthy. 2003. "Excitatory Actions of GABA in Developing Brain Are Mediated by L-Type Ca2+ Channels and Dependent on Age, Sex, and Brain Region." *Neuroscience*. https://doi.org/10.1016/S0306-4522(02)00794-7.

Perry, John R.B., Felix Day, Cathy E. Elks, Patrick Sulem, Deborah J. Thompson, Teresa

Ferreira, Chunyan He, et al. 2014. “Parent-of-Origin-Specific Allelic Associations among 106 Genomic Loci for Age at Menarche.” *Nature* 514 (7520). https://doi.org/10.1038/nature13545.

Pertegal, Miriam, Francisco J. Fenoy, Barbara Bonacasa, Jaime Mendiola, Juan L. Delgado, Moises Hernández, Miguel G. Salom, Vicente Bosch, and Isabel Hernández. 2015. “2-Methoxyestradiol Plasma Levels Are Associated with Clinical Severity Indices and Biomarkers of Preeclampsia.” *Reproductive Sciences*. https://doi.org/10.1177/1933719114537716.

Pierce, Karen, and Elizabeth Redcay. 2008. “Fusiform Function in Children with an ASD Is a Matter of ‘Who.’” *Biological Psychiatry* 64 (7): 552. https://doi.org/10.1016/J.BIOPSYCH.2008.05.013.

Pinar, H., C. J. Sung, C. E. Oyer, and D. B. Singer. 1996. “Reference Values for Singleton and Twin Placental Weights.” *Pediatric Pathology and Laboratory Medicine* 16 (6). https://doi.org/10.1080/15513819609168713.

Pinto, Dalila, Elsa Delaby, Daniele Merico, Mafalda Barbosa, Alison Merikangas, Lambertus Klei, Bhooma Thiruvahindrapuram, et al. 2014. “Convergence of Genes and Cellular Pathways Dysregulated in Autism Spectrum Disorders.” *American Journal of Human Genetics* 94 (5): 677–94. https://doi.org/10.1016/j.ajhg.2014.03.018.

Pitteloud, Nelly, Andrew A. Dwyer, Suzzunne DeCruz, Hang Lee, Paul A. Boepple, William F. Crowley, and Frances J. Hayes. 2008. “Inhibition of Luteinizing Hormone Secretion by Testosterone in Men Requires Aromatization for Its Pituitary but Not Its Hypothalamic Effects: Evidence from the Tandem Study of Normal and Gonadotropin-Releasing Hormone-Deficient Men.” *Journal of Clinical Endocrinology and Metabolism*. https://doi.org/10.1210/jc.2007-2156.

Pohl, A, S Cassidy, B Auyeung, and S Baron-Cohen. 2014. “Uncovering Steroidopathy in Women with Autism: A Latent Class Analysis.” *Mol Autism* 5: 27. https://doi.org/10.1186/2040-2392-5-27.

Polimanti, Renato, and Joel Gelernter. 2017. “Widespread Signatures of Positive Selection in Common Risk Alleles Associated to Autism Spectrum Disorder.” *PLoS Genetics* 13 (2). https://doi.org/10.1371/journal.pgen.1006618.

Pote, Inês, Siying Wang, Vaheshta Sethna, Anna Blasi, Eileen Daly, Maria Kuklisova-Murgasova, Sarah Lloyd-Fox, et al. 2019. “Familial Risk of Autism Alters Subcortical and Cerebellar Brain Anatomy in Infants and Predicts the Emergence of Repetitive Behaviors in Early Childhood.” *Autism Research* 12 (4): 614–27. https://doi.org/10.1002/aur.2083.

Power, Michael L., and Jay Schulkin. 2012. *The Evolution of the Human Placenta*. *The Evolution of the Human Placenta*. https://doi.org/10.5860/choice.50-6213.

Puts, N A J, E L Wodka, A D Harris, D Crocetti, M Tommerdahl, S H Mostofsky, and R A E Edden. 2017. “Reduced GABA and Altered Somatosensory Function in Children with Autism Spectrum Disorder.” *Autism Res* 10 (4): 608–19. https://doi.org/10.1002/aur.1691.

Quartier, A, L Chatrousse, C Redin, C Keime, N Haumesser, A Maglott-Roth, L Brino, et al.

2018. “Genes and Pathways Regulated by Androgens in Human Neural Cells, Potential Candidates for the Male Excess in Autism Spectrum Disorder.” *Biol Psychiatry*. https://doi.org/10.1016/j.biopsych.2018.01.002.

Quast, Anja, Volker Hesse, Johannes Hain, Peter Wermke, and Kathleen Wermke. 2016. “Baby Babbling at Five Months Linked to Sex Hormone Levels in Early Infancy.” *Infant Behavior and Development* 44 (August): 1–10. https://doi.org/10.1016/j.infbeh.2016.04.002.

Raz, Aviad E., Yael Amano, and Stefan Timmermans. 2019. “Coming to Terms with the Imperfectly Normal Child: Attitudes of Israeli Parents of Screen-Positive Infants Regarding Subsequent Prenatal Diagnosis.” *Journal of Community Genetics* 10 (1). https://doi.org/10.1007/s12687-018-0361-9.

Raz, Raanan, Andrea L. Roberts, Kristen Lyall, Jaime E. Hart, Allan C. Just, Francine Laden, and Marc G. Weisskopf. 2015. “Autism Spectrum Disorder and Particulate Matter Air Pollution before, during, and after Pregnancy: A Nested Case–Control Analysis within the Nurses’ Health Study II Cohort.” *Environmental Health Perspectives*. https://doi.org/10.1289/ehp.1408133.

Regev, Ohad, Gal Cohen, Amnon Hadar, Jenny Schuster, Hagit Flusser, Analya Michaelovski, Gal Meiri, Ilan Dinstein, Reli Hershkovitch, and Idan Menashe. 2021. “Association Between Abnormal Fetal Head Growth and Autism Spectrum Disorder.” *Journal of the American Academy of Child and Adolescent Psychiatry* 60 (8). https://doi.org/10.1016/j.jaac.2020.11.019.

Reshef Tal Richard O Burney, Stephen B Mooney, Linda C Giudice, Hugh S Taylor. 2015. “Endocrinology of Pregnancy.” In *Endotext*, edited by G Chrousos. South Dartmouth, MA: MDTEXT.COM,INC. https://www.ncbi.nlm.nih.gov/books/NBK278962/.

Risal, Sanjiv, Yu Pei, Haojiang Lu, Maria Manti, Romina Fornes, Han Pin Pui, Zhiyi Zhao, et al. 2019. “Prenatal Androgen Exposure and Transgenerational Susceptibility to Polycystic Ovary Syndrome.” *Nature Medicine*. https://doi.org/10.1038/s41591-019-0666-1.

Roberts, Andrea L., Kristen Lyall, Janet W. Rich-Edwards, Alberto Ascherio, and Marc G. Weisskopf. 2013. “Association of Maternal Exposure to Childhood Abuse with Elevated Risk for Autism in Offspring.” *JAMA Psychiatry*. https://doi.org/10.1001/jamapsychiatry.2013.447.

———. 2016. “Maternal Exposure to Intimate Partner Abuse before Birth Is Associated with Autism Spectrum Disorder in Offspring.” *Autism*. https://doi.org/10.1177/1362361314566049.

Robertson, C E, D J Kravitz, J Freyberg, S Baron-Cohen, and C I Baker. 2013a. “Slower Rate of Binocular Rivalry in Autism.” *J Neurosci* 33 (43): 16983–91. https://doi.org/10.1523/JNEUROSCI.0448-13.2013.

———. 2013b. “Tunnel Vision: Sharper Gradient of Spatial Attention in Autism.” *J Neurosci* 33 (16): 6776–81. https://doi.org/10.1523/JNEUROSCI.5120-12.2013.

Robinson, Elise B., Paul Lichtenstein, Henrik Anckarsäter, Francesca Happé, and Angelica Ronald. 2013. “Examining and Interpreting the Female Protective Effect against Autistic Behavior.” *Proceedings of the National Academy of Sciences of the*

United States of America 110 (13): 5258–62. https://doi.org/10.1073/pnas.1211070110.

Rochira, V, and C Carani. 2009. "Aromatase Deficiency in Men: A Clinical Perspective." *Nat Rev Endocrinol* 5 (10): 559–68. https://doi.org/10.1038/nrendo.2009.176.

Rodprasert, Wiwat, Helena E. Virtanen, Juho Antti Mäkelä, and Jorma Toppari. 2020. "Hypogonadism and Cryptorchidism." *Frontiers in Endocrinology*. https://doi.org/10.3389/fendo.2019.00906.

Roman-Urrestarazu, Andres, Carolina Yáñez, Claudia López-Garí, Constanza Elgueta, Carrie Allison, Carol Brayne, Mónica Troncoso, and Simon Baron-Cohen. 2020. "Autism Screening and Conditional Cash Transfers in Chile: Using the Quantitative Checklist (Q-CHAT) for Early Autism Detection in a Low Resource Setting." *Autism*, December, 136236132097227. https://doi.org/10.1177/1362361320972277.

Romero-Garcia, Rafael, Varun Warrier, Edward T. Bullmore, Simon Baron-Cohen, and Richard A.I. Bethlehem. 2019. "Synaptic and Transcriptionally Downregulated Genes Are Associated with Cortical Thickness Differences in Autism." *Molecular Psychiatry* 24 (7). https://doi.org/10.1038/s41380-018-0023-7.

Rooij, Daan van, Evdokia Anagnostou, Celso Arango, Guillaume Auzias, Marlene Behrmann, Geraldo F. Busatto, Sara Calderoni, et al. 2018. "Cortical and Subcortical Brain Morphometry Differences Between Patients With Autism Spectrum Disorder and Healthy Individuals Across the Lifespan: Results From the ENIGMA ASD Working Group." *The American Journal of Psychiatry* 175 (4): 359. https://doi.org/10.1176/APPI.AJP.2017.17010100.

Rosenfeld, Cheryl S. 2020. "The Placenta-Brain-Axis." *Journal of Neuroscience Research*. https://doi.org/10.1002/jnr.24603.

Rossignol, Daniel, and Richard Frye. 2014. "Melatonin in Autism Spectrum Disorders." *Current Clinical Pharmacology*. https://doi.org/10.2174/15748847113086660072.

Rotem, Ran S., Vy T. Nguyen, Gabriel Chodick, Michael Davidovitch, Varda Shalev, Russ Hauser, Brent A. Coull, Andrea Bellavia, and Marc G. Weisskopf. 2021. "Associations of Maternal Androgen-Related Conditions with Risk of Autism Spectrum Disorder in Progeny and Mediation by Cardiovascular, Metabolic, and Fertility Factors." *American Journal of Epidemiology* 190 (4). https://doi.org/10.1093/aje/kwaa219.

Rubenstein, J L R, and M M Merzenich. 2003. "Model of Autism: Increased Ratio of Excitation/Inhibition in Key Neural Systems." *Genes, Brain and Behavior* 2 (5): 255–67. https://doi.org/10.1046/j.1601-183X.2003.00037.x.

Ruigrok, Amber N.V., Gholamreza Salimi-Khorshidi, Meng Chuan Lai, Simon Baron-Cohen, Michael V. Lombardo, Roger J. Tait, and John Suckling. 2014. "A Meta-Analysis of Sex Differences in Human Brain Structure." *Neuroscience and Biobehavioral Reviews*. https://doi.org/10.1016/j.neubiorev.2013.12.004.

Ruta, L, E Ingudomnukul, K Taylor, B Chakrabarti, and S Baron-Cohen. 2011. "Increased Serum Androstenedione in Adults with Autism Spectrum Conditions." *Psychoneuroendocrinology* 36 (8): 1154–63. https://doi.org/10.1016/j.psyneuen.2011.02.007.

Ruta, Liliana, Flavia Chiarotti, Giuseppe Maurizio Arduino, Fabio Apicella, Elisa Leonardi, Roberta Maggio, Cristina Carrozza, et al. 2019. "Validation of the Quantitative Checklist for Autism in Toddlers in an Italian Clinical Sample of Young Children With Autism and Other Developmental Disorders." *Frontiers in Psychiatry* 10 (JUN): 488. https://doi.org/10.3389/fpsyt.2019.00488.

Ruth, Katherine S., Felix R. Day, Jessica Tyrrell, Deborah J. Thompson, Andrew R. Wood, Anubha Mahajan, Robin N. Beaumont, et al. 2020. "Using Human Genetics to Understand the Disease Impacts of Testosterone in Men and Women." *Nature Medicine*. https://doi.org/10.1038/s41591-020-0751-5.

Rutter, Micheal. 1978. "Diagnosis and Definition of Autism 139." *Journal of Autism and Childhood Schizophrenia*.

Sanchez-Ferrer, M L, J Mendiola, A I Hernandez-Penalver, S Corbalan-Biyang, A Carmona-Barnosi, M T Prieto-Sanchez, A Nieto, and A M Torres-Cantero. 2017. "Presence of Polycystic Ovary Syndrome Is Associated with Longer Anogenital Distance in Adult Mediterranean Women." *Hum Reprod* 32 (11): 2315–23. https://doi.org/10.1093/humrep/dex274.

Santos, Hudson P. Jr, Arjun Bhattacharya, Robert M. Joseph, Lisa Smeester, Karl C. K. Kuban, Carmen J. Marsit, T. Michael O'Shea, and Rebecca C. Fry. 2020. "Evidence for the Placenta-Brain Axis: Multi-Omic Kernel Aggregation Predicts Intellectual and Social Impairment in Children Born Extremely Preterm." *Molecular Autism 2020 11:1* 11 (1): 1–16. https://doi.org/10.1186/S13229-020-00402-W.

Sarachana, T, and V W Hu. 2013. "Genome-Wide Identification of Transcriptional Targets of RORA Reveals Direct Regulation of Multiple Genes Associated with Autism Spectrum Disorder." *Mol Autism* 4 (1): 14. https://doi.org/10.1186/2040-2392-4-14.

Satterstrom, F. Kyle, Jack A. Kosmicki, Jiebiao Wang, Michael S. Breen, Silvia De Rubeis, Joon Yong An, Minshi Peng, et al. 2020. "Large-Scale Exome Sequencing Study Implicates Both Developmental and Functional Changes in the Neurobiology of Autism." *Cell*. https://doi.org/10.1016/j.cell.2019.12.036.

Schaadt, Gesa, Volker Hesse, and Angela D. Friederici. 2015. "Sex Hormones in Early Infancy Seem to Predict Aspects of Later Language Development." *Brain and Language* 141 (February): 70–76. https://doi.org/10.1016/J.BANDL.2014.11.015.

Schaer, Marie, Marie Christine Ottet, Elisa Scariati, Daniel Dukes, Martina Franchini, Stephan Eliez, and Bronwyn Glaser. 2013. "Decreased Frontal Gyrification Correlates with Altered Connectivity in Children with Autism." *Frontiers in Human Neuroscience*, no. NOV. https://doi.org/10.3389/fnhum.2013.00750.

Scherrer, Benoit, Anna K Prohl, Maxime Taquet, Kush Kapur, Jurriaan M Peters, Xavier Tomas-Fernandez, Peter E Davis, et al. 2020. "The Connectivity Fingerprint of the Fusiform Gyrus Captures the Risk of Developing Autism in Infants with Tuberous Sclerosis Complex." *Cerebral Cortex (New York, NY)* 30 (4): 2199. https://doi.org/10.1093/CERCOR/BHZ233.

Schork, Andrew J., Hyejung Won, Vivek Appadurai, Ron Nudel, Mike Gandal, Olivier Delaneau, Malene Revsbech Christiansen, et al. 2019. "A Genome-Wide Association

Study of Shared Risk across Psychiatric Disorders Implicates Gene Regulation during Fetal Neurodevelopment." *Nature Neuroscience* 22 (3). https://doi.org/10.1038/s41593-018-0320-0.

Schreurs, Malou P. H., Emily M. Houston, Victor May, and Marilyn J. Cipolla. 2012. "The Adaptation of the Blood-brain Barrier to Vascular Endothelial Growth Factor and Placental Growth Factor during Pregnancy." *The FASEB Journal*. https://doi.org/10.1096/fj.11-191916.

Schröder, Yvonne, Daniela Michelle Hohmann, Tina Meller, Ulrika Evermann, Julia-Katharina Pfarr, Andreas Jansen, Inge Kamp-Becker, Sarah Grezellschak, and Igor Nenadić. 2021. "Associations of Subclinical Autistic-like Traits with Brain Structural Variation Using Diffusion Tensor Imaging and Voxel-Based Morphometry." *European Psychiatry* 64 (1). https://doi.org/10.1192/j.eurpsy.2021.15.

Schultz, Robert T., Isabel Gauthier, Ami Klin, Robert K. Fulbright, Adam W. Anderson, Fred Volkmar, Pawel Skudlarski, Cheryl Lacadie, Donald J. Cohen, and John C. Gore. 2000. "Abnormal Ventral Temporal Cortical Activity during Face Discrimination among Individuals with Autism and Asperger Syndrome." *Archives of General Psychiatry* 57 (4): 331–40. https://doi.org/10.1001/archpsyc.57.4.331.

Schumann, Cynthia M., Cinnamon S. Bloss, Cynthia Carter Barnes, Graham M. Wideman, Ruth A. Carper, Natacha Akshoomoff, Karen Pierce, et al. 2010. "Longitudinal Magnetic Resonance Imaging Study of Cortical Development through Early Childhood in Autism." *Journal of Neuroscience* 30 (12): 4419–27. https://doi.org/10.1523/JNEUROSCI.5714-09.2010.

Schutz, Chris K., Diana Polley, Paula D. Robinson, Maryse Chalifoux, Fabio Macciardi, Bradley N. White, and Jeanette J.A. Holden. 2002. "Autism and the X Chromosome: No Linkage to Microsatellite Loci Detected Using the Affected Sibling Pair Method." *American Journal of Medical Genetics* 109 (1). https://doi.org/10.1002/ajmg.10138.

Schwarz, Jaclyn M., Shu Ling Liang, Scott M. Thompson, and Margaret M. McCarthy. 2008. "Estradiol Induces Hypothalamic Dendritic Spines by Enhancing Glutamate Release: A Mechanism for Organizational Sex Differences." *Neuron*. https://doi.org/10.1016/j.neuron.2008.03.008.

Scott, Julia A., Piotr A. Habas, Kio Kim, Vidya Rajagopalan, Kia S. Hamzelou, James M. Corbett-Detig, A. James Barkovich, Orit A. Glenn, and Colin Studholme. 2011. "Growth Trajectories of the Human Fetal Brain Tissues Estimated from 3D Reconstructed in Utero MRI." *International Journal of Developmental Neuroscience* 29 (5): 529. https://doi.org/10.1016/J.IJDEVNEU.2011.04.001.

Sebat, Jonathan, B. Lakshmi, Dheeraj Malhotra, Jennifer Troge, Christa Lese-Martin, Tom Walsh, Boris Yamrom, et al. 2007. "Strong Association of de Novo Copy Number Mutations with Autism." *Science* 316 (5823). https://doi.org/10.1126/science.1138659.

Sellers, Katherine J., Matthew C.S. Denley, Atsushi Saito, Evangeline M. Foster, Irene Salgarella, Alessio Delogu, Atsushi Kamiya, and Deepak P. Srivastava. 2020. "Brain-synthesized Estrogens Regulate Cortical Migration in a Sexually Divergent Manner."

European Journal of Neuroscience. https://doi.org/10.1111/ejn.14755.

Sellers, Katherine J., Filippo Erli, Pooja Raval, Iain A. Watson, Ding Chen, and Deepak P. Srivastava. 2015. "Rapid Modulation of Synaptogenesis and Spinogenesis by 17β-Estradiol in Primary Cortical Neurons." *Frontiers in Cellular Neuroscience* 9: 137. https://doi.org/10.3389/fncel.2015.00137.

Shi, Lei, Qiang Lin, and Bing Su. 2015. "Estrogen Regulation of Microcephaly Genes and Evolution of Brain Sexual Dimorphism in Primates." *BMC Evolutionary Biology 2015 15:1* 15 (1): 1–10. https://doi.org/10.1186/S12862-015-0398-X.

Shin, J. S., M. Y. Choi, M. S. Longtine, and D. M. Nelson. 2010. "Vitamin D Effects on Pregnancy and the Placenta." *Placenta*. https://doi.org/10.1016/j.placenta.2010.08.015.

Shpigler, Hagai Y., Michael C. Saul, Frida Corona, Lindsey Block, Amy Cash Ahmed, Sihai D. Zhao, and Gene E. Robinson. 2017. "Deep Evolutionary Conservation of Autism-Related Genes." *Proceedings of the National Academy of Sciences of the United States of America* 114 (36). https://doi.org/10.1073/pnas.1708127114.

Shum, Carole, Sara C. Macedo, Katherine Warre-Cornish, Graham Cocks, Jack Price, and Deepak P. Srivastava. 2015. "Utilizing Induced Pluripotent Stem Cells (IPSCs) to Understand the Actions of Estrogens in Human Neurons." *Hormones and Behavior*. https://doi.org/10.1016/j.yhbeh.2015.06.014.

Simantov, Tslil, Alexa Pohl, Alexandros Tsompanidis, Elizabeth Weir, Michael V Lombardo, Amber Ruigrok, Paula Smith, Carrie Allison, Simon Baron-Cohen, and Florina Uzefovsky. 2021. "Medical Symptoms and Conditions in Autistic Women." *Autism*. https://doi.org/10.1177/13623613211022091.

Simpson, E R, M S Mahendroo, G D Means, M W Kilgore, C J Corbin, and C R Mendelson. 1993. "Tissue-Specific Promoters Regulate Aromatase Cytochrome P450 Expression." *Clinical Chemistry* 39 (2): 317–24. http://www.ncbi.nlm.nih.gov/pubmed/8432022.

Sirianni, Rosa, Khurram S. Rehman, Bruce R. Carr, C. Richard Parker, and William E. Rainey. 2005. "Corticotropin-Releasing Hormone Directly Stimulates Cortisol and the Cortisol Biosynthetic Pathway in Human Fetal Adrenal Cells." *Journal of Clinical Endocrinology and Metabolism* 90 (1). https://doi.org/10.1210/jc.2004-0865.

Skuse, David, Richard Warrington, Dorothy Bishop, Uttom Chowdhury, Jennifer Lau, William Mandy, and Maurice Place. 2004. "The Developmental, Dimensional and Diagnostic Interview (3di): A Novel Computerized Assessment for Autism Spectrum Disorders." *Journal of the American Academy of Child and Adolescent Psychiatry*. https://doi.org/10.1097/00004583-200405000-00008.

Slappendel, Geerte, William Mandy, Jan van der Ende, Frank C. Verhulst, Ad van der Sijde, Jorieke Duvekot, David Skuse, and Kirstin Greaves-Lord. 2016. "Utility of the 3Di Short Version for the Diagnostic Assessment of Autism Spectrum Disorder and Compatibility with DSM-5." *Journal of Autism and Developmental Disorders*. https://doi.org/10.1007/s10803-016-2713-9.

Smith, Roger, E. Jean Wickings, Maria E. Bowman, Anne Belleoud, Guy Dubreuil, Joanne J. Davies, and Gemma Madsen. 1999. "Corticotropin-Releasing Hormone in

Chimpanzee and Gorilla Pregnancies." *Journal of Clinical Endocrinology and Metabolism* 84 (8). https://doi.org/10.1210/jcem.84.8.5906.

Sollberger, Silja, and Ulrike Ehlert. 2016. "How to Use and Interpret Hormone Ratios." *Psychoneuroendocrinology* 63 (January): 385–97. https://doi.org/10.1016/J.PSYNEUEN.2015.09.031.

Somel, Mehmet, Henriette Franz, Zheng Yan, Anna Lorenc, Song Guo, Thomas Giger, Janet Kelso, et al. 2009. "Transcriptional Neoteny in the Human Brain." *Proceedings of the National Academy of Sciences of the United States of America* 106 (14). https://doi.org/10.1073/pnas.0900544106.

Sovio, Ulla, Francesca Gaccioli, Emma Cook, Martin Hund, David Stephen Charnock-Jones, and Gordon C.S. Smith. 2018. "505: Fetal Sex Differences in Biochemical and Ultrasound Indices of Placentation." *American Journal of Obstetrics and Gynecology* 218 (1). https://doi.org/10.1016/j.ajog.2017.11.031.

Spencer, K., J. N. Macri, D. A. Aitken, and J. M. Connor. 1992. "Free β-HCG as First-Trimester Marker for Fetal Trisomy." *The Lancet*. https://doi.org/10.1016/0140-6736(92)92073-O.

Srivastava, D. P., K. M. Woolfrey, F. Liu, N. J. Brandon, and P. Penzes. 2010. "Estrogen Receptor Beta Activity Modulates Synaptic Signaling and Structure." *Journal of Neuroscience* 30 (40): 13454–60. https://doi.org/10.1523/JNEUROSCI.3264-10.2010.

Stanyon, Roscoe, and Francesca Bigoni. 2014. "Sexual Selection and the Evolution of Behavior, Morphology, Neuroanatomy and Genes in Humans and Other Primates." *Neuroscience and Biobehavioral Reviews*. https://doi.org/10.1016/j.neubiorev.2014.10.001.

Steier, Johan Arnt, Ole L. Myking, and Per B. Bergsjø. 1999. "Correlation between Fetal Sex and Human Chorionic Gonadotropin in Peripheral Maternal Blood and Amniotic Fluid in Second and Third Trimester Normal Pregnancies." *Acta Obstetricia et Gynecologica Scandinavica*. https://doi.org/10.1080/j.1600-0412.1999.780504.x.

Stewart-Williams, Steve, and Andrew G. Thomas. 2013. "The Ape That Thought It Was a Peacock: Does Evolutionary Psychology Exaggerate Human Sex Differences?" *Psychological Inquiry* 24 (3). https://doi.org/10.1080/1047840X.2013.804899.

Stoltenberg, C., S. Schjølberg, M. Bresnahan, M. Hornig, D. Hirtz, C. Dahl, K. K. Lie, et al. 2010. "The Autism Birth Cohort: A Paradigm for Gene-Environment-Timing Research." *Molecular Psychiatry*. https://doi.org/10.1038/mp.2009.143.

Straughen, J K, D P Misra, G Divine, R Shah, G Perez, S VanHorn, V Onbreyt, et al. 2017. "The Association between Placental Histopathology and Autism Spectrum Disorder." *Placenta* 57: 183–88. https://doi.org/10.1016/j.placenta.2017.07.006.

Studholme, Colin, Christopher D. Kroenke, and Manjiri Dighe. 2020. "Motion Corrected MRI Differentiates Male and Female Human Brain Growth Trajectories from Mid-Gestation." *Nature Communications 2020 11:1* 11 (1): 1–16. https://doi.org/10.1038/s41467-020-16763-y.

Tache, Veronique, Rebecca J. Baer, Robert J. Currier, Chin Shang Li, Dena Towner, L. Elaine Waetjen, and Laura L. Jelliffe-Pawlowski. 2014. "Population-Based Biomarker Screening and the Development of Severe Preeclampsia in California." *American Journal of Obstetrics and Gynecology* 211 (4): 377.e1-377.e8. https://doi.org/10.1016/j.ajog.2014.03.026.

Talge, N. M., C. Holzman, P. K. Senagore, M. Klebanoff, and R. Fisher. 2011. "Biological Indicators of the In-Utero Environment and Their Association with Birth Weight for Gestational Age." *Journal of Developmental Origins of Health and Disease* 2 (05): 280–90. https://doi.org/10.1017/S2040174411000298.

Tan, D W, S Z Gilani, M T Maybery, A Mian, A Hunt, M Walters, and A J O Whitehouse. 2017. "Hypermasculinised Facial Morphology in Boys and Girls with Autism Spectrum Disorder and Its Association with Symptomatology." *Sci Rep* 7 (1): 9348. https://doi.org/10.1038/s41598-017-09939-y.

Tan, D W, S N Russell-Smith, J M Simons, M T Maybery, D Leung, H L Ng, and A J Whitehouse. 2015. "Perceived Gender Ratings for High and Low Scorers on the Autism-Spectrum Quotient Consistent with the Extreme Male Brain Account of Autism." *PLoS One* 10 (7): e0131780. https://doi.org/10.1371/journal.pone.0131780.

Tan, Diana Weiting, Murray T. Maybery, Syed Zulqarnain Gilani, Gail A. Alvares, Ajmal Mian, David Suter, and Andrew J.O. Whitehouse. 2020. "A Broad Autism Phenotype Expressed in Facial Morphology." *Translational Psychiatry*. https://doi.org/10.1038/s41398-020-0695-z.

Tang, Guomei, Kathryn Gudsnuk, Sheng Han Kuo, Marisa L. Cotrina, Gorazd Rosoklija, Alexander Sosunov, Mark S. Sonders, et al. 2014. "Loss of MTOR-Dependent Macroautophagy Causes Autistic-like Synaptic Pruning Deficits." *Neuron* 83 (5). https://doi.org/10.1016/j.neuron.2014.07.040.

Terloyeva, Dina, Alexander J. Frey, Bo Y. Park, Elizabeth M. Kauffman, Leny Mathew, Anna Bostwick, Erika L. Varner, et al. 2020. "Meconium Androgens Are Correlated with ASD-Related Phenotypic Traits in Early Childhood in a Familial Enriched Risk Cohort." *Molecular Autism*. https://doi.org/10.1186/s13229-020-00395-6.

Tsai, S., N. E. Hardison, A. H. James, A. A. Motsinger-Reif, S. R. Bischoff, B. H. Thames, and J. A. Piedrahita. 2011. "Transcriptional Profiling of Human Placentas from Pregnancies Complicated by Preeclampsia Reveals Disregulation of Sialic Acid Acetylesterase and Immune Signalling Pathways." *Placenta* 32 (2). https://doi.org/10.1016/j.placenta.2010.11.014.

Tsompanidis, A., E. Aydin, E. Padaigaitė, G. Richards, C. Allison, G. Hackett, T. Austin, R. Holt, and S. Baron-Cohen. 2021. "Maternal Steroid Levels and the Autistic Traits of the Mother and Infant." *Molecular Autism* 12 (1). https://doi.org/10.1186/s13229-021-00453-7.

Underwood, Mark A, William M Gilbert, and Michael P Sherman. 2005. "Amniotic Fluid: Not Just Fetal Urine Anymore." *Journal of Perinatology* 25 (5): 341–48. https://doi.org/10.1038/sj.jp.7211290.

Vacher, Claire-Marie, Helene Lacaille, Jiaqi J. O'Reilly, Jacquelyn Salzbank, Dana Bakalar,

Sonia Sebaoui, Philippe Liere, et al. 2021. “Placental Endocrine Function Shapes Cerebellar Development and Social Behavior.” *Nature Neuroscience 2021*, August, 1–10. https://doi.org/10.1038/s41593-021-00896-4.

Verburg, Petra E., Graeme Tucker, Wendy Scheil, Jan Jaap H.M. Erwich, Gus A. Dekker, and Claire Trelford Roberts. 2016. “Sexual Dimorphism in Adverse Pregnancy Outcomes - A Retrospective Australian Population Study 1981-2011.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0158807.

Vinkhuyzen, A A E, D W Eyles, T H J Burne, L M E Blanken, C J Kruithof, F Verhulst, V W Jaddoe, H Tiemeier, and J J McGrath. 2018. “Gestational Vitamin D Deficiency and Autism-Related Traits: The Generation R Study.” *Mol Psychiatry* 23 (2): 240–46. https://doi.org/10.1038/mp.2016.213.

Walker, Cheryl K., Kaitlin W. Anderson, Kristin M. Milano, Saier Ye, Daniel J. Tancredi, Isaac N. Pessah, Hertz Picciotto Irva, and Harvey J. Kliman. 2013. “Trophoblast Inclusions Are Significantly Increased in the Placentas of Children in Families at Risk for Autism.” *Biological Psychiatry*. https://doi.org/10.1016/j.biopsych.2013.03.006.

Walker, Cheryl K., Paula Krakowiak, Alice Baker, Robin L. Hansen, Sally Ozonoff, and Irva Hertz-Picciotto. 2015. “Preeclampsia, Placental Insufficiency, and Autism Spectrum Disorder or Developmental Delay.” *JAMA Pediatrics* 169 (2): 154. https://doi.org/10.1001/jamapediatrics.2014.2645.

Wallace, Gregory L., Nathan Dankner, Lauren Kenworthy, Jay N. Giedd, and Alex Martin. 2010. “Age-Related Temporal and Parietal Cortical Thinning in Autism Spectrum Disorders.” *Brain* 133 (12): 3745–54. https://doi.org/10.1093/BRAIN/AWQ279.

Wan, Hongquan, Chunguo Zhang, He Li, Shuxin Luan, and Chang Liu. 2018. “Association of Maternal Diabetes with Autism Spectrum Disorders in Offspring.” *Medicine (United States)*. https://doi.org/10.1097/MD.0000000000009438.

Warrier, Varun, David M. Greenberg, Elizabeth Weir, Clara Buckingham, Paula Smith, Meng Chuan Lai, Carrie Allison, and Simon Baron-Cohen. 2020. “Elevated Rates of Autism, Other Neurodevelopmental and Psychiatric Diagnoses, and Autistic Traits in Transgender and Gender-Diverse Individuals.” *Nature Communications*. https://doi.org/10.1038/s41467-020-17794-1.

Warrier, Varun, Roberto Toro, Hyejung Won, Claire S. Leblond, Freddy Cliquet, Richard Delorme, Ward De Witte, et al. 2019. “Social and Non-Social Autism Symptoms and Trait Domains Are Genetically Dissociable.” *Communications Biology*. https://doi.org/10.1038/s42003-019-0558-4.

Watterberg, K. L., and S. M. Scott. 1995. “Evidence of Early Adrenal Insufficiency in Babies Who Develop Bronchopulmonary Dysplasia.” *Pediatrics* 95 (1). https://doi.org/10.1542/peds.95.1.120.

Welberg, Leonie A.M., K. V. Thrivikraman, and Paul M. Plotsky. 2005. “Chronic Maternal Stress Inhibits the Capacity to Up-Regulate Placental 11β-Hydroxysteroid Dehydrogenase Type 2 Activity.” *Journal of Endocrinology* 186 (3). https://doi.org/10.1677/joe.1.06374.

Welsh, Michelle, Hiroko Suzuki, and Gen Yamada. 2014. “The Masculinization

Programming Window." In *Endocrine Development*, 27:17–27. https://doi.org/10.1159/000363609.

Werler, Steffi, Andreas Poplinski, Jörg Gromoll, and Joachim Wistuba. 2011. "Expression of Selected Genes Escaping from X Inactivation in the 41, XXY* Mouse Model for Klinefelter's Syndrome." *Acta Paediatrica, International Journal of Paediatrics* 100 (6). https://doi.org/10.1111/j.1651-2227.2010.02112.x.

Werling, Donna M, and Daniel H Geschwind. 2015. "Recurrence Rates Provide Evidence for Sex-Differential, Familial Genetic Liability for Autism Spectrum Disorders in Multiplex Families and Twins." *Molecular Autism* 6 (1): 1–14. https://doi.org/10.1186/s13229-015-0004-5.

Whitehouse, A J, E Mattes, M T Maybery, C Dissanayake, M Sawyer, R M Jones, C E Pennell, J A Keelan, and M Hickey. 2012. "Perinatal Testosterone Exposure and Autistic-like Traits in the General Population: A Longitudinal Pregnancy-Cohort Study." *J Neurodev Disord* 4 (1): 25. https://doi.org/10.1186/1866-1955-4-25.

Whitehouse, Andrew J. O., Gail A. Alvares, Dominique Cleary, Alexis Harun, Angela Stojanoska, Lauren J. Taylor, Kandice J. Varcin, and Murray Maybery. 2018. "Symptom Severity in Autism Spectrum Disorder Is Related to the Frequency and Severity of Nausea and Vomiting during Pregnancy: A Retrospective Case-Control Study." *Molecular Autism* 9 (1): 37. https://doi.org/10.1186/s13229-018-0223-7.

Whitehouse, Andrew J.O., Syed Zulqarnain Gilani, Faisal Shafait, Ajmal Mian, Diana Weiting Tan, Murray T. Maybery, Jeffrey A. Keelan, et al. 2015. "Prenatal Testosterone Exposure Is Related to Sexually Dimorphic Facial Morphology in Adulthood." *Proceedings of the Royal Society B: Biological Sciences* 282 (1816). https://doi.org/10.1098/rspb.2015.1351.

Wichstrøm, Lars. 2000. "Predictors of Adolescent Suicide Attempts: A Nationally Representative Longitudinal Study of Norwegian Adolescents." *Journal of the American Academy of Child and Adolescent Psychiatry* 39 (5). https://doi.org/10.1097/00004583-200005000-00014.

Wilkins, Jon F., and David Haig. 2003. "What Good Is Genomic Imprinting: The Function of Parent-Specific Gene Expression." *Nature Reviews Genetics*. https://doi.org/10.1038/nrg1062.

Windham, Gayle C., Kristen Lyall, Meredith Anderson, and Martin Kharrazi. 2016. "Autism Spectrum Disorder Risk in Relation to Maternal Mid-Pregnancy Serum Hormone and Protein Markers from Prenatal Screening in California." *Journal of Autism and Developmental Disorders* 46 (2): 478–88. https://doi.org/10.1007/s10803-015-2587-2.

Wright, D., K. Spencer, K. Kagan, N. Tørring, O. B. Petersen, A. Christou, J. Kallikas, and K. H. Nicolaides. 2010. "First-Trimester Combined Screening for Trisomy 21 at 7-14 Weeks' Gestation." *Ultrasound in Obstetrics and Gynecology*. https://doi.org/10.1002/uog.7755.

Xiang, Anny H., Xinhui Wang, Mayra P. Martinez, Johanna C. Walthall, Edward S. Curry, Kathleen Page, Thomas A. Buchanan, Karen J. Coleman, and Darios Getahun. 2015. "Association of Maternal Diabetes with Autism in Offspring." *JAMA - Journal of the*

American Medical Association. https://doi.org/10.1001/jama.2015.2707.

Xiao, Zhou, Ting Qiu, Xiaoyan Ke, Xiang Xiao, Ting Xiao, Fengjing Liang, Bing Zou, et al. 2014. "Autism Spectrum Disorder as Early Neurodevelopmental Disorder: Evidence from the Brain Imaging Abnormalities in 2-3 Years Old Toddlers." *Journal of Autism and Developmental Disorders* 44 (7): 1633–40. https://doi.org/10.1007/s10803-014-2033-x.

Yeh, Shuyuan, Hiroshi Miyamoto, Hiroki Shima, and Chawnshang Chang. 1998. "From Estrogen to Androgen Receptor: A New Pathway for Sex Hormones in Prostate." *Proceedings of the National Academy of Sciences of the United States of America* 95 (10): 5527. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC20411/.

Yuan, Bo, Davut Pehlivan, Ender Karaca, Nisha Patel, Wu Lin Charng, Tomasz Gambin, Claudia Gonzaga-Jauregui, et al. 2015. "Global Transcriptional Disturbances Underlie Cornelia de Lange Syndrome and Related Phenotypes." *Journal of Clinical Investigation* 125 (2). https://doi.org/10.1172/JCI77435.

Yuen, Tracy, Melanie Penner, Melissa T. Carter, Peter Szatmari, and Wendy J. Ungar. 2018. "Assessing the Accuracy of the Modified Checklist for Autism in Toddlers: A Systematic Review and Meta-Analysis." *Developmental Medicine and Child Neurology*. https://doi.org/10.1111/dmcn.13964.

Yushkevich, Paul A., Joseph Piven, Heather Cody Hazlett, Rachel Gimpel Smith, Sean Ho, James C. Gee, and Guido Gerig. 2006. "User-Guided 3D Active Contour Segmentation of Anatomical Structures: Significantly Improved Efficiency and Reliability." *NeuroImage* 31 (3): 1116–28. https://doi.org/10.1016/j.neuroimage.2006.01.015.

Zambrano, Elena, Carolina Guzmán, Guadalupe L. Rodríguez-González, Marta Durand-Carbajal, and Peter W. Nathanielsz. 2014. "Fetal Programming of Sexual Development and Reproductive Function." *Molecular and Cellular Endocrinology*. https://doi.org/10.1016/j.mce.2013.09.008.

Zechner, Ulrich, Monika Wilda, Hildegard Kehrer-Sawatzki, Walther Vogel, Rainald Fundele, and Horst Hameister. 2001. "A High Density of X-Linked Genes for General Cognitive Ability: A Run-Away Process Shaping Human Evolution?" *Trends in Genetics*. https://doi.org/10.1016/S0168-9525(01)02446-5.

Zerbo, Ousseny, Yinge Qian, Cathleen Yoshida, Judith K. Grether, Judy Van de Water, and Lisa A. Croen. 2015. "Maternal Infection During Pregnancy and Autism Spectrum Disorders." *Journal of Autism and Developmental Disorders*. https://doi.org/10.1007/s10803-013-2016-3.

Zhou, N, L Sun, H Yang, Q Chen, X Wang, H Yang, L Tan, et al. 2016. "Anogenital Distance Is Associated with Serum Reproductive Hormones, but Not with Semen Quality in Young Men." *Hum Reprod* 31 (5): 958–67. https://doi.org/10.1093/humrep/dew052.

Zhu, Yihui, Charles E. Mordaunt, Dag H. Yasui, Ria Marathe, Rochelle L. Coulson, Keith W. Dunaway, Julia M. Jianu, et al. 2019. "Placental DNA Methylation Levels at CYP2E1 and IRS2 Are Associated with Child Outcome in a Prospective Autism Study." *Human Molecular Genetics*. https://doi.org/10.1093/hmg/ddz084.

Appendix

Appendix 1 provides details on the neurodevelopmental instruments for autistic traits that were used in this thesis.

Appendices 2 - 5 include Supplementary Material from the first three studies on prenatal steroids and hormone markers (Chapters 2, 3 and 4 respectively).

Appendix 1 - Measures of autistic traits

1.1 The Quantitative Checklist of Autism in Toddlers - (Q-CHAT)

Reference:

- Allison, C., Baron-Cohen, S., Wheelwright, S., Charman, T., Richler, J., Pasco, G., & Brayne, C. (2008). The Q-CHAT (Quantitative Checklist for Autism in Toddlers): a normally distributed quantitative measure of autistic traits at 18-24 months of age: preliminary report. *J Autism Dev Disord*, *38*(8), 1414–1425.

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child echo things s/he hears (e.g things that you say, lines from songs or movies, sounds)?	4	3	2	1	0

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child use simple gestures (e.g wave goodbye)?	0	1	2	3	4

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child make unusual finger movements near his/her eyes?	4	3	2	1	0

	Always	Usually	Sometimes	Rarely	Never
Does your child spontaneously look at your face to check your reaction when faced with something unfamiliar?	0	1	2	3	4

	Most of the day	Several hours	Half an hour	Ten minutes	A couple of minutes
How long can your child's interest be maintained by just one or two objects?	4	3	2	1	0

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child twiddle objects repetitively (e.g pieces of string)	4	3	2	1	0

	Always	Usually	Sometimes	Rarely	Never
Does your child seem oversensitive to noise?	4	3	2	1	0

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child stare at nothing with no apparent purpose?	4	3	2	1	0

	Always	Usually	Sometimes	Rarely	Never
Does your child look at you when you call his/her name?	0	1	2	3	4

	Very easy	Quite easy	Quite difficult	Very difficult	Impossible
How easy is it for you to get eye contact with your child?	0	1	2	3	4

	Always	Usually	Sometimes	Rarely	Never
When your child is playing alone, does s/he line objects up?	4	3	2	1	0

	Always	Usually	Sometimes	Rarely	Never	My child does not speak
Can other people easily understand your child's speech?	0	1	2	3	4	4

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child point to indicate that s/he wants something (e.g a toy that is out of reach)?	0	1	2	3	4

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child point to share interest with you (e.g pointing at an interesting sight)?	0	1	2	3	4

	Several hours	Half an hour	Ten minutes	A couple of minutes	Less than a minute
How long can your child's interest be maintained by a spinning object (e.g washing machine, electric fan, toy car wheels)?	4	3	2	1	0

	None – s/he has not started speaking yet	Less than 10 words	10 – 50 words	51 – 100 words	Over 100 words
How many words can your child say?	4	3	2	1	0

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child pretend (eg care for dolls, talk on a toy phone)?	0	1	2	3	4

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child follow where you're looking?	0	1	2	3	4

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
How often does your child sniff or lick unusual objects?	4	3	2	1	0

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child place your hand on an object when s/he wants you to use it (e.g on a door handle when s/he wants you to open the door, on a toy when s/he wants you to activate it)?	4	3	2	1	0

	Always	Usually	Sometimes	Rarely	Never
Does your child walk on tiptoe?	4	3	2	1	0

	Very easy	Quite easy	Quite difficult	Very difficult	Impossible
How easy is it for your child to adapt when his/her routine changes or when things are out of their usual place?	0	1	2	3	4

	Always	Usually	Sometimes	Rarely	Never
If you or someone else in the family is visibly upset, does your child show signs of wanting to comfort them (e.g stroking their hair, hugging them)?	0	1	2	3	4

	Many times a day	A few times a day	A few times a week	Less than once a week	Never
Does your child do the same thing over and over again (e.g running the tap, turning the light switch on and off, opening and closing doors)?	4	3	2	1	0

	Very typical	Quite typical	Slightly unusual	Very unusual	My child doesn't speak
Would you describe your child's first words as:	0	1	2	3	4

1.2 The Autism Spectrum Quotient (AQ - Adult)

Reference:

- S. Baron-Cohen, S. Wheelwright, R. Skinner, J. Martin and E. Clubley, (2001) The Autism Spectrum Quotient (AQ) : Evidence from Asperger Syndrome/High Functioning Autism, Males and Females, Scientists and Mathematicians Journal of Autism and Developmental Disorders 31:5-17

Responses that score 1 point are marked. Other responses score 0. For total score, sum all items.

		definitely agree	**slightly agree**	**slightly disagree**	**definitely disagree**
1.	I prefer to do things with others rather than on my own.			1	1
2.	I prefer to do things the same way over and over again.	1	1		
3.	If I try to imagine something, I find it very easy to create a picture in my mind.			1	1
4.	I frequently get so strongly absorbed in one thing that I lose sight of other things.	1	1		
5.	I often notice small sounds when others do not.	1	1		
6.	I usually notice car number plates or similar strings of information.	1	1		
7.	Other people frequently tell me that what I've said is impolite, even though I think it is polite.	1	1		
8.	When I'm reading a story, I can easily imagine what the characters might look like.			1	1
9.	I am fascinated by dates.	1	1		
10.	In a social group, I can easily keep track of several different people's conversations.			1	1
11.	I find social situations easy.			1	1
12.	I tend to notice details that others do not.	1	1		

		definitely agree	slightly agree	slightly disagree	definitely disagree
13.	I would rather go to a library than a party.	1	1		
14.	I find making up stories easy.			1	1
15.	I find myself drawn more strongly to people than to things.			1	1
16.	I tend to have very strong interests which I get upset about if I can't pursue.	1	1		
17.	I enjoy social chit-chat.			1	1
18.	When I talk, it isn't always easy for others to get a word in edgeways.	1	1		
19.	I am fascinated by numbers.	1	1		
20.	When I'm reading a story, I find it difficult to work out the characters' intentions.	1	1		
21.	I don't particularly enjoy reading fiction.	1	1		
22.	I find it hard to make new friends.	1	1		
23.	I notice patterns in things all the time.	1	1		
24.	I would rather go to the theatre than a museum.			1	1
25.	It does not upset me if my daily routine is disturbed.			1	1
26.	I frequently find that I don't know how to keep a conversation going.	1	1		
27.	I find it easy to "read between the lines" when someone is talking to me.			1	1
28.	I usually concentrate more on the whole picture, rather than the small details.			1	1
29.	I am not very good at remembering phone numbers.			1	1
30.	I don't usually notice small changes in a situation, or a person's appearance.			1	1
31.	I know how to tell if someone listening to me is getting bored.			1	1
32.	I find it easy to do more than one thing at once.			1	1

		definitely agree	slightly agree	slightly disagree	definitely disagree
33.	When I talk on the phone, I'm not sure when it's my turn to speak.	1	1		
34.	I enjoy doing things spontaneously.			1	1
35.	I am often the last to understand the point of a joke.	1	1		
36.	I find it easy to work out what someone is thinking or feeling just by looking at their face.			1	1
37.	If there is an interruption, I can switch back to what I was doing very quickly.			1	1
38.	I am good at social chit-chat.			1	1
39.	People often tell me that I keep going on and on about the same thing.	1	1		
40.	When I was young, I used to enjoy playing games involving pretending with other children.			1	1
41.	I like to collect information about categories of things (e.g., types of car, types of bird, types of train, types of plant, etc.).	1	1		
42.	I find it difficult to imagine what it would be like to be someone else.	1	1		
43.	I like to plan any activities I participate in carefully.	1	1		
44.	I enjoy social occasions.			1	1
45.	I find it difficult to work out people's intentions.	1	1		
46.	New situations make me anxious.	1	1		
47.	I enjoy meeting new people.			1	1
48.	I am a good diplomat.			1	1
49.	I am not very good at remembering people's date of birth.			1	1
50.	I find it very easy to play games with children that involve pretending.			1	1

1.3 The Social Responsiveness Scale - Abridged version

References:

- Consantino JN. *Social Responsiveness Scale (SRS), Manual*. Los Angeles: Western Psychological Services; 2002.
- Blanken, L. M. E., Dass, A., Alvares, G., van der Ende, J., Schoemaker, N. K., El Marroun, H., … Whitehouse, A. (2018). A prospective study of fetal head growth, autistic traits and autism spectrum disorder. *Autism Res*, *11*(4), 602–612. doi:10.1002/aur.1921

Probes were rated on a 4-point Likert scale:
0 (not true) / 1 (sometimes true) / 2 (often true)/ 3 (almost always true) / 4 (true)

1. Is unable to pick up on any of the meaning of conversations of older children or adults.
2. Is slow or awkward in turn-taking interactions with peers.
3. Is able to understand the meaning of other people's tone of voice and facial expressions.
4. Avoids eye contact or has unusual eye contact.
5. Does not attempt to interact with the other children when on the playground or in a group with other young children.
6. Has strange ways of playing with toys.
7. Has more difficulty than other children with changes in his/her routine.
8. Is regarded by other children as odd or weird.
9. Has trouble keeping up with the flow of a normal interaction with other children.
10. Has difficulty "relating" to peers.
11. Has a restricted (or unusually narrow) range of interests.
12. Is imaginative, good at pretending (without losing touch with reality).
13. Has repetitive odd behaviors such as hand flapping or rocking.
14. Responds to clear, direct questions in ways that don't seem to make any sense.
15. Talks to people with an unusual tone of voice (for example, talks like a robot).
16. Concentrates too much on parts of things rather than "seeing the whole picture" (for example, spins the wheels of a toy car, but doesn't play with it as a car, or plays with doll's hair but not with the whole doll).
17. Is inflexible, has a hard time changing his/her mind.
18. Gives unusual or illogical reasons for doing things.

Appendix 2 - Study 1 Supplementary Material

Laboratory Methods

Prior to liquid chromatography tandem mass spectroscopy (LC-MS/MS), analytes were extracted from the amniotic fluid samples. Amniotic fluid was diluted with 125 µL of solution containing internal standards for mass spectroscopy. An Oasis WAX elution plate was used for solid phase extraction. Prior to addition of samples, the plate was washed with 200 µL of methanol (Fischer Scientific, Waltham, MA) and 200 µL of water purified with an in-house MilliQ purifier (EMD Millipore, Billerica, MA). The diluted amniotic fluid samples were added, followed by 200 µL 2% formic acid (Merck, Whitehouse Station, NJ) and 200 µL 10% methanol. The plate was dried under a vacuum for 1 hour, and then analytes were eluted twice with 25 µL of a solution containing 5% ammonium hydroxide (Sigma-Aldrich, St. Louis, MO) 45% acetonitrile (Sigma-Aldrich, St. Louis, MO), and 50% methanol. This extract was further diluted into 50 µL of water.

35 µL of diluted extract was injected into the LC-MS/MS setup. The LC-MS/MS setup consisted of a Waters Acquity Ultra Performance Liquid Chromatography machine with an Acquity sample manager and an Acquity sample organizer, followed by Xevo TQ-S triple quadrupole mass spectrometer equipped with an electron spray ionization probe. Samples were separated on a Poroshell 120 Phenyl Hexyl 2.7µ column (Algilent, Santa Clara, CA) at 50◦ C. A linear gradient of 0.1% ammonium hydroxide in water (A) to 0.1% of ammonium hydroxide in methanol (B) was used to separate the analytes. The elution gradient followed a pattern of 0.5 min at 80% A and 20% B, 0.5 min at 50% A and 50% B, 3.5 min at 30% A and 70% B, and 5 minutes at 100% B. The quadrupole mass spectrometer was operated in negative mode. Transitions and internal standards are given in Supplementary Table 1. Coefficients of variation (CV) were all 10% or less within the working range of the setup (50 pmol/L to 50 nmol/L).

Appendix Table 2.1: *Transitions and internal standards used in mass spectroscopy*

Analyte	Transition	Internal Standard	Transition
17β-estradiol	271.2/145.1	17β-estradiol-D3	274.2/145.1
estriol	287.2/171.1		
estrone	269.1/145	Estrone-D4	273.1/147.1
estrone-sulfate	269.1/145.1	estrone-sulfate-D4	273.1/147.1

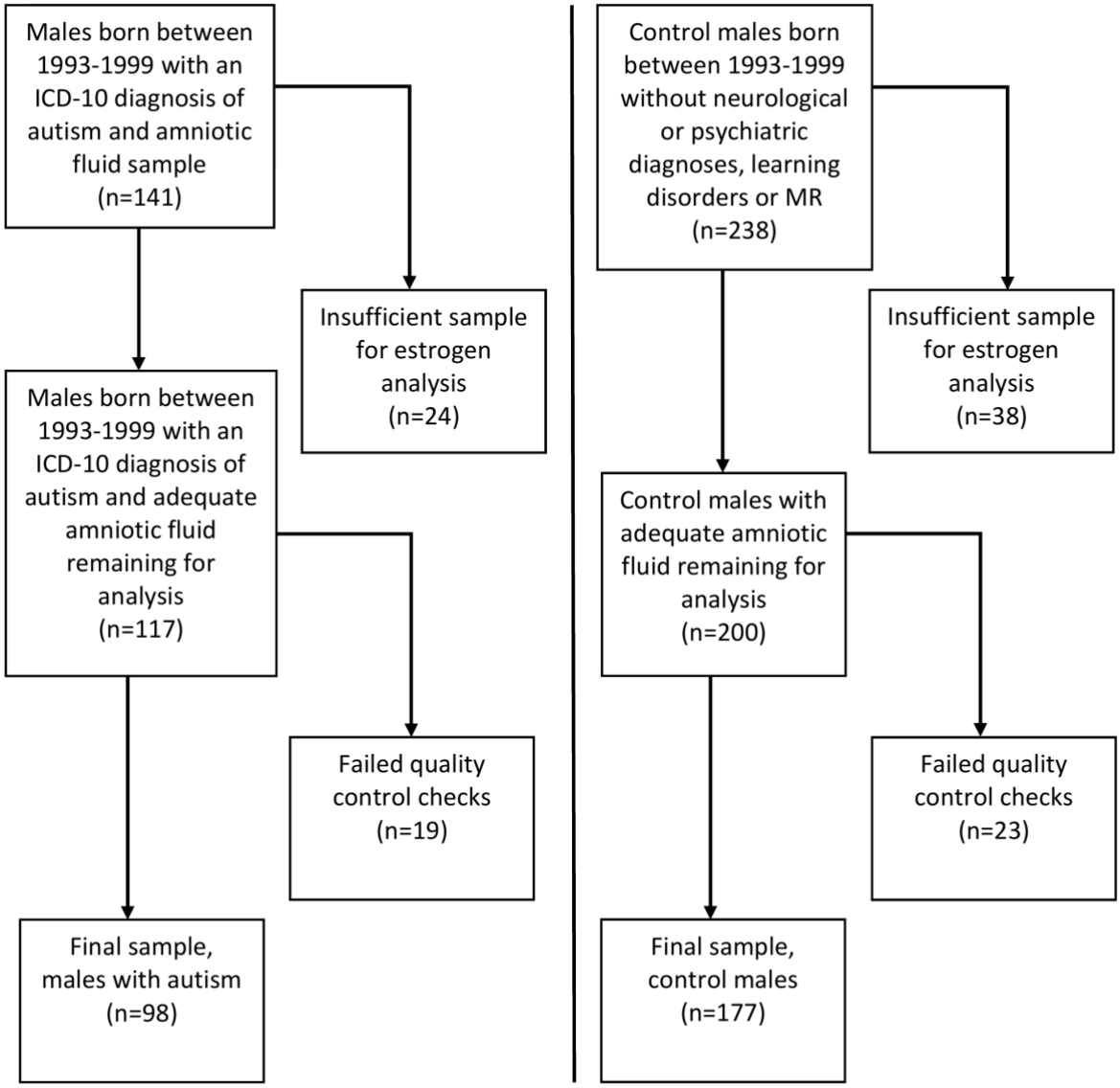
Appendix Table 2.2: *Descriptive statistics of estrogen data. IQR: interquartile range*

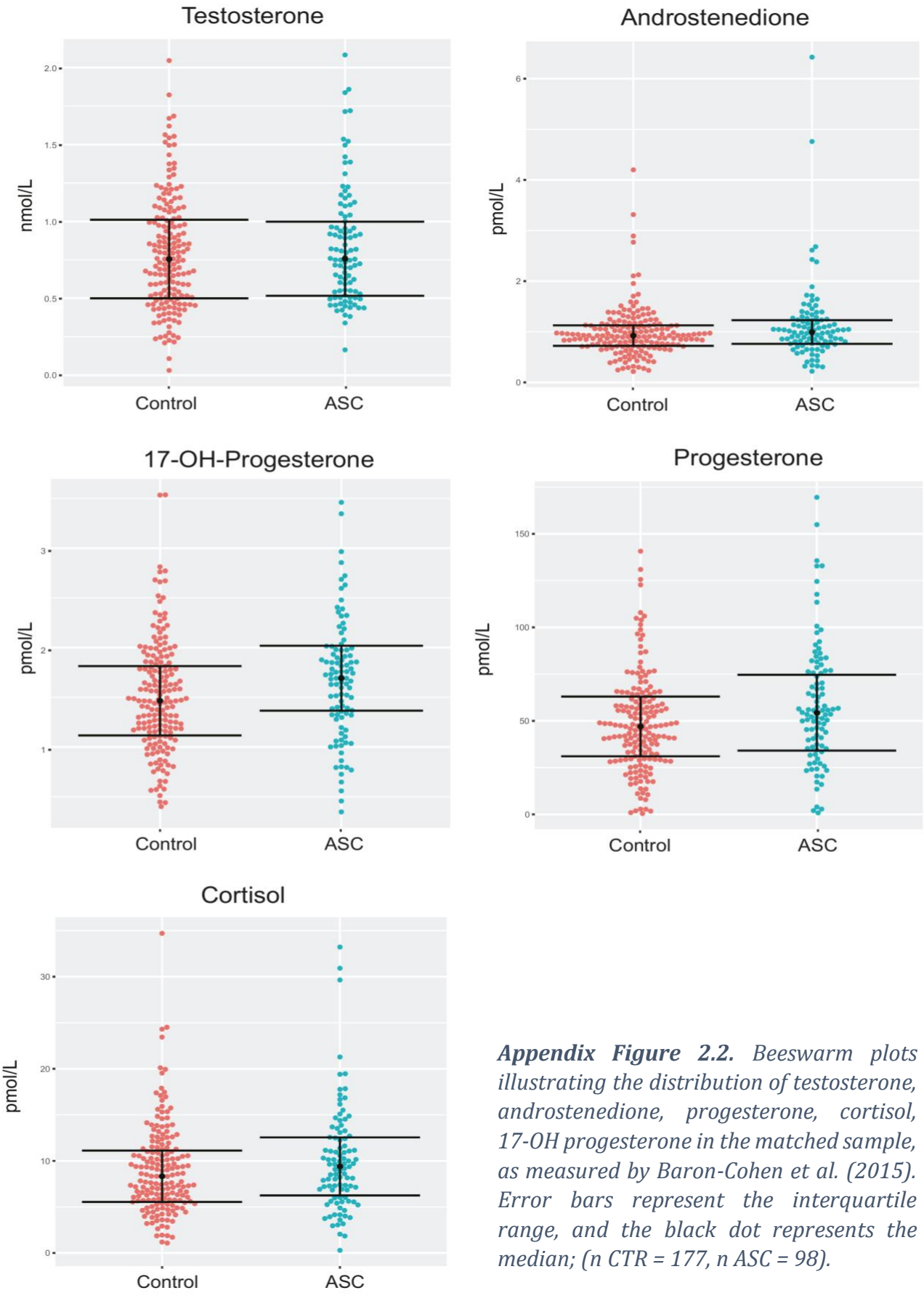
	Control		**Case**	
	n=177		n=98	
	median	IQR	median	IQR
Estriol	2106	[1122, 3091]	2548	[1698, 3399]
Estradiol	181	[32, 330]	245	[70, 421]
Estrone	664	[291, 1036]	877	[431, 1323]
Estrone Sulfate	8895	[4541, 13249]	10605	[5283, 15926]

Appendix Table 2.3: *Pearson's correlation matrix of amniotic fluid steroid hormones. Asterisk denotes statistical significance (q<0.05), following correction via FDR. T: Testosterone; A: Androstenedione; P: Progesterone; P-OH: 17OH-Progesterone; E2: Estradiol E-S: Estrone sulfate*

	A	**P-OH**	**P**	**Cortisol**	**E2**	**Estriol**	**Estrone**	**E-S**
T	0.37278 *	0.359*	0.29175 *	0.2646*	0.0068	-0.0414	-0.0220	-0.0327
A	1	0.3737*	0.59317 *	0.2906*	0.0481	0.1415	0.2360*	0.0463
P-OH		1	0.44315 *	0.4355*	-0.0488	0.2829*	0.1774*	0.0267
P			1	0.2274*	0.10558	0.2584*	0.2974*	0.0969
Cortisol				1	-0.0532	0.363*	0.2234*	0.0325
E2					1	0.08792	0.5473*	0.4846*
Estriol						1	0.5578*	0.1953*
Estrone							1	0.589*

Appendix Figure 2.1. *Flowchart illustrating selection of final sample. The initial sample matches the sample from Baron-Cohen et al. (2015) prior to the application of data quality selection criteria (i.e., the second to last row in the cohort selection flowchart). We removed cases and controls which had insufficient remaining volume for estrogen analysis. Finally, we re-applied the data quality selection criteria used in Baron-Cohen et al. (2015) (e.g., removal of outliers >99th%, removal of duplicate assays not within 3SD), and obtained a final sample of 98 males with autism and 177 control males.*





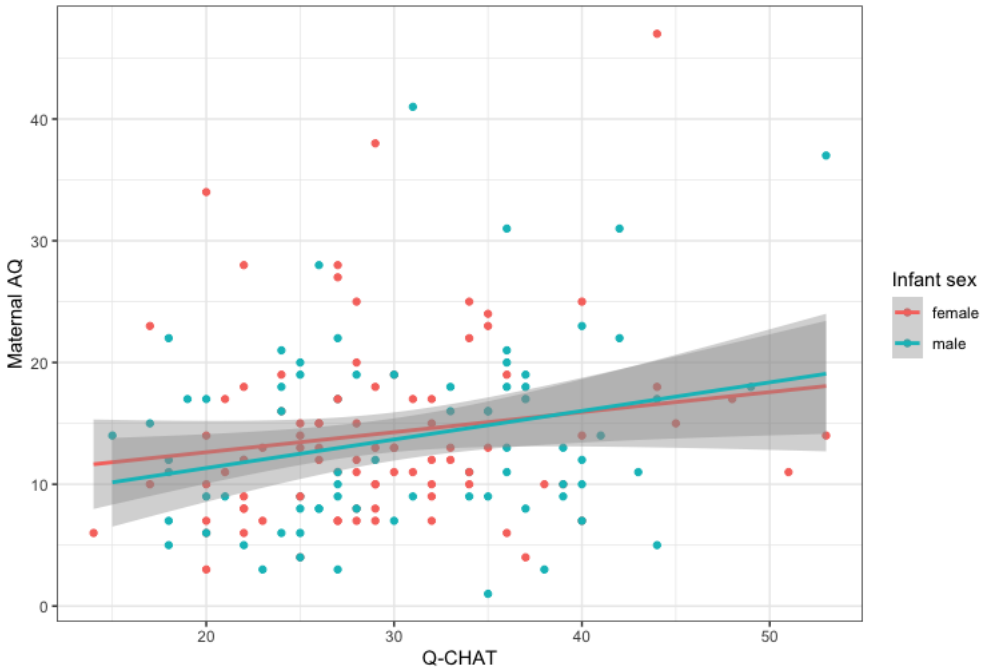
Appendix Figure 2.2. *Beeswarm plots illustrating the distribution of testosterone, androstenedione, progesterone, cortisol, 17-OH progesterone in the matched sample, as measured by Baron-Cohen et al. (2015). Error bars represent the interquartile range, and the black dot represents the median; (n CTR = 177, n ASC = 98).*

Appendix 3 - Study 2 Supplementary Material

Appendix Table 3.1*: Assay details, as conducted for research purposes by the Core Biochemical Assay Laboratory (CBAL) of CUH NHS Foundation Trust.*

	Chemiluminescence Immunoassay Assays by DiaSorin	Units of Measurement	Lower limit of detection	Imprecision In-House Measurements
Estradiol	Estradiol II Gen No.310680 (issued 6-2016)	pmol/L	36.7	2.9% at 425 pmol/L, & 1.9% at 1223 pmol/L
Testosterone	Testosterone No.310410 (issued 12-2014)	nmol/L	0.17	4.9% at 6.37 nmol/L, & 4.4% at 18.2 nmol/L
DHEAS	DHEA-S, No.310430, (issued 04-2016)	ug/L	1.0	4.9% at 6.4 ųg/L, & 4.4% at 18.2 ųg/L
Progesterone	Progesterone II Gen No.310690, (issued 06-2016)	ng/L	0.12	4.2% at 2.2 ng/L, & 2.5% at 21.4 ng/L
SHBG	SHBG, No. 319020, (issued 03-2017)	nmol/L	0.2	9.9% at 43 nmol/L, & 7.2% at 190 nmol/L

Appendix Figure 3.1: Maternal AQ and Q-CHAT of the same mother-infant pairs, showing a high degree of correlation between them.



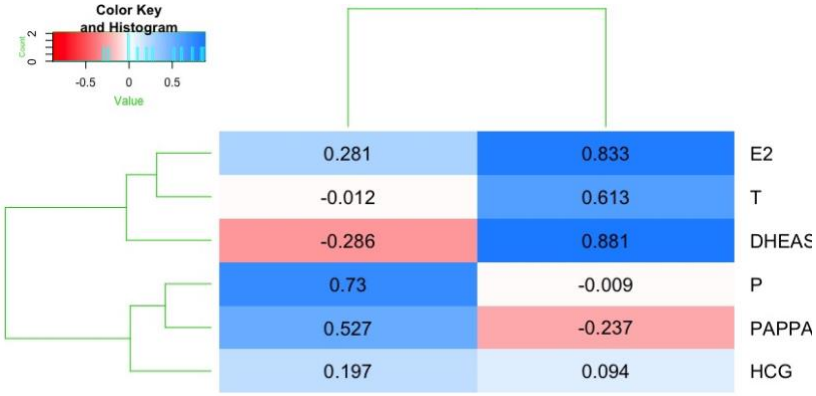
***Appendix Table 3.2**: Multiple regression model of the associations between maternal hirsutism score and AQ.*

	Coefficient	SE	Semipartial	p-value
Intercept	20.36	3.86		<0.0001
Hirsutism	1.91	0.67	0.21	0.005
PCOS	-0.35	2.69	-0.01	0.90
Hirs*PCOS	0.32	1.78	0.01	0.86
Maternal Age	-0.25	0.12	-0.16	0.04
Autism in Family	15.61	2.1	0.49	<0.0001
			Adjusted R2=0.24	**Model p**<0.0001

***Appendix Table 3.3**: Pairwise correlation coefficients (Pearson's r) for each pair of prenatal factors, with asterisks denoting statistical significance.*

	DHEAS	Progesterone	Estradiol	Testosterone	hCG	PAPP-A
DHEAS	1					
Progesterone	-0.214*	1				
Estradiol	0.655***	0.199*	1			
Testosterone	0.540***	-0.037	0.51***	1		
hCG MoM	0.019	0.179	0.101	0.214*	1	
PAPP-A MoM	-0.36***	0.380***	-0.041	-0.159	0.048	1

***Appendix Figure 3.2**: Heatmap and dendrogram of the loadings of each of the tested variables on latent factors. Factor 1 includes all sex steroids measured in the current study*



***Appendix Table 3.4**: Associations between circulating hormones/peptides and maternal factors.*

	Maternal PCOS		**Hirsutism Score (0 to 2+ areas)**		**Maternal Age**	
	Effect size	*p*	*Effect size*	*p*	*Effect size*	*p*
Estradiol	**D=1.06**	**0.008**	**D=0.47**	**0.034**	r=-0.1	0.274
Testosterone	D=0.35	0.247	D=0.21	0.328	**r=-0.37**	**<0.001**
DHEAS	D=0.13	0.54	D=0.33	0.127	**r=-0.30**	**<0.001**
Progesterone	**D=0.92**	**0.018**	D=0.001	0.995	**r=0.31**	**<0.001**
SHBG	**D=0.62**	**0.017**	D=0.03	0.870	r=0.06	0.51
FEI	D=0.58	0.100	**D=0.51**	**0.025**	r=-0.17	0.08
FTI	D=0.03	0.888	D=0.25	0.268	**r=-0.38**	**<0.001**
Steroid Factor	D=0.50	0.135	**D=0.50**	**0.028**	**r=-0.22**	**0.015**
hCG MoM	D=0.07	0.762	D=0.11	0.542	r=-0.04	0.61
PAPP-A MoM	D=0.18	0.291	**D=0.38**	**0.016**	r=0.08	0.261

***Appendix Table 3.5**: Sex differences among infant clinical characteristics*

	Males		**Females**			
	Mean	*SD*	*Mean*	*SD*	*Coeff*	*p*
Q-CHAT	30.36	8.13	29.63	7.58	-0.62	0.538
Age at follow-up	575.49	21.54	570.60	25.65	-1.26	0.210
Birth Weight	3461.8	481.49	3363.35	538.99	-1.34	0.183
Maternal age	32.68	4.83	32.16	4.27	-0.85	0.396
Maternal AQ	14.15	8.06	15.04	8.18	0.751	0.453
	Males		**Females**			
	N - with	*N - without*	*N - with*	*N - without*	*Coeff*	*p*
PCOS	17	87	9	106	3.02	0.082
Autism in Family	9	95	8	107	0.05	0.83

***Appendix Table 3.6**: Full model results of the association of FEI with maternal AQ*

	Coefficient	**SE**	**Semipartial**	**p-value**
Intercept	12.81	4.63		0.007
FEI	1.15	0.50	0.22	0.023
maternal age	-0.06	0.13	-0.05	0.631
PCOS	0.97	1.67	0.06	0.564
			Adjusted R2=0.04	**Model p=**0.055

Appendix Table 3.7: *Full model of the association of estradiol (E2) with infant Q-CHAT*

	Coefficient	**SE**	**Semipartial**	**p-value**
Intercept	114.21	35.21		0.002
Estradiol (E2)	-4.00	2.31	-0.19	0.087
Sex	-72.96	37.27	-0.22	0.054
E2 * Sex	8.27	4.08	0.23	0.036
Infant Age	-0.04	0.04	-0.12	0.287
Birth Weight	-0.002	0.002	-0.11	0.347
Maternal Age	-0.59	0.04	-0.30	0.007
Maternal AQ	0.10	0.16	0.07	0.544
PCOS	-2.04	2.83	-0.08	0.473
			Adjusted R2=0.09	**Model p=**0.052

Appendix Table 3.8*: Multiple linear regression model tests for residual normality and model homoscedasticity.*

	AQ		**Q-CHAT**	
	Normality of Residuals	Homoscedasticity (Breusch-Pagan)	Normality of Residuals (Shapiro-Wilk)	Homoscedasticity (Breusch-Pagan)
Estradiol				
Test statistic	W= 0.98	BP=1.19	W=0.98	BP=0.95
p-value	p=0.175	p=0.755	p=0.152	p=0.996
Testosterone				
Test statistic	W=0.98	BP=0.93	W=0.98	BP=12.8
p-value	p=0.105	p=0.817	p=0.154	p=0.119
DHEAS				
Test statistic	W=0.98	BP=1.09	W=0.98	BP=14.14
p-value	p=0.105	p=0.78	p=0.213	p=0.08
Progesterone				
Test statistic	W=0.98	BP=0.55	W=0.97	BP=9.80
p-value	p=0.09	p=0.91	p=0.05	p=0.27
hCG MoM				
Test statistic	**W=0.96**	BP=0.85	W=0.98	BP=10.58
p-value	**p=0.0003**	p=0.65	p=0.167	p=0.226
PAPP-A MoM				
Test statistic	**W=0.96**	BP=0.86	W=0.98	BP=12.36
p-value	**p=0.0004**	p=0.65	p=0.104	p=0.136
Composite scores				
Free Estradiol Index				
Test statistic	W=0.98	BP=1.68	W=0.98	BP=13.22
p-value	p=0.64	p=0.641	p=0.491	p=0.104
Free Testosterone Index				
Test statistic	W=0.98	BP=1.00	W=0.98	BP=11.72
p-value	p=0.08	p=0.801	p=0.129	p=0.164
Steroid Factor				
Test statistic	W=0.98	BP=0.58	W=0.99	BP=15.79
p-value	p=0.142	p=0.902	p=0.512	p=0.066

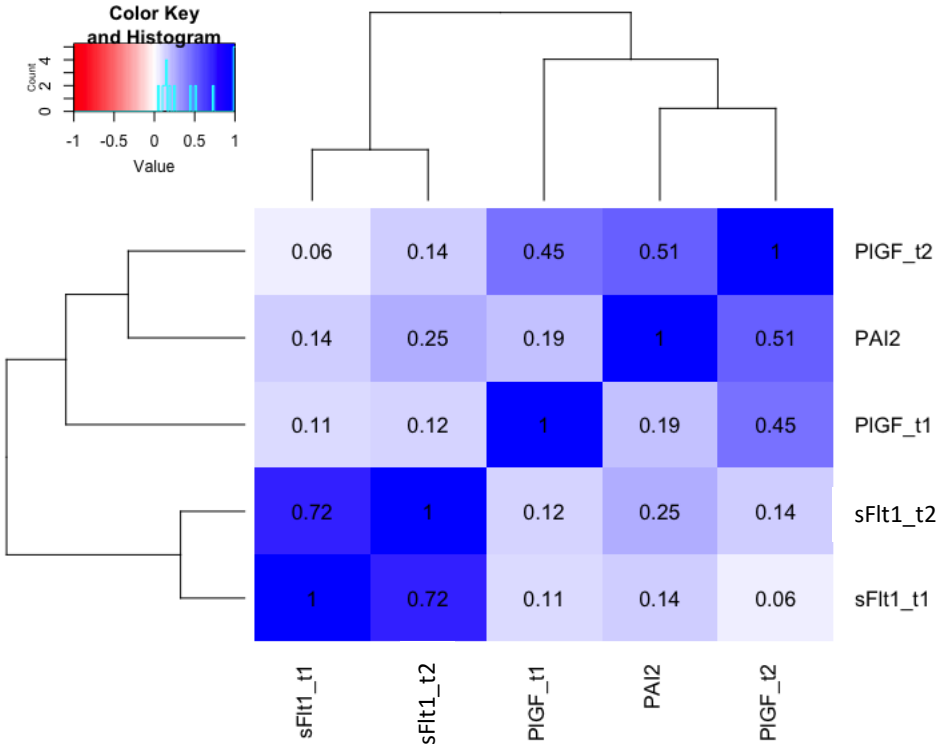
***Appendix Table 3.9**: Sensitivity analysis for hormone-to-autistic trait associations, which excludes participants that conceived via IVF. Effects are marginally non-significant in multiple regression models (AQ-FEI: p=0.065 / QCHAT-E2: p=0.052) but the trend is consistent with the reported findings of the main study.*

	AQ			
	Standardised Coefficient	**Standard Error**	**Partial Correlation Coeff**	**p - value**
Estradiol				
Pearson's	r=0.11			p=0.242
MR [1]: intercept	r=11.63	12.29		p=0.35
: predictor	r=0.70	1.24	r=0.05	p=0.58
Testosterone				
Pearson's	r=0.11			p=0.25
MR [1]: intercept	r=17.24	4.37		p=0.00
: predictor	r=0.61	1.10	r=0.05	p=0.58
FEI				
Pearson's	r=0.24			**p=0.016**
MR [1]: intercept	r=14.10	4.55		p=0.002
: predictor	r=0.92	0.50	r=0.18	p=0.065
FAI				
Pearson's	r=0.20			**p=0.041**
MR [1]: intercept	r=13.00	5.00		p=0.011
: predictor	r=6.60	3.83	r=0.17	p=0.090
	Q-CHAT			
Estradiol				
MR [1]intercept:	r=114.39	36.48		p=0.002
hormone:	r=-3.87	2.45	r=-0.17	p=0.119
hormone-by-sex:	r=8.19	4.19	r=0.21	p=0.052
Testosterone				
MR [1]intercept:	r=81.70	29.61		p=0.010
hormone:	r=-1.90	2.16	r=-0.10	p=0.380
hormone-by-sex:	r=0.38	3.41	r=0.01	p=0.912
FEI				
MR [1]intercept:	r=83.52	29.65		p=0.006
hormone:	r=-0.78	1.47	r=-0.06	p=0.597
hormone-by-sex:	r=1.89	1.77	r=0.11	p=0.289
FAI				
MR [1]intercept:	r=79.34	29.73		p=0.009
hormone:	r=2.05	8.94	r=0.02	p=0.819
hormone-by-sex:	r=-10.04	11.87	r=-0.09	p=0.410

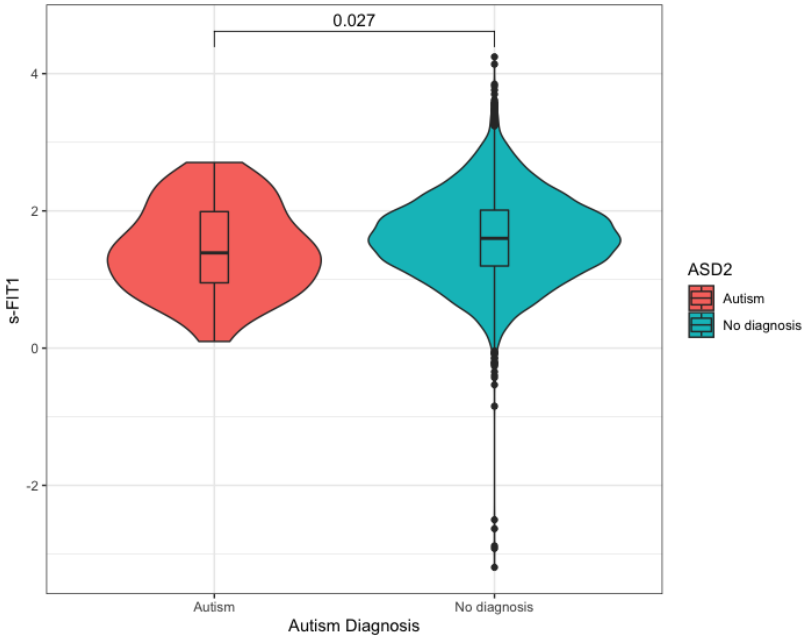
Appendix 4 - Study 3 Supplementary Material

Appendix Figures

Appendix Figure 4.1: Heatmap of the pairwise correlations of all assayed placental markers in maternal plasma. All values are Pearson's correlation coefficients, significant at p<0.001. t1: 1st trimester measurement / t2: 2nd trimester measurement.



Appendix Figure 4.2: Values of sFlt-1 (log-transformed). Males diagnosed with autism have significantly lower levels of sFlt-1 in maternal plasma in the 2nd trimester (U-test: p=0.027).



Appendix Tables

***Appendix Table 4.1**: Maternal characteristics in the Generation R cohort, their differences according to fetal sex (p-value for Mann Whitney U-test) and their association with z-scores of SRS Scores (values for univariate linear regression models). Further details in previous publications. *BMI at the start of pregnancy.*

	Age of child (months)	*Maternal age (years)*	*Maternal BMI**	*Maternal Education*	*Birth Weight (grams)*
Cohort	mean=74.02 SD=5.8	mean=31.36 SD=4.69	mean=24.89 SD=4.56	1: Primary or less, n=151 2: Secondary, n=1845 3: Higher, n=3063	mean=3409.5 SD=563.7
Males	mean=74.05 SD=5.87	mean= 31.41 SD=4.72	mean=24.80 SD=4.49	1: n=81 2: n=912 3: n=1547	mean= 3470.4 SD=580.14
Females	mean=73.98 SD=5.71	mean= 31.31 SD=4.66	mean=24.97 SD=4.63	1: n=78 2: n=929 3: n=1512	mean= 3347 SD= 539.3
p for sex	0.651	0.420	0.123	0.534	**<0.0001**
SRS Scores					
β to SRS	**0.10**	**-0.127**	**0.087**	**-0.248**	**-0.058**
p-value	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

Appendix Table 4.2: *Maternal characteristics in the Generation R cohort, and their correlation to placental marker concentrations in the 1st and 2nd trimester (via Pearson's) . *BMI at the start of pregnancy.*

	*Gestational age**	*Maternal age*	*Maternal BMI**	*Placental Weight*	*Birth Weight*
1st trimester					
PAI					
β	**0.622**	0.018	**-0.161**	**0.07**	0.012
p-value	**< 0.0001**	0.304	**< 0.0001**	**< 0.0001**	0.510
PlGF					
β	**0.713**	0.019	0.007	**0.059**	0.008
p-value	**< 0.0001**	0.277	0.60	**0.0001**	0.644
Sflt-1					
β	**0.071**	-0.027	**-0.142**	**0.130**	**0.084**
p-value	**< 0.0001**	0.123	**< 0.0001**	**< 0.0001**	**< 0.0001**
2nd trimester					
PlGF					
β	**0.315**	-0.01	**-0.119**	**0.072**	**0.047**
p-value	**< 0.0001**	0.541	**< 0.0001**	**< 0.0001**	**0.003**
Sflt-1					
β	**0.024**	**-0.084**	**-0.154**	**0.068**	0.018
p-value	**0.044**	**1.046e-07**	**< 0.0001**	**< 0.0001**	0.264
Longitudinal					
PlGF - e					
β	NA	-0.008	**-0.10**	**0.047**	-0.004
p-value		0.645	**< 0.0001**	**0.004**	0.835

Appendix Table 4.3*: Full linear Regression models for sex differences of placental marker concentrations, controlling for placental weight and gestational age. Marker levels have been log-transformed. 'PlGF-e': the rate of PlGF increase between measurements*

1st trimester	*N*	*Sex*	*Placental Weight*	*Gestational Age*	*MR Model*
PAI-2	5900	β=-0.0715	β=0.0002	β=0.1268	Adj.R^2=0.3952
		p<0.0001	p<0.0001	p<0.0001	p<0.0001
PlGF	5963	β=-0.0461	β=0.0002	β=0.2389	Adj.R^2= 0.5174
		p=0.0011	p < 0.0001	p < 0.0001	p= <0.0001
sFlt-1	5951	β=-0.0859	β=0.0005	β=0.0227	Adj.R^2=0.0289
		p<0.0001	p<0.0001	p<0.0001	p<0.0001
2nd trimester					
PlGF	7294	β=0.055	β=0.0002	β=0.1408	Adj.R^2=0.098
		p=0.0001	p<0.0001	p<0.0001	p<0.0001
sFlt-1	7292	β=-0.0776	β=0.0003	β=0.0084	Adj.R^2=0.0081
		p<0.0001	p<0.0001	p=0.256	p<0.0001
Longitudinal					
PlGF - e	5250	β=1.6421	β=0.0058	N/A	Adj.R^2=0.0036
		p=0.0072	p=0.0051		p=0.0004

***Appendix Table 4.4**: Sensitivity Analyses for PlGF at the 2nd trimester, in association with autistic traits (SRS z-scores). Placental markers have been log-transformed and adjusted for placental weight and gestational age. Linear regression models for SRS also control for age of child at the time of SRS measurement. 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, birth weight adjusted for gestational age and maternal education level.*

**European ethnicities include the following categories: Dutch, American-western, Asian-western, Turkish, European, Oceanian.*

***Complications excluded include: Pregnancy-induced Hypertension, Preeclampsia, Born small for gestational age and Spontaneous Preterm Birth.*

PlGF to SRS	*MALES*				*FEMALES*			
Cohort Subsets	N	β	p	Model Adj-R^2	N	β	p	Adj-R^2
European ethnicities*								
Model 1	1037	0.012	0.849	0.006	1009	**0.120**	**0.011**	0.007
Model 2		0.034	0.584	0.067		**0.120**	**0.011**	0.039
No autism diagnosis								
Model 1	1884	0.072	0.154	0.020	1842	**0.150**	**0.001**	0.014
Model 2		0.071	0.147	0.114		**0.129**	**0.002**	0.094
No complications*								
Model 1	1707	0.059	0.325	0.008	1652	**0.190**	**0.000**	0.019
Model 2		0.070	0.076	0.074		**0.123**	**0.000**	**0.078**

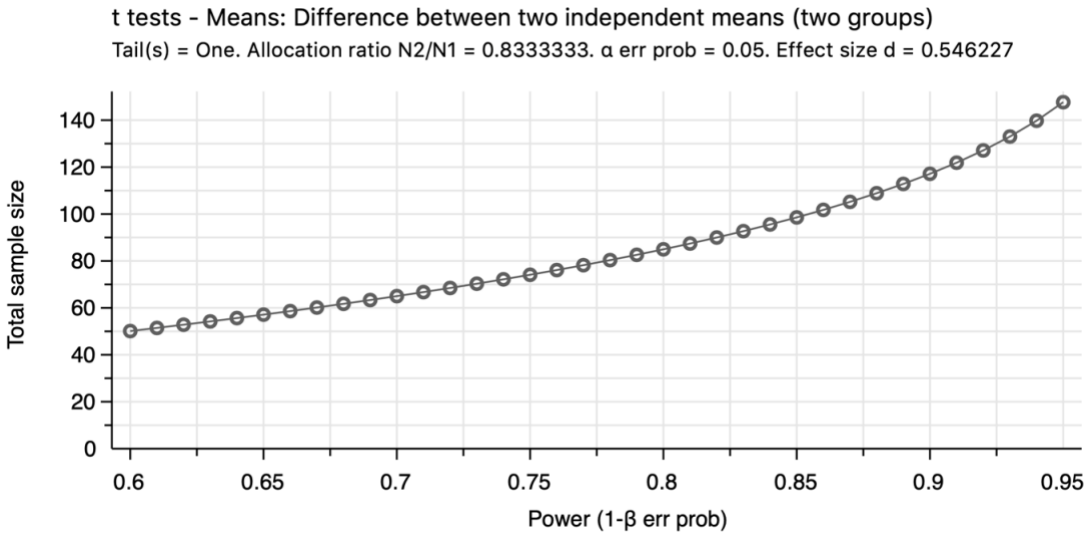
Appendix Table 4.5: *Full logistic Regression model of sFlt-1 levels, in association with a diagnosis of autism in both males and females. SFlt-1 levels have been log-transformed and adjusted for placental weight and gestational age at time of measurement.*

2nd trimester sFlt-1 to autism	***Autism Diagnosis N=59 males / N=9 females / N=7227 undiagnosed***			
	Coefficient	SE	z-value	p-value
Intercept	β=--4.61	1.20	-3.84	0.0001
Sex	β=-4.76	1.64	-2.91	0.003
sFlt-1	β=-0.53	0.26	-2.08	0.038
sex*sFlt-1	β=1.66	0.83	2.00	0.045
birth weight	β=0.02	0.01	1.23	0.219
				Model AIC: 424.85

Appendix 5: Chapter 4 Power Analysis

Based on the salivary testosterone distributions for males and females, their respective means and standard deviations, a power analysis was conducted using software G*Power (Appendix Figure 4.). For the projected effect size for Cohen's D=0.55, a total sample size of n>86 would be required for a statistically significant result in two-tailed Student's t-test. This is consistent with previous publications (Appendix Table 4.) on salivary testosterone measurements, where no difference was found when n<35 (Auyeung et al, 2012).

Appendix Figure 5.1: *Power analysis plot with the projected sample size required to achieve a statistically significant sex difference in salivary testosterone levels (n>86), given the properties of the measured distributions in Study 4 (Cohen's D=0.55). Obtained via G*Power.*



Appendix Table 5.1: *Comparison of sample sizes and significance of sex differences in the published studies of mini-puberty testosterone levels (T) and autistic traits in infants.*

	n - males	n - females	Effect size	p - value
Studies of salivary T during mini-puberty ***Auyeung et al 2012***	15	20	d=0.31	p>0.05
Kung et al 2016	39	47	d=0.55	**p=0.01**
Study 4	18	15	d=0.55	p=0.14