Fetal Biometry and Early Behavioural Development

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“There are times in life when people must know when not to let go. Balloons are designed to teach small children this.”
Terry Pratchett

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 preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution, except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit of 60,000 words

Ezra Aydin
December 2019

Preface

The ultrasound and follow-up data used throughout this thesis were collected by myself, research students and ultra-sonographers at the Rosie Maternity Hospital.

Chapter 3 and **4** has been published in Prenatal Diagnosis as: Fetal anogenital distance using ultrasound: A replication study. Aydin, E., Holt, R., Chaplin, D., Hawkes, R., Allison, C., Hackett, G., Austin, T., Tsompanidis, A., Gabis, L., Ziv, S., & Baron-Cohen, S. (**2019**)

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The last four years have been filled with hard work, laugher and adventure. It takes a village to raise a child…it took the love and support of a village to help me write this PhD. There wasn't a way I was going to be able to write this without a glass of wine in my hand. So, glass filled, let's begin shall we?

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This thesis is dedicated to all the lovely people I met during my time at Cambridge, and to the memory of Bradfield who I met for the last time.

Abstract

The overall aim of this thesis is to explore the use of standardised fetal biometric measurements and their relationship, if any, with later infant outcomes. In addition, the possible influence of fetal sex and several maternal conditions (polycystic ovary syndrome (PCOS), hirsutism and autism) on fetal growth measurements, are explored. The biometrics include anogenital distance (AGD), several brain measurements (transcerebellar diameter (TCD), ventricular atrium (VA)) and head circumference (HC). AGD was included as a proxy for prenatal sex steroid hormones, given the importance of the latter as an influence of brain development and for a number of autistic traits postnatally. The fetuses were assessed using ultrasound at 12-, 20- and between 26-30 weeks gestation and followed up at 18-20 months' of age to see if these fetal biometric measurements are associated with later language and sensory development, as well as early autistic traits.

Chapter 1 gives an overview of current research on fetal development, as well as the different methodologies used. This includes an introduction to the maternal conditions considered in this thesis and the fetal biometrics (HC, VA, TCD and AGD) that are measured. **Chapter 2** explores potential sex differences and the influence that maternal conditions (PCOS, hirsutism and autism) may have on developing fetal brain structure. Results indicate no significant sex differences between fetal brain measurements, or growth velocity. In addition, there was no relationship between maternal conditions and the fetal growth measurements. **Chapter 3** explores the feasibility of measuring AGD *in utero* (as a proxy for prenatal sex steroid hormones). Further, it examines the influence of maternal conditions such as PCOS (which is associated with increased testosterone) on AGD *in utero*. Significant sex differences in AGD were demonstrated, supporting previous findings. Results showed no relationship between fetal AGD and maternal testosterone related conditions (autism, PCOS and hirsutism). **Chapter 4** examines population based differences in fetal biometry and the applicability of findings from **Chapter 3** to an Israeli population. Results indicated that there were significant differences in AGD between Israeli and UK populations, potentially attributed to ethnicity. This supports the need for population-based or customisable growth charts if this measure is to be used clinically. **Chapter 5** explores the relationship between fetal brain measures and early behavioural

development at 18-20 months. The outcome measures include the Quantitative Checklist for Autism in Toddlers (Q-CHAT), the MacArthur-Bates Communicative Development Inventory (MB-CDI) short form and the Infant/Toddler Sensory Profile (ITSP). Results indicated a significant positive relationship between TCD and VA size at 20 weeks and Q-CHAT scores at 18-20 months' of age, which remained significant for females only when examining sex differences. There were no significant associations between the other fetal brain measurements and the MB-CDI short form, Q-CHAT or the ITSP. **Chapter 6** explores the relationship between fetal AGD and early behavioural development at 18-20 months. No significant relationships were found between fetal AGD length and infant development.

The results from the thesis are summarised in **Chapter 7** where broader theoretical and clinical implications of the findings are discussed. From the results presented in this thesis, it is apparent that one biometric measure (AGD) displays sex-based differences and will require sex-specific growth charts if it is to be used clinically. However further research is warranted to assess the clinical usefulness of AGD as a measurement. Additionally, for the first time a relationship between gross fetal brain structures (TCD and VA) and early autistic traits was measured. In conclusion, this thesis discussed the possible use of novel biometrics (AGD) for research and clinical use. Additionally, the potential for using fetal biometrics to help assess later infant outcomes, in particular autistic traits is presented.

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Abbreviations List:

AC ..... Abdominal Circumference

AGD ..... Anogenital Distance

BPD ..... Biparietal Diameter

BW ..... Birth Weight

CAH ..... Congenital Adrenal Hyperplasia

CAST ..... Childhood Autism Spectrum Test

CDI ..... Communication Development Inventory

CUSP ..... Cambridge Ultrasound Siblings and Parents Study

EFW ..... Estimated Fetal Weight

FL ..... Femur Length

fT ..... Fetal Testosterone

GA ..... Gestational Age

HC ..... Head Circumference

ITSP ..... Infant/Toddler Sensory Profile

IUGR ..... *Intrauterine* Growth Restriction

LBW ..... Low Birth Weight

LGA ..... Large-for-Gestational-Age

MB-CDI ..... MacArthur Bates Communication Development Inventory

MCA ..... Middle Cerebral Artery

MRI ..... Magnetic Resonance Imaging

NICU ..... Neonatal Intensive Care Unit

PI ..... Pulsatility Index

PCOS ..... Polycystic Ovary Syndrome

Q-CHAT ..... Quantitative Checklist for Autism in Toddlers

SGA ..... Small-for-gestational-age

TCD ..... Transcerebellar Diameter

UA ..... Umbilical Artery

VA ..... Ventricular Atrium

1 Introduction

1.1 Fetal Development: An Overview

Until the 1990's, research into early experiences in humans referred only to the postnatal stages of development. It was also believed that the fetus, whilst *in utero*, was insulated and protected from harm from outside influences via the maternal-fetal unit (Alkandari et al., 2015)[*]. Since then, this view has shifted, and research has reflected the increased awareness of the influences of the *intrauterine* environment. This included exploring fetal development[†] and its links to later cognitive, social and perceptual development (Leitner, Fattal-Valevski, Geva, Eshel, Toledano-Alhadef, Rotstein, Bassan, Radianu, Bitchonsky, Jaffa, & Harel, 2007; Lewis, Austin, Knapp, Vaiano, & Galbally, 2015; Rees & Harding, 2004). Epidemiological studies have linked conditions such as schizophrenia (Messias et al., 2007; Opler & Susser, 2005) and affective disorders (Brown, 2000) which become apparent after birth, with the prenatal environment, showing this period of development is critical to later life (Lewis et al., 2015; Mulder et al., 2002). During prenatal development, the unborn child has the potential to be negatively influenced by a variety of factors such as (but not limited to) maternal infections (Debnath et al., 2015), maternal emotional state, and teratogens (Mulder et al., 2002). Such factors in the *intrauterine* environment shape later biological, neurodevelopmental and

[*]Maternal-fetal unit is the ''structurally and functionally interconnected metabolic unit shared by a mother and fetus through the placenta, which determines the levels of drugs and hormones in the mother and fetus during gestation." (*Maternal-Placental-Foetal Unit | Definition of Maternal-Placental-Foetal Unit by Medical Dictionary*, n.d.)

[†] In this thesis, the term 'fetal development' will be used when referring to the development of the fetus that also has implications in later cognitive and behavioural functioning. The term 'fetal growth' will be used when observing discussing only fetal biometric measurements.

physiological outcomes. For example, alterations* in the *intrauterine* environment have been linked to later cognitive functioning (e.g., memory and learning) (Leitner et al, 2007; Pereira & Ferreira, 2015) and the onset of schizophrenia (Boksa, 2008; Debnath et al., 2015). Research in fetal development has highlighted the importance of early identification of potential influences (Alkandari et al., 2015) to support early diagnosis, treatment and neurodevelopment of infants from the postnatal stages of development, if we are to optimise their long-term outcomes.

'Developmental programming', a term coined to encompass all early postnatal influences (fetal, prenatal and *intrauterine*), is the view that alternations within the *intrauterine* environment can have influences on a variety of biological mechanisms (e.g., metabolism and neuroendocrine pathophysiology) influencing the individual in later life. These influences can be maternal, fetal and/or placental in nature, and this has led to a plethora of research exploring the mediating and moderating effects of external environmental influences (e.g., famine (Painter et al., 2005) and pollution (Tang et al., 2014)), adverse *intrauterine* environments (e.g., *intrauterine* growth restriction (IUGR) (Sharma et al., 2016)) and maternal conditions and behaviours (e.g., prenatal maternal stress (Lou et al., 2008; Mulder et al., 2002) and drug use (Mick et al., 2002). However due to essential ethical constraints on human research, our current knowledge about the prenatal determinants of childhood outcomes and adult disease is limited. For example, current knowledge about malnutrition and stress during pregnancy comes from studies observing the influence of natural disasters, catastrophic events (e.g., Zika virus (Oliveira Melo et al., 2016), ice storms (King et al., 2012) and earthquakes (Tan et al., 2009)) and limited 'rare' populations (e.g., maternal smokers (Iñiguez et al., 2013; Reissland, Francis, Kumarendran, & Mason, 2015)).

Animal models have been invaluable for exploring prenatal influences and to help bridge the gap in our understanding and give direction to research in humans. Experimental manipulations of conditions in human fetal, neonatal and infant research is constrained and, is in most

* These alternations range anywhere from intrauterine growth restriction (IUGR) to maternal infections. IUGR has been linked to later cognitive functioning (Leitner et al, 2007) and can be caused by a number of factors such as maternal substance use, maternal medication (e.g., anticonvulsants) as well as ethnicity or race (Sharma et al., 2016). Maternal infection during pregnancy has also been linked to later outcome in that it is considered a plausible risk factor for schizophrenia (Boksa, 2008; Debnath et al., 2015). Animal models have demonstrated maternal infection during pregnancy has the ability to impact the central nervous system development in the fetus, effecting the overall structure, function and behaviour (Minakova & Warner, 2018).

instances, rightly unethical. For example, animal models have allowed researchers and clinicians to explore the influences of IUGR on the developing fetus, as well as the outcome of interventions (Swanson & David, 2015) (e.g., protein restriction of the mother (Resnick et al., 1982)). Research in fetal development and fetal growth use animal models which helps shape, derive and test developmental models and theories, which can be applied in research with human fetuses in a non-invasive and ethical manner, for example through ultrasound. The work discussed within this thesis will focus on research derived from observations into human fetal development.

During development the fetus experiences periods of fetal vulnerability, and adversities (e.g., restriction in food) that lead to variations and long term effects can be observed in later development (Kinney et al., 2008). During early to mid-gestation, the placental barrier modulates fetal exposure to circulating maternal hormones. Animal models have suggested that this barrier becomes less active towards the end of mid-gestation (approximately 19-26 weeks gestational age (GA) for the human fetus, coinciding with the end of neurogenesis[*]), making the fetus more vulnerable to fluctuations in hormone levels (Seckl, 2006). Research has identified a period of fetal vulnerability that can influence the onset of later neurological, neurodevelopmental and physical issues. However, this period of vulnerability appears to range from the first trimester of pregnancy through to the last 10 weeks of gestation (Reissland & Kisilevsky, 2016) (between 30- and 40- weeks GA). In the last 20 years, fetal growth and fetal development research has shed light on potential links between prenatal development and later adult outcomes. In turn it has helped develop our understanding about potential mechanisms associated with altered fetal growth and later neurological deficits, non-communicable diseases and reproductive problems. Inconsistencies in findings and limited research into fetal development and fetal growth show the difficulties researchers and clinicians face, when attempting to draw conclusions and devise appropriate interventions. However, continued research into this area will help inform support-based interventions (beginning from the prenatal stages of development), which in turn may ultimately improve the health of the population and reduce the burden on our healthcare system.

[*] Neurogenesis is defined as the "formation of new neurons from the neural stem and progenitor cells which occurs in various brain regions such as the subgranular zone of dentate gyrus in the hippocampus and the subventricular zone of lateral ventricles" (Begega et al., 2017).

1.1.1 Fetal Development and Sex Differences

It is important to acknowledge sex differences in fetal development and the influence this may have on development across an individual's lifespan. There are sex differences in how this environment may influence outcomes (Bale, 2011; Di Renzo et al., 2007). Whilst the impact of biological sex on the developing fetus (such as the increase fetal sex has on vulnerability or protection) is still largely unknown, sex differences in development (from infancy to adulthood) have been well documented. Studies consistently demonstrate that more male fetuses are born preterm (Zeitlin, 2002), have higher risk of obstetric complications (Di Renzo et al., 2007), motor and cognitive deficits and increased numbers of developmental diagnoses (Kraemer, 2000). This discrepancy between the sexes is potentially due to the greater ability of female fetuses on average to be able to adapt to early adversity *in utero* (Sandman et al., 2013). However, it is argued that this adaptation observed in females on average comes at a price, as an increase in vulnerability in later development (e.g., affective conditions including anxiety (Sandman et al., 2013)). In a recent review DiPetro and Voegtline (2017) concluded that it is largely unknown how 'average' sex differences influence fetal development due to the limited number of studies that report sex differences. Due to the nature of fetal development research, limitations are a common feature in this field (for example, important ethical restrictions, and only using retrospective study designs).

1.2 Methods for Studying Fetal Development

Numerous methods have been used to study early physical development of the human fetus. These methods can be differentiated by the type of study design (i.e. retrospective or prospective studies) and the tools utilised for the measurement (i.e. magnetic resonance imaging (MRI) and ultrasound). Each design and technical measurement tool has their own merits and limitations.

1.2.1 Retrospective Studies

One of the simplest methods is the use of retrospective research. Retrospective studies provide insight into the influence of exposures during the prenatal period, such as maternal smoking and drug use (Jaakkola & Gissler, 2004; Knopik et al., 2016). However, retrospective studies are limited to standardised measures (e.g., those that can be found on medical records) and can be subjective (e.g., collecting pregnancy information from parents post pregnancy). For

example, Jaakkola et al. (2004) observed the relationship between maternal smoking, fetal growth and childhood asthma. Data was obtained retrospectively from 5 national administrative health registries in Finland. Researchers were constrained by maternal self-report of the number of cigarettes smoked on average per day*. This limits the generalisability of the conclusions the researchers were able to draw in relation to maternal smoking. In contrast, MRI and ultrasound research allow for 'real time' prospective development data to be collected, which can be compared to the infant's later development. A limitation of these two latter methods is that, unlike retrospective methods, they are time-consuming, expensive, often have small sample sizes, and are prone to high drop-out rates.

1.2.2 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is a useful tool for identifying risk of complications in expectant mothers, such as atypical fetal brain development (Prayer et al., 2006; Triulzi et al., 2005), and placental abnormalities (Girardi, 2016). Whilst both MRI and ultrasound can detect subtle anatomical details of the fetal brain (Achiron et al., 2004; Pistorius et al., 2010), MRI allows for the layers of the parenchyma to be imaged with more clarity than can be done using ultrasound (Prayer et al., 2006), enabling easier recognition of physical abnormality of the fetus. This may be useful in instances where growth abnormalities have already been identified in the expectant mother using ultrasound. MRI is recognised as the gold standard for examining brain development. However, MRI is not advised for use before 24-26 weeks GA because of the small size brain structures which it is not able to image, lack of spatial resolution (Garel et al., 2011)† and there is some debate about the potential for harm to the fetus caused by MRI. Research examining the potential consequences of MRI on fetal stress (Leithner et al., 2008), noise and thermal influences on the fetus (Arulkumaran et al., 1992; Gowland & De Wilde, 2008), on rate of genetic mutations (Schreiber et al., 2001) and longitudinal follow up studies (Clements et al., 2000; Kok, de Vries, Heerschap, & van den Berg, 2004) have reported no

* In research of this nature it has been previously observed that it is likely that parents might not accurately remember the number of cigarettes smoked on average per day. Additionally there a number of other variables that could influence fetal smoking exposure such as (1) the nicotine content is known to differ between brands, (2) how deeply the individual inhales and (3) amount of the cigarette smoked (Klebanoff et al., 1998). Such variables are not possible to take into account in retrospective research.

† 'Lack spatial resolution' is used refer to the limited definition (between white and grey matter) and structural growth of the fetal brain at this point in development.

significant influence of MRI on the developing fetus. However, as a research method, MRI is viewed as more invasive (it is noisy and often takes at least 30 minutes) and is therefore less welcomed by expectant mothers when it is not an essential procedure.

In contrast, the use of multiplanar visualisation in ultrasound, along with advances in its spatial resolution, has made it possible diagnose cerebral malformations through the use of ultrasound alone (Garel et al., 2011). Therefore, for the purposes of this thesis I focus only on ultrasound. It should be noted that MRI is being used in a parallel study, the Cambridge Human Infant Longitudinal Imaging (CHILD) study, and the participants in the study described in this thesis, the Cambridge Ultrasound Sibling and Parents (CUSP) study[*] are invited to take part in both studies.

1.2.3 Ultrasound

The use of transabdominal ultrasound in research is reliable[†], non-invasive, and can be used in the general population. Ultrasound is a medical imaging technique that uses high frequency sound waves and their reflections. High frequency sound waves are passed into the body using a probe, which hit the boundary between tissues (e.g., between soft tissue and bone, fluid and soft tissue). They are then reflected into the probe, and the machine uses these reflected sound waves to calculate the distance from the probe to the tissue and/or organ. Using these distances, the machine creates the scan image. A large number of studies have used ultrasound to examine the influence of the prenatal period on later child and adult outcomes (such as blood pressure in childhood (Blake et al., 2000)). These studies have used fetal growth measurements (e.g., Head Circumference (HC), Abdominal Circumference (AC), Femur Length (FL) and Estimated Fetal Weight (EFW)). In the UK, all measures are taken during a standard ultrasound (given at 12- and again at 20-weeks) and for research purposes data can be collected both prospectively and retrospectively.

[*] The longitudinal data (from 12 weeks gestation to 18-20 months' of age) collected from the CUSP study will be the subject of this thesis.

[†] Across the world ultrasound measures are standardised and are measured using various landmarks. This enables the data collected across various sites and/or countries to be directly comparable without the need for inter- and intra-rater reliability. Measurements are done by a trained sonographer.

The commercial introduction of 2D ultrasound around the 1980's (Campbell, 2013) allowed for more novel research, such as exploration into the ontogeny of spontaneous fetal behaviour and movement. General fetal movements (movements of body, limbs and head) have been linked to development of the central nervous system and fetal sleep regulation, as well as brain activity (from the third trimester onwards) (Visser & Mulder, 2009). Observations of general fetal movements have provided insights into potential environmental influences and pregnancy complications such as preterm labour and prenatal maternal stress, based on the quality and quantity of these movements (Sival et al., 1992). However, due to intra- and inter-individual differences, fetal general movements are a poor predictor of fetal well-being (Nijhuis et al., 1999). In addition, the study of general fetal movements is considered time-consuming and impractical to be included as a routine screening method (Lebit & Vladareanu, 2011; Reissland & Kisilevsky, 2016).

The introduction of 3D and 4D ultrasound has been useful in maternal and paternal fetal bonding in instances of antepartum depression or when physical fetal abnormalities have been detected (e.g., cleft lip and palate) (Escalon et al., 2010; Ji et al., 2005). Ultrasound is offered routinely for mothers and is therefore more welcomed (compared to MRI) by women during pregnancy. The relatively low cost and increase in resolution over the decades has enabled ultrasound to be a valuable method to study fetal development non-invasively, with the potential of implementing research findings into routine clinical care[*]. There are many commonly taken measurements using ultrasound during pregnancy (e.g., HC, FL and AC). In addition to these, researchers have begun to explore the use of novel biometrics in predicting later child and adult outcomes (e.g., anogenital distance (AGD) (Castaño-Vinyals et al., 2012; Eisenberg & Lipshultz, 2015) and transcerebellar diameter (TCD) (Atallah et al., 2019).[†]

Whilst ultrasound is more cost effective than MRI, welcomed by mothers and a standard procedure in pregnancy, ultrasound is incapable of assessing prenatal to neonatal growth

[*] For example, clip lip and/or cleft palate is prevalent in 9.92 per 10,000 pregnancies (IPDTOC Working Group, 2011). The use of 3D ultrasound has been a valuable tool to help assess the severity but also help counsel families during the pregnancy. This gives consultants and families a chance to see the fetus, discuss causes of the abnormality and the corrective procedures involved (should they wish to pursue) (Davalbhakta & Hall, 2000).

[†] Whilst in the UK, TCD is commonly measured during 20 weeks gestation, it is still a novel measure across the rest of the world (Atallah et al., 2019).

longitudinally. Researchers utilise ultrasound to bridge the gap in our understanding between the prenatal and neonatal periods. However, unlike MRI, this method is not transferable to the neonate due to the absence of corresponding standard ultrasound planes (Koning, Roelants, et al., 2017)* after birth. Whilst we acknowledge this limitation of ultrasound when observing longitudinal growth (e.g., in brain development), for the purposes of this study, ultrasound allows novel and exploratory fetal growth measures to be linked to later behavioural development.

1.3 Maternal influence on fetal development

The introduction of the concept of "fetal origins of adult disease" (FOAD) by Barker (2004) led to an increase in the amount of fetal research focusing on potential prenatal determinants related to later health and disease. As highlighted earlier, one determinant that has been well documented by researchers is the influence of the mother on the developing fetus. This influence ranges from intrinsic (genetic) to environmental (*intrauterine*) factors, such as maternal emotional health (Kinsella & Monk, 2009; Weinstock, 2005) and smoking during pregnancy (Blake et al., 2000; Jaakkola & Gissler, 2004; Reissland et al., 2015). These factors have been associated with having pre- and postnatal influences on the fetus from altering central nervous system development (Kinsella & Monk, 2009; Wadhwa et al., 2001) to preterm birth (Staneva et al., 2015). Maternal influences can be separated into two main components, 'maternal health-related behaviours' and 'maternal conditions.'

Maternal health-related behaviours can be categorised into involving behaviours such as drug consumption (both prescribed and recreational), smoking, and prenatal maternal stress during pregnancy. These have been shown to have long-lasting effects on child development. Further research has demonstrated that prenatal maternal stress or anxiety during pregnancy alters the developing fetus' physiological development (Lou et al., 2008; Mulder et al., 2002). In this thesis, I will not be exploring the impact of potential health-related behaviours on fetal development†.

*Compared to MRI, cranial ultrasound uses standard protocol of acquiring images on the coronal, midsagittal and parasagittal planes (Rademaker, 2005). Whilst MRI uses sagittal, coronal and transverse planes. The difference in planes used to acquire these images makes it difficult to use as comparison.

†Mothers within the study were screened for drug use, smoking and alcohol consumption, and pregnancy records checked for prenatal maternal stress and anxiety diagnosis. Based on previous research they are known

A large body of research focuses on the influence of maternal conditions (e.g., maternal HIV or PCOS diagnosis) on the pregnancy itself (e.g., complications such as hypertension and IUGR (Kjerulff et al., 2011; Puttabyatappa et al., 2016)), birthing complications (e.g., assisted delivery (Qin et al., 2013)) or neonatal outcomes (e.g., low birth weight (LBW) (Ticconi et al., 2003)). However, to date, research into 'maternal conditions' are yet to include the influences of maternal psychiatric or neurodevelopmental diagnoses (such as autism or schizophrenia).

Due to the range of potential influences on prenatal development, for the purposes of this thesis, the focus will be on the influence of a maternal endocrine conditions and a neurodevelopmental condition on fetal development. One of the most common endocrine conditions affecting women of reproductive age is polycystic ovary syndrome (PCOS) (Gilbert et al., 2018; Lizneva et al., 2016). Whilst diabetes mellitus (both type 1 and 2) and gestational diabetes are also just as prevalent in women of reproductive age (Al-Rifai & Aziz, 2018; Lawrence et al., 2008), serious complications as a result of diabetes on fetal development are only present when left untreated (Bratila, 2016). During pregnancy all women in the UK are tested for gestational diabetes and, if necessary, treated. Autism spectrum conditions (hereafter autism) is a neurodevelopmental condition that has also been associated with endocrine dysregulation related to testosterone. This thesis will observe the influence of both maternal conditions on fetal development. To our knowledge, no research has explored the influence maternal autism has on the developing fetus.

1.3.1 Maternal Endocrine and Neurodevelopmental Conditions

Progression in assisted reproductive techniques (e.g., IVF) has allowed for more women with endocrine conditions (e.g., PCOS) to have successful pregnancies. However, this has produced unique therapeutic challenges for clinicians, as these women require consistent monitoring and treatment throughout their pregnancy (Calina et al., 2019). Maternal endocrine conditions can fall under two categories; conditions diagnosed before pregnancy and those diagnosed during pregnancy. Conditions left untreated or insufficiently treated can negatively influence fetal

to influence fetal development, therefore it was chosen not to include any of these behaviours. For example, maternal smoking has been associated to a reduction in BW (da S. Pereira, da Mata, Figueiredo, de Andrade, & Pereira, 2017) and growth during the second trimester (Jaddoe et al., 2007)

development (e.g., SGA in maternal PCOS (Sir-Petermann et al., 2005) and LBW in hyperthyroidism (Phoojaroenchanachai et al., 2001)).

During the first trimester of pregnancy the fetus is entirely dependent on the mothers' endocrine function. It is only during the second trimester, when the fetus' own hormonal glands begin to function that the fetus becomes less dependent (Monticone et al., 2012). However, these glands continue to develop in function and morphology until birth. Therefore, the mother's endocrine system influences the developing fetus throughout pregnancy.

Many maternal endocrine disorders (e.g., diabetes and hyperthyroidism) have been linked with a range of developmental complications in pregnancy such as spontaneous abortion, premature birth and pre-eclampsia (Calina et al., 2019). However, due to ongoing monitoring and treatment during pregnancy, few mothers experience the above complications as a result of an endocrine disorder. Conversely rarer endocrine conditions such as 'adrenal disorders' (e.g., congenital adrenal hyperplasia (CAH)[*]) are harder to diagnose due to the changes in the maternal endocrine system during typical pregnancies. These are associated with maternal and fetal mortality and morbidity if not promptly diagnosed and treated (Monticone et al., 2012). For the purposes of this thesis the focus will be on one of the most common endocrine conditions: PCOS in women who were given a diagnosis prior to pregnancy[†].

1.3.1.1 Polycystic Ovary Syndrome (PCOS)

PCOS is one of the most common endocrine disorders affecting 4-21% women of reproductive age (dependant on diagnostic criteria[‡]) (Lizneva et al., 2016). Polycystic ovary syndrome (PCOS) is a set of symptoms due to elevated androgens, particularly testosterone. Increased testosterone in the mother who has PCOS has been shown to influence the overall development

[*] CAH is a condition resulting from prenatally elevated androgens as a result of 'reduced efficiency of cortisol synthesis in the adrenal gland (Ruta et al., 2011).

[†] Within the CUSP study no mothers were diagnosed with PCOS during pregnancy and all mothers were receiving treatment for their diagnosis.

[‡] According to NHS guidelines PCOS is diagnosed if at least 2 of the following 3 criteria is fulfilled: (1) irregular periods, (2) high levels of testosterone in blood and (3) scans showing polycystic ovaries (*Polycystic Ovary Syndrome - Diagnosis*, 2017)

of her offspring. Recent research has demonstrated physical changes (e.g., lengthened anogenital distance (AGD) in female offspring (Barrett et al., 2018)) and cognitive developmental changes (e.g., falling behind peers in motor, personal-social and communication development in both males and females (Bell et al., 2018)) as well as increased likelihood of a later autism diagnosis in the offspring in both sexes (Cherskov et al., 2018; Kosidou et al., 2016).

Women diagnosed with PCOS often also suffer from a range of co-morbidities such as infertility and metabolic complications (Gilbert et al., 2018). PCOS is activated during puberty, with a diagnosis typically being made after menarche. The physiological changes in luteinizing hormones and insulin, combined with existing genetic and environmental factors, predisposes a percentage of women to a later diagnosis of PCOS (Franks, 2002). Whilst clinicians and researchers have identified the need for early diagnosis, the heterogeneous nature of the condition and the spectrum of clinical features creates variation in presentation of the condition across a woman's lifespan (Rosenfield, 2007; Teede et al., 2010). This can, therefore, cause problems in the diagnosis of PCOS before or during adolescence.

1.3.1.2 PCOS and Pregnancy

The pathophysiology of PCOS (i.e. elevated levels of androgens) leads to a number of reproductive issues, such as subfertility due to the irregularity of anovulatory cycles (Kamalanathan et al., 2013; McDonnell & Hart, 2017). Whilst mentioned above, the advances in medicine have allowed women to have successful pregnancies. However, they are not without their risks or complications. One of the most common complications experienced in women with PCOS is early pregnancy loss (EPL). This is defined as a miscarriage during the first trimester of pregnancy. Within the general population ELP occurs in 10-15% of women, compared with 30-50% of women with a PCOS diagnosis (McDonnell & Hart, 2017). Difficulties in conception and ELP have been attributed to hyperandrogenism and the hyperinsulinemic environment created as a result of the condition (McDonnell & Hart, 2017).

Alongside difficulties in conception and ELP, pregnant women with a PCOS diagnosis have been shown to be at a greater risk of gestational diabetes (GD), very preterm birth, preeclampsia, giving birth to baby's large-for-gestational age (LGA) and a greater likelihood of needing a caesarean section (CS)(Joham et al., 2016; Qin et al., 2013). It has been suggested

that the hyperinsuliemic environment as a result of the condition may directly influence vascular functioning, creating resistance and resulting in the observed increase in pregnancy complications (Joham et al., 2016). Currently little research has been conducting looking at the impact PCOS has on fetal growth, instead focusing on outcomes such as, increased in congenital anomalies (e.g. cardio and gastrointestinal anomalies), LGA and increased rate of hospital visits in childhood and adolescence (Doherty et al., 2015).

1.3.1.3 Hirsutism

Hirsutism* is defined as coarse hairs appearing on women in a male like pattern (such as bodily hair) (Azziz et al., 2000). The excessive hair growth is related to increased levels of circulating testosterone. Whilst hyperandrogenism is often caused by PCOS, this is not always the case. Approximately half of women who suffer from mild hirsutism have idiopathic hirsutism (Rosenfield, 2005). This is defined as having 'normal ovulatory function and normal circulating androgen concentrations' (Azziz et al., 2000). When the hirsutism is more moderate or severe then PCOS may be suspected and androgen levels assessed via blood tests (Rosenfield, 2005).Therefore, in this thesis I also include women with suspected hirsutism, as a separate sub-group to women with a PCOS diagnosis.

1.3.1.4 PCOS, Hirsutism and Fetal Development

Animal experiments have suggested that PCOS is related to excess androgen exposure, which disrupts the hypothalamus-pituitary-gonad (HPG) axis (Wu et al., 2010). These experiments have also shown an association between *in utero* hormonal exposure and a subsequent diagnosis of PCOS, which suggests that endocrine disruption occurs during prenatal development of the mother. Whilst the exact causes of PCOS is unknown, fetal androgen excess (in particular fetal testosterone (fT)) may be related to the aetiology of PCOS (Dumesic et al., 2007), the variation and timing of this excess explaining the diversity seen in the adult phenotype (Abbott et al., 2005). Studies are yet to confirm these speculations. However, elevated levels of prenatal exposure to testosterone increases later likelihood of neuroendocrine, ovarian and metabolic deficits in girls born to women with a PCOS diagnosis (Goodarzi et al., 2011). Barrett et al. (2018) demonstrated an on-average physiological

* Hirsutism affects approximately ~ 10% of the population (higher in certain ethnicities, e.g., east Asian) and has been linked to undiagnosed PCOS.

difference in girls born to women with a diagnosis of PCOS or hirsutism, where the AGD appears to be more masculinised in length, likely due to the increased exposure to testosterone *in utero*. The positive association between PCOS and AGD length is also observed in adult women with a diagnosis (further discussed in **Chapter 3**, *section 3.1.5.1*). PCOS also affects pregnancy and delivery complications such as higher rates of neonatal intensive care unit (NICU) admission, gestational diabetes and of pregnancy-induced hypertension (Boomsma et al., 2006). However, to date, the conclusions from these studies have been inconsistent and there is no research observing the physical development of the human fetus in mothers with a diagnosis of PCOS or hirsutism.

1.3.2 Autism Spectrum Conditions ('Autism')

Autism is a neurodevelopmental condition, diagnosed by impairments in social interaction, communication alongside unusually repetitive behaviours and narrow interests (American Psychiatric Association, 2013). Current prevalence for autism is estimated to be 1 in 59 (Baio et al., 2018)[*]. Explanations for this increase in prevalence of autism include improved recognition, greater awareness, the growth of services and clinics for autism, and changes in the diagnostic criteria (e.g. a dimensional 'spectrum' approach for autism) (Hansen et al., 2015). The introduction and acceptance of the heterogeneity of autism has contributed to the planning and implication of more successful intervention and educational services.

Autism is considered to be a multifactorial disorder. It is in part genetic (Abrahams & Geschwind, 2008), with an atypical neurodevelopmental trajectory (Elsabbagh & Johnson, 2010) and influenced by gene-environment interactions (Chaste & Leboyer, 2012) including potential prenatal developmental factors (Bonnet-Brilhault et al., 2018). Which in turn makes it difficult to determine which of these three factors could be considered the primary cause for each individual. In recent years' the prenatal sex steroid theory has gathered support (Baron-Cohen, 2002). The prenatal sex steroid theory proposes that elevated levels of prenatal androgens or estrogens *in utero* contribute to differences in brain development that are linked to the atypical cognitive traits observed in autism (Auyeung et al., 2009; Auyeung, Taylor, Hackett, & Baron-Cohen, 2010; Baron-Cohen et al., 2015).

[*] Baio et al. (2018) also state that this prevalence of 16.8 per 1,000 (1 in 59) also varied between the assessed cites, from 13.1-29.3 per 1,000. They suggest this variability may be due to sex, ethnicity, and other variables (Baio et al., 2018).

1.3.2.1 Sex differences in autism

Rates of autism diagnosis is consistently higher in males compared to females. The sex differences in ratio (estimated to be two to three males being diagnosed for every female (Lai et al., 2015)). Whilst this increase in prevalence is still debated, one suggestion is linked to the prenatal sex steroid theory (further discussed in *section 1.3.2.3*). A more recent line of research suggests a 'female protective effect' (FPE). The FPE theory attempts to explain the differences in number and severity of diagnosed cases in terms of genetic variability. This model suggests males display a greater genetic variability during development, resulting in an increase in the number of males with a diagnosis but with a decreased severity of autism. Whilst females may be more sensitive to genetic disruptions (implying genetic disruptions may lead to less female fetuses surviving to term) decreasing the number with a diagnosis of autism but increasing the severity (Ferri et al., 2018). Overall more recent research has shown a gene, epigenetic and environment interaction that contributes to not only an autism diagnosis, but this observed male bias. For example, when considering genetics, the x-inactivation genes are potentially thought to be protective (females), and the Y chromosomes haplotypes to potentially increase autism risk (males)(Ferri et al., 2018).

Currently research suggests that this difference in diagnosis rates may also be a product of the current diagnostic norms, as these have been prominently developed using male sample (Halladay et al., 2015). Research has suggested that the cut-off values created may not accurately capture females (Lundström et al., 2019). Instead only capturing females who represent the more extreme end of the spectrum (Lundström et al., 2019). Females have been observed to demonstrate fewer stereotyped and repetitive behaviour's and have subtler socio-communicative difficulties. Emerging evidence suggests female are more likely to 'internalise' their difficulties (Mandy et al., 2012) and adopt 'masking' or 'camouflaging' behaviour's (Hull et al., 2017), increasing anxiety and negatively impacting their own mental health (Mandy et al., 2012).

The theories discussed above demonstrate an important need for more representative research, inclusive of both sexes, observing longitudinal development incorporating a design including methods to explore gene, epigenetic and environmental influences and/or predictors of autism. Advances in techniques and methodologies have enabled researchers to begin exploring the

multifactorial aspects of autism. The combination of neurological differences, environmental factors and genetic characteristics are hoped to improve autism diagnosis and care.

1.3.2.2 Autism and Fetal Development

Early detection of autism is hoped will improve long term outcomes for children and families of autistic people. Early identification of autism or autistic traits in 'high risk' infants is now possible from 12 months' (e.g., using expressive, receptive language ability and the 1-year well-baby check-up (Pierce et al., 2011)) and 18 months' of age (e.g. using the Quantitative Checklist for Autism in Toddlers (Q-CHAT)(Allison et al., 2008)).

More recently, potential autism related biomarkers from the fetal stages of development has been explored. However, the association between fetal growth, pregnancy outcomes and later autism risk is inconsistent (Gardener et al., 2009). Accelerated head growth during the first year of life in children later diagnosed with autism (Courchesne, Campbell, & Solso, 2011; Courchesne, Redcay, Morgan, & Kennedy, 2005), in particular between 6-9 months' of age has been observed, only in males (Fukumoto et al., 2011). To date, only a handful of studies have used ultrasound to measure fetal growth in relation to autism. All studies have examined general fetal growth measures (HC, biparietal diameter (BPD) and FL) and findings have been mixed. A large-scale study found that deviance from expected fetal growth was related to increased risk of autism (Abel et al., 2013). However, EFW was used to assess fetal growth and compared to existing growth charts. As will be discussed in **Chapter 4**, these growth charts are now outdated and therefore these findings should be interpreted with caution. Additionally, this study was unable to suggest any underlying mechanism for the association between growth deviance and autism likelihood. This could be due to the study not controlling for a number of maternal influences linked to fetal growth such as nutrition and substance/alcohol use.

Hobbs et al. (2007), using 2D ultrasound data collected at 12- and 20-weeks, concluded there were no significant differences between autistic children and neurotypical children on all general fetal growth measures (p values ranging from .027 – .684). Similarly, a recent prospective longitudinal study using 2D ultrasound (Unwin et al., 2016) examined general fetal growth in pregnancy in a 'high risk' (existing sibling with an autism diagnosis) vs a 'low risk' group at multiple gestation points. They found that there were no significant differences in fetal

growth between the two groups (p values ranging from .09 - .78). None of the 'high risk'* fetuses were followed up later in development. Unwin et al. (2016) concluded that future research should examine the growth of sub-regions in the brain implicated in autism (e.g., corpus callosum or cerebellum), as opposed to only HC and general growth measures. A limitation of prospective ultrasound studies is their sample sizes. The study by Unwin et al. (2016) only had a sample of 23 'high risk' and 36 'low risk' pregnancies. Retrospective ultrasound studies are more varied in their sample sizes: 45 autism and 222 controls (Hobbs et al., 2007); 14 'high risk' and 56 'low risk' (Whitehouse et al., 2011), and 4,283 autism and 36,588 case matched controls (Abel et al., 2013). However, retrospective research is limited by what measures were taken at the initial ultrasound (such as the standard HC, AC and FL) and is, therefore, not the preferred method for furthering our understanding in this area.

1.3.2.3 Autism: A Maternal Endocrine Condition?

Research into fetal testosterone (fT) in autism was motivated by the documentation of a greater prevalence of autism in males than females (Leitner et al. 2007; Pereira & Ferreira, 2015). This observed sex ratio (estimated to be at least 2 or 3 males for every one female) may provide a clue into the underlying aetiology of the condition (Debnath et al., 2015). Prior research has explored the relationship between testosterone exposure *in utero* and autistic traits using measures of fT from amniotic fluid as the only ethical way to measure fT without affecting the fetus (Auyeung et al., 2010). Exposure to elevated levels of fT during pregnancy is associated with atypical brain development (Geschwind, 2011) that may later manifest itself in the atypical behaviours seen in autism (Baron-Cohen et al., 2015). However, to date, research using amniotic fluid samples are opportunistic, potentially biased by maternal age and taken only in pregnancies deemed to be 'high risk'. Therefore, this research is not representative and cannot be generalised to all pregnancies.

Due to the sex ratio observed in autism it may be that the underlying aetiological mechanisms are influenced during the prenatal stages of development and may present itself physiologically *in utero*. Therefore, in this thesis, autism will be included alongside PCOS and hirsutism as a potential influence on fetal growth when measuring novel fetal biometrics. As discussed above (*section 1.3.2.1*) autism research could benefit from a multifaceted approach (exploration of

*'High risk' was defined as having a sibling with a diagnosis. For the purposes of this thesis 'high risk' will also include families whether the mother has a diagnosis of autism.

genetic, epigenetic and environmental predictors). Due to the limitations imposed by a doctoral project, I will only be observing what would be considered as environmental factors (e.g. fT and fetal growth). Future directions taking into consideration genes and epigenetics will be discussed in **Chapter 7**.

1.4 A Novel Measure of the Influence of fT on Fetal Development: Anogenital Distance

A physical characteristic that has been used as an indirect measure of exposure to fT levels is the 2D:4D digit ratio (Manning, 2011; Manning et al., 2001; Markoulakis et al., 2012). This is the ratio of the second to the fourth digit, which is typically sexually dimorphic, is set during the prenatal period and correlates with exposure to testosterone and estrogens (i.e. a higher 2D:4D ratio is associated with lower fT and higher fetal estrogen level, whilst the opposite is observed when the 2D:4D ratio is lower) *in utero* (Lutchmaya, Baron-Cohen, Raggatt, Knickmeyer, & Manning, 2004). However, 2D:4D digit ratio is also influenced by postnatal and pubertal steroid levels, and is therefore subject to change throughout development (Dean & Sharpe, 2013). Anogenital distance (AGD) has been suggested as an alternative measure reflecting prenatal dihydrotestosterone levels (Dean & Sharpe, 2013). AGD is the distance between the anus and the scrotum or vulva (perineum). Although AGD continues to increase after birth, the differences observed between the sexes in AGD are maintained (Papadopoulou et al., 2013). AGD length is established during the prenatal 'masculinization programming window' (MacLeod et al., 2010)[*], and postnatal hormonal exposure does not reprogram AGD (Kita et al., 2016; R. T. Mitchell et al., 2015). ADG is thus a more robust biomarker of fT levels than 2D:4D digit ratio in both animal studies (Clemens et al., 1978) and in human infancy (Papadopoulou et al., 2013). Decreased AGD is associated with a variety of male reproductive outcomes (e.g., sperm count (Eisenberg & Lipshultz, 2015) and non-communicable disease's such as prostate cancer (Castaño-Vinyals et al., 2012)) and endocrine influences (e.g., phthalate exposure (Swan et al., 2005) and PCOS (Barrett et al., 2018)). In females the influences of endocrine conditions such as PCOS (Barrett et al., 2018) is associated with an increased AGD. The potential clinical utility of this measurement has resulted in

[*] The 'masculinization programming window' is thought to occur between 8-14 weeks' GA in humans. During this period in development sexual differentiation occurs, indifferent gonads and genitalia are transformed into sex-specific structures. This differentiation is driven by the production various hormones, primarily testosterone.

researchers exploring its usefulness in determining retrospective early life androgen disruption and its ability to predict a range of later outcomes. Currently, there is very limited research examining AGD length (Wainstock et al., 2017).

Anogenital distance is measured using several different landmarks (*see figure* 1). In males, AGD is measured from the centre of the anus to the posterior base of the scrotum (AGD_{AS}) or to the cephalad insertion of the penis (AGD_{AP}). In females AGD, is measured from the centre of the anus to the posterior convergence of the fourchette (AGD_{AF}) or to the anterior tip of the clitoral hood (AGD_{AC}). In epidemiological studies, the most commonly used is AGD_{AS} (anoscrotal distance) and AGD_{AF} (anofourchette distance). Therefore, in this thesis, this measurement shall be used when measuring AGD distance.

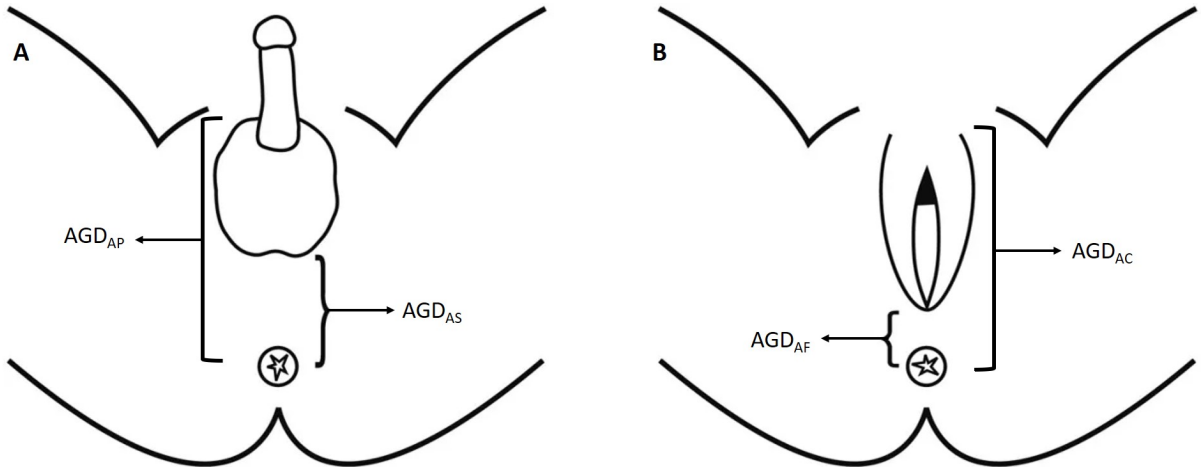

Figure 1: Landmarks for the different anogenital distance measurement in males (A) and females (B).

1.4.1 Fetal Anogenital Distance in Ultrasound

Researchers have shifted their attention to the applicability of measuring AGD in the developing fetus to study potential androgen disruption prospectively. In a landmark study, Gilboa et al. (2014) successfully measured AGD in 2D ultrasound scans of fetuses of 424 pregnant women between 20-35 weeks of pregnancy. Based on these measures, Gilboa et al. (2014) were able to plot AGD distance based on GA. Since the sexually dimorphic nature of this measurement has been demonstrated during prenatal development, suggesting its link to early androgen exposure, researchers have begun to explore the uses of this measurement from

the prenatal stages of development and links to later outcomes. Differences in fetal AGD has been found to be associated with hypospadias in men (Gilboa et al., 2017) and to be useful in determining fetal sex from 11 weeks GA (Arfi et al., 2016; Sipahi, Tokgöz, & Tosun, 2019). Research is yet to explore fetal AGD in relation to maternal endocrine influence (such as PCOS) and later child behavioural development (see **Chapter 3**, *section 3.2.4* for outlining practicalities around AGD imaging).

1.5 Novel Measure of the Influence of fT on Fetal Development: Fetal Brain Development

Several weeks after conception, the formation of the cerebral hemispheres can be observed (Muller & O'Rahilly, 1988), with spontaneous movements of the head and trunk being seen from as early as 9 weeks' gestation (de Vries et al., 1982). By the time a fetus is 37-40 weeks' GA, considered full term, it is able to demonstrate a level of cognitive capacity, such as the ability to discriminate between sounds (Kadic & Kurjak, 2018) and to anticipate directed movements (Reissland, Francis, Aydin, Mason, & Schaal, 2014). It is during these first 40 or so weeks of gestation that the foundations for later cognition and behaviour are created. During this period of prenatal development, the brain is susceptible to a range of factors, from the fetus' own circulating hormones to the impact of maternal influences. This thesis explores potential sex differences and the influence maternal conditions may have on developing brain structures.

1.5.1 The Influence of Hormones on Fetal Brain Development

1.5.1.1 Fetal Hormonal Influences and Sexual Differentiation

Sexual differentiation begins *in utero* via the secretion and exposure of the fetus to sex specific hormones (e.g., testosterone) (Swaab, 2007). During this period, the testicles and ovaries also develop between 6-12 weeks' gestation. The production of testosterone and dihydrotestosterone by the developing testes is essential for the development of the male reproductive organs. Conversely, female sexual organs develop through the absence of these circulating hormones. Once genital differentiation has occurred during the first trimester, sexual differentiation in the developing brain is observed from the second trimester onwards (Swaab, 2007). During this period in pregnancy, testosterone has a direct influence on brain

growth; it is this first peak of hormones that aids in the development of brain structures and circuits. When the second peak of hormones occurs (seen in puberty) these circuits are activated and certain behavioural patterns and conditions become apparent (e.g., schizophrenia) (Swaab, 2007).

On average sex differences in the brain have been well documented in child and adult MRI studies (Goldstein, 2001; Lenroot et al., 2007). For example, prenatal testosterone has been documented as having a measurable organisational effect on the developing brain. Lombardo et al. (2012) demonstrated a positive relationship between fT levels (measured via amniocentesis) and grey matter in several regions of the brain that during later development* showed clear sexually dimorphic differences. Using these findings authors suggest this demonstrates fT influences early organisation of a number of brain areas (e.g., hypothalamus) which later display sexually dimorphic differences. Potentially explaining the behavioural and cognitive differences observed between the sexes. However, the exact emergence of these differences, and whether emergence begins *in utero* remains unknown (Wheelock et al., 2019). Currently, standardised fetal growth charts used by clinicians (which measure HC, TCD and ventricular atrium (VA) development as early markers for developmental delays) do not always take into account average sex differences.

Research into sexual dimorphism *in utero* has produced mixed findings. To our knowledge, only two studies have observed sex differences in the fetal brain using MRI. Scott et al. (2011) found no difference between the sexes in total brain or hemispheric volumes in fetuses between 20 and 31 weeks GA. More recently, Wheelock et al. (2019) demonstrated differences in functional connectivity *in utero* between sexes in fetuses between 25 and 39 weeks GA. Both studies had relatively small sample sizes; 39 fetuses (19 male and 20 female) and 118 fetuses (70 male and 48 female) respectively. Small samples sizes combined with the large GA range suggest that these results should be interpreted with caution and further exploration is needed. It is of interest that the 'Developing Human Connectome' project(*Developing Human Connectome Project (DHCP) | The Developing Human Connectome Project*, n.d.) at Kings College London will have a sample size of 1000 fetuses, which should be adequately powered to detect any sexual dimorphism if it exists. However, this study is still ongoing.

* The sample within this study consisted of 217 children ages between 8-11 years (101 males and 116 females). fT levels were collected between 13-20 weeks' gestation via amniocentesis (Lombardo et al., 2012).

Other studies have explored sex differences using general fetal biometrics (i.e. AC, HC and FL) (Broere-Brown et al., 2016; Melamed et al., 2013) and including HC as a proxy measurement for brain development and size (Joffe et al., 2005). These studies have shown a significant difference in HC size from as early as the second trimester (Broere-Brown et al., 2016; Joffe et al., 2005; Melamed et al., 2013; Smulian et al., 1995). However, conclusions of findings have been mixed and inconclusive on whether males (Broere-Brown et al., 2016; Smulian et al., 1995) or females (Melamed et al., 2013) have a slower growth rate or if sexual dimorphism of HC largely emerges during the postnatal period of development (Joffe et al., 2005). Whilst research has identified a significant difference in HC (as well as other growth measures) between the sexes, the use of sex-based growth charts is still under debate. Researchers have acknowledged that whilst a statistically significant difference does exist, this difference may not be enough for clinical application. For example, Smulian et al. (1995) when measuring gross fetal biometry (e.g. AC, HC) found for the greatest difference between the sexes to occur at 21 weeks' gestation in the BPD. This difference was 1.15mm between males and females. Whilst this is statistically significant, the limited difference in range of this measure would not be and has not been considered to be significant enough to warrant sex-based growth charts.

In sum, there is very limited research examining differences in sex and whether sex hormones (e.g., fT) play a role in fetal HC or brain development. Our existing knowledge of early brain development suggests that the gross development of brain structures during the prenatal period is critical to an individual's later development. However, during this period of development, alongside the fetus' own hormonal influence, maternal influences also play a large role on shaping the fetal brain.

1.5.1.2 Maternal Influences

From 11-12 weeks' gestation onwards, maternal nutrients*, and maternal hormones including thyroxine and sex steroids, are transferred from the placenta into the fetus' circulation (de Escobar et al., 2008). During the first trimester of pregnancy, the mother is the main source of

* Maternal nutrients refer to the nutrients passed through the placenta into fetal circulation. These nutrients include glucose, amino and fatty acids and are essential to sustain appropriate fetal development(Brett et al., 2014).

hormones for the developing fetus and brain (de Escobar, Obregón, & Rey, 2004). This has both mediating and moderating effects on the fetus during these critical prenatal stages of development. As discussed earlier, alterations to the mothers' emotional, physical and psychological states can alter the fetal environment (Boksa, 2008; Minakova & Warner, 2018; M. Pereira & Ferreira, 2015; Sharma et al., 2016), which is observed through an increase in the circulating levels of hormones in both the mother and the fetus. Research has begun to explore the potential impact that increasing levels of maternal circulating hormones may have on pre- and postnatal brain development. Whilst studies have observed the associations between maternal influences and fetal brain development, to date there is no research looking at direct measurements of fetal brain growth *in utero* in relation to maternal influences.

Previous research has observed a variety of maternal influences on fetal brain development, with most research observing maternal immune activation. The term maternal immune activation refers to the activation of 'the innate and adaptive immune system (in the mother) after infection, stress, inflammation or poor physical health (Spann et al., 2018).' Maternal immune activation is associated with reductions in postnatal (Smith et al., 2007) and infant (Spann et al., 2018) functional (neural) connectivity. However, all of this research examining these maternal immune activations influences on fetal brain development has measured differences or abnormality in prenatal growth from early life observations coupled with maternal serum samples. The vast majority of previous research on prenatal brain development has reported abnormal growth, stunting of brain development and neurological deficits in relation to outcomes related to weight and size, such as LBW, preterm birth, and IUGR, without direct examination of the fetal brain (Gutbrod, 2000; Tolsa et al., 2004; Walhovd et al., 2012). To our knowledge, no studies have measured brain growth using ultrasound in mothers with PCOS, hirsutism or 'high risk' autism populations.

1.6 Fetal Biometric Measures and Later Infant Outcomes

Longitudinal research observing the relationship between fetal growth and development in relation to later outcomes is still in its infancy. LBW is a common predictor of adverse fetal and infant outcomes. Numerous studies have explored the relationship between deviation in fetal growth (e.g., small for gestational age (SGA), IUGR) and BW with mixed findings for later child outcomes. For example, Llurba et al. (2013) reported no relationship between IUGR (also referred to as fetal growth restriction) and differences in cognitive functioning or

developmental delay, while Leitner et al. (2007) showed IUGR influences neurodevelopmental performance and cognition. Furthermore, research has linked preterm birth, LBW and very low birth weight (VLBW) to increased likelihood of developing sensory issues (e.g., sensory processing disorder (Machado, Oliveira, Magalhães, Miranda, & Bouzada, 2017)), autism (Beranova et al., 2017; Pyhälä et al., 2014) and cognitive deficits (e.g., delays in speech and language development) (Kok, Lya den Ouden, Verloove-Vanhorick, & Brand, 1998), maternal selective serotonin reuptake inhibitor (SSRI) use during pregnancy to autistic traits (Marroun et al., 2014) and prenatal maternal stress to increased incidence of ADHD (Ronald et al., 2011). However, the majority of research examining the relationship between the prenatal period and later outcomes has only measured pregnancy outcomes (e.g., BW) with very limited research measures of the longitudinal influence of fetal growth and development on early infant outcomes.

Currently, fetal biometrics are used to predict fetuses who may be at risk for LBW, IUGR, SGA or large for gestational age (LGA). These risks have been identified as predictors for later outcome. Very few studies have explored the individual biometry measurements and whether they are associated with later infant outcomes. Ventriculomegaly (defined as the ventricular atrium (VA) measuring >10mm) has been associated with later developmental difficulties (e.g., lower attention span (Leitner et al., 2009) and higher prevalence of ADHD (Ball et al., 2013)). However, findings are inconsistent, with Signorelli et al. (2004) reporting no associated differences in development. An overall decrease in fetal growth measures has also been linked to later mental and psychomotor development (Hahn et al., 2016). It should be noted that this relationship was observed when using the EFW of the fetus (a proxy for later BW), and no association was found between individual growth measures (e.g., FL or HC) and later developmental outcomes.

A limitation of research observing fetal growth in relation to later outcomes is the standardisation of fetal biometry measures to normative growth charts (Chitty, Altman, Henderson, & Campbell, 1994b, 1994a; Hadlock, Harrist, Sharman, Deter, & Park, 1985). In recent years, these growth charts have come under criticism as they were created over a decade ago on a small, mainly white, middle class sample. The potential issues related to these growth charts will be further explored in **Chapter 4**.

1.6.1 Maternal Influences on Infant Outcomes

As discussed in previous sections, the maternal influence over fetal development and offspring outcomes is something that has been extensively studied by researchers. Exploring influences such as prescribed medication (e.g. SSRI's (Marroun et al., 2014)), nutrition, maternal immune activation (Smith et al., 2007; Spann et al., 2018) and social determinants (A. M. Mitchell & Christian, 2017) have given researchers and clinicals alike insights into the influence the mother has on their unborn child and later development.. For example, one of the most widely researched topics in relation to later infant outcomes is maternal mental health (e.g. stress, anxiety and depression). Huizink and colleagues (2003) found increased maternal stress in late pregnancy resulted in poor cognitive and motor outcomes in infants at three and eight months' of ages. Maternal influences during pregnancy are thought to influence prenatal programming, during sensitive periods of development resulting in long term effects of cognitive, social and physical development of the offspring (Doherty et al., 2015; Knopik et al., 2016; Ronald et al., 2011; Weinstock, 2005). In the case of maternal stress, the increase in cortisol experienced by the fetus has been shown to alter typical brain development (Weinstock, 2005). Whilst it is important to acknowledge maternal influences *in utero* have an influence on later infant outcomes, in this thesis, the focus will be on fetal growth and potential relationships between fetal biometrics and infant outcomes in relation to early language development and autistic traits.

Maternal influences were chosen not to be explored within this thesis for a number of reasons linked to maternal population and feasibility. The population of Cambridgeshire is predominately white (90.3%), with the average weekly earnings being 3.13% higher than the rest of the UK (Demographic, n.d.). In addition, according to records healthy lifestyle and nutrition in Cambridge is significantly different than the rest of the UK population. Adult smoking (17%) and obesity (17%) is significantly lower than the rest of the UK population at 22% and 24% respectively (Demographic, n.d.). Furthermore, nutritionally the consumption of five or more portions of fruit or veg a day has also been reported as being 8% higher than the rest of the UK (Demographic, n.d.). Therefore, it was not considered plausible to observe social disparities or nutritional influences. It was also chosen not to ask expectant mothers about experienced stress or depression, as I did not wish to elicit or highlight any negative feelings the mother may be experiencing when it wasn't a primary outcome of this current study, particularly when working with mothers with PCOS and autism diagnosis. Lastly, whilst

prescribed medication was requested and taken into account, as researchers were not actively seeking this group of expectant mothers. Therefore, it would not be possible to draw any comparisons or observe any influence of these medications on the developing fetus and later infant outcomes.

1.7 The Current Study

This thesis explores the use of standardised novel biometric measurements and their potential to differentiate between maternal conditions and predict later infant outcomes. The novel biometrics included AGD and several brain measurements (HC*, TCD and VA) size. The sample was followed up at 18-20 months' age to see if these fetal biometric measurements are associated with later language development and autistic traits. This study followed longitudinally a sample of 219 families (115 female and 104 male fetuses) from 12 weeks GA until 20 months' of age. Within the families 108 mothers had no maternal conditions, 65 had hirsutism but no PCOS, 26 had a PCOS diagnosis and 17 had a maternal or sibling diagnosis of autism.

In addition, population-based differences (between the UK and Israel) in these novel biometric measurements were measured, where possible. Fetal growth is known to differ between populations due to a variety of factors (e.g., ethnicity, genetics), however, it is yet to be explored whether this difference extends to these novel measures.

1.7.1 Importance of Research

In the last decade the importance of fetal development and its research has come to light, particularly in relation to early diagnosis and intervention. The review of literature within this chapter has highlighted that the prenatal period of development has been largely unexplored in relation to maternal endocrine conditions, autism and later infant behavioural outcomes.

Previous research has explored the relationship between general fetal biometric measures (e.g. FL and AC) with a particular focus on HC and autism (Blanken et al., 2018; Fukumoto et al.,

* Whilst HC is not a novel fetal biometric, it is a common measurement used in this field of research and has been shown to be a reliable proxy for brain size measurement (Joffe et al., 2005).

2011; Unwin et al., 2016; Whitehouse et al., 2011). Findings from these studies have been mixed, with Unwin et al (2016) suggesting the need to explore specific growth in brain regions in the fetus, as opposed to these standardized measurements. Further discussed in **Chapter 2** (*section 2.1.2*) the use of MRI would help assist this gap in the literature, however the use of MRI during pregnancy is still a topic of discussion amongst medical professionals (Bulas & Egloff, 2013; Ray et al., 2016) and therefore is not advised unless other visualisation techniques, such as ultrasound, are not adequate (Gonçalves et al., 2016). Therefore, this thesis will utilise 2D and 3D ultrasound alongside novel biometric measures that will enable the observation of specific brain areas (e.g. TCD). Assessing their feasibility and reliability, as well as taking a more detailed look at fetal brain growth in relation to maternal conditions and later infant outcomes, such as the early emergence of autistic traits.

Similarly, researchers and clinicians alike are exploring the use of specific measurements (i.e. AGD and TCD) as early predictors of later conditions. AGD is associated with a variety of male reproductive outcomes (e.g., sperm count (Eisenberg & Lipshultz, 2015) and non-communicable disease's such as prostate cancer (Castaño-Vinyals et al., 2012)) and endocrine influences (e.g., phthalate exposure (Swan et al., 2005) and PCOS (Barrett et al., 2018)). As discussed in *section 1.4*, the potential clinical utility of this measurement has resulted in researchers exploring its usefulness in determining retrospective early life androgen disruption and its ability to predict a range of later outcomes. Currently, there is very limited research examining AGD length (Wainstock et al., 2017). Several groups (Arfi et al., 2016 & Gilboa et al., 2014) have explored the feasibility of taking this measurement in utero, with great success. This thesis will aim to develop on existing literature, exploring the feasibility and reliability of this measure in a UK population, whilst also exploring the potential need for population-based fetal growth charts for AGD. However, similar to other fetal biometrics, if AGD is to be used clinically potential differences between populations need to be explored before reference charts can be created for clinical use.

1.7.2 Novelty of Research

Currently, there is no longitudinal research examining the potential influence of a maternal PCOS or familial autism diagnosis on fetal biometrics and early emergence of autistic traits from fetal to postnatal life. Research such as this in defining atypical development *in utero* may help establish the relationship between direct measures of hormones and physical

characteristics, from the prenatal stages of development to adult life. Additionally, amniocentesis is now being phased out of medical care. Therefore, a reliable non-invasive and indirect measure of fT should be examined to reduce the potential harm to mother and baby* and be more accessible to mothers needing additional observations during pregnancy. A reliable non-invasive measure will also be beneficial for research purposes when investigating the relationship between fetal hormonal environment and later development.

1.7.3 Methodological Considerations

The adopted methods and reasoning will be discussed throughout the thesis. However, based on previous work and time constraints a number of initial considerations have been taken into account. To reduce the burden on participants, maternal influence (i.e. whether the mother had an autism diagnosis or maternal endocrine condition) was based off existing medical records, no additional testing (e.g. ADOS or maternal serum) was conducted. Secondly, ultrasound was used to observe fetal growth. This method was chosen due to its ease of use, acceptance by parents and the existence of standardised measures/training. The decision to use ultrasound led to some limitations, such as which areas of the brain were observable and reliability measurable as well as the gestational age of the fetus. Researchers would be unable to scan mothers past a certain gestational age (approx. 32 weeks' gestation) as the older the fetus became, the larger and less free amniotic fluid around the baby. Making ultrasound visualisation limited and difficult. Therefore, for ease and reliability researchers defined a gestation within late second and early third trimester for this additional scan. The practical challenges faced when using ultrasound are discussed in **Chapter 2**, *section 2.3.5*.

1.7.4 Aims and Objectives

The overall aim of this these was to provide initial insight into the relationship (if any) between fetal growth measurements and later infant behavioural outcomes. In addition, the feasibility and reliability of using more 'novel' ultrasounds measurements of the fetus (i.e. TCD and AGD) was observed. Below I have outlined overall aim for each chapter (see *Table 1*)

* Amniocentesis is associated with increased risks of miscarriage (1 in a 100 post 15 weeks gestation), infection (1 in 1,000) and increased risk of a baby developing club foot (*Amniocentesis - Risks,* 2018).

	Aim
Chapter 2	The aim of this chapter was to explore fetal brain growth observing differences in fetal sex and potential influence of maternal endocrine conditions.
Chapter 3	The aim of this chapter was to explore AGD observing differences in fetal sex and potential influence of maternal endocrine conditions.
Chapter 4	The overall aim of this chapter was to explore whether AGD requires population-based charts (similar to other fetal biometric measures), if it is to be used to predict fetal and potentially postnatal developmental outcomes.
Chapter 5	This chapter explored the relationship (if any) between fetal brain growth and later behavioural outcomes in the infant aged 18-20 months.
Chapter 6	This chapter explored the relationship (if any) fetal AGD and later behavioural outcomes in the infant aged 18-20 months.

Table 1: Summary for each chapter within thesis.

2 The Fetal Brain: Sexual differentiation and maternal influences

2.1 Introduction

This chapter will explore whether there are sex differences in the developing fetal brain structures and whether maternal endocrine or neurodevelopmental conditions such as polycystic ovary syndrome (PCOS), hirsutism and autism influence these developmental changes.

2.1.1 Overview

Advances in imaging techniques have led to the ability to visualise brain development. Until the last decade, research investigating the developing brain explored the maturation of neuronal networks and grey and white matter from the first years of life. Only recently have researchers and clinicians become aware of the importance of the very early phases of brain development prenatally (in the 40 weeks of gestation) and its influence on later cognition and the emergence of neurological disorders (Reissland & Kisilevsky, 2016). Research has now shifted the definition of the 'developing brain' to include characterising brain growth in both the pre- and postnatal brain period since significant changes in morphology take place during these periods.

Our understanding of the developing brain is still in its infancy, with most research focused on gross anatomical growth during the prenatal period. These investigations have provided

researchers with an opportunity to explore the relationship between fetal and neonatal brain development (in relation to later cognitive, motor, language and social skills and physical development) observed from infancy to adulthood. As discussed in **Chapter 1** (*section 1.1*), it is during this period of development that the brain is especially susceptible to a range of factors (e.g., famine (Painter et al., 2005)).

2.1.2 Methodology to examine fetal brain development

As discussed in the **Chapter 1** (*section 1.2*), research investigating the developing brain has used a combination of magnetic resonance imaging (MRI) and ultrasound techniques. MRI is widely accepted as being the gold standard for studying the human brain. Although there are no known risks of MRI during pregnancy (whether imaging mother or fetus) it is still a topic of discussion amongst medical professionals (Bulas & Egloff, 2013; Ray et al., 2016) and therefore is not advised unless other visualisation techniques, such as ultrasound, are not adequate (for example, in cases of high maternal BMI or oligohydramnios (Gonçalves et al., 2016)[*]).

Compared to MRI, ultrasound has a lower resolution. However, advances in ultrasound technology over recent years have resulted in the ability to identify subtle anatomical details of the fetal brain (Achiron & Achiron, 2001; Toi et al., 2004). Ultrasound is therefore the primary tool used to assess fetal growth and gross structural brain development. Growth charts have been created for neonatal brain development which has enabled clinicians to identify babies and infants at risk of later developmental difficulties and abnormalities (e.g., World Health Organisation (WHO) growth charts for neonatal and infant close monitoring(*UK-WHO Growth Charts - Neonatal and Infant Close Monitoring (NICM) | RCPCH*, n.d.)). However, currently no similar fetal brain growth charts exist. Instead, clinicians use head circumference (HC) as a proxy for brain development (Joffe et al., 2005) and later risk identification.

2.1.3 Autism and brain development

Previous findings examining brain structures have identified a wide range of areas implicated in autism, such as increased amygdala volume (Schumann et al., 2004), enlarged brain volume

[*]Oligohydramnios is a condition during pregnancy characterised by a less than expected volume of amniotic fluid surrounding the fetus.

(Courchesne et al., 2001) and a larger cerebellum (Becker & Stoodley, 2013). The cerebellum co-ordinates and regulates muscle movement and connects cortical and subcortical areas. Through these connections the cerebellum acts as a modulator for many emotional and behavioural functions (Schmahmann, 1997) including memory and language. There are many studies linking structural differences in the cerebellum with autism, with the majority demonstrating larger cerebellum volumes in autistic children and young autistic adults (ages 2-16 years) (Courchesne et al., 2001; Hardan, Minshew, Harenski, & Keshavan, 2001; Sparks et al., 2002). Cerebral volume has been correlated to conditions such as schizophrenia (Okada et al., 2016), premature birth (Monson et al., 2016), and is also correlated with lateral ventricular volume (Karacan, Kosar, Çimen, Solak, & Sahin, 2013). Research studying cerebral volume in autistic individuals has found a significant increase in the autism group, compared to controls ($p = .03$) (Hardan, Minshew, Harenski, et al., 2001). However, research focusing on lateral ventricular volumes in autistic individuals versus controls has been contradictory. Using MRI, Hardan et al. (2001) found no significant differences between the two groups (autism sample n=16: controls n=19, $p = .46$). Conversely, Turner et al. (2016) found a significant enlargement of the lateral ventricle (autism sample 472: controls 538, $p = .001$). These contradictory findings may be due to the imbalance between the groups within the sample (35 participants (Hardan, et al. 2001) compared to 1010 participants (Turner et al., 2016)). The small sample sizes within Hardan et al. (2001) may have been insufficient at detecting differences between the groups.

More recently, research has explored potential differences in the early developing brain in autism. During the early years (up to 4 years of age) of development, an enlarged total brain volume has been consistently observed in infants later diagnosed with autism (Courchesne et al., 2007; Redcay & Courchesne, 2005). A longitudinal study by Hazlett et al. (The IBIS Network et al., 2017) identified that this enlargement begins as early as 12 months' of age, as well as a hyper expansion of the cortical surface in the areas associated with sensory processing. Hazlett et al. (2005) also examined HC, finding no difference at birth between autistic individuals and controls. However, this study found that HC growth curves began to diverge between autistic and typically developing children from approximately 2 years of age. Researchers concluded that the differences seen in brain growth associated with autism begin postnatally from around 12 months' of age. Based on previous autism findings, the study reported below will focus on cerebral volume (using HC as a proxy) (Chang et al., 2000), the

cerebellum (Rutten et al., 2009) and the ventricular atrium (VA) (Achiron et al., 2004; Kim et al., 2008) which can be easily measured using a 2D or 3D ultrasound.

The studies reported above include children or adults using MRI, while in the current study 2D ultrasound will be used during the fetal period to measure gross brain anatomy. Advances in ultrasound imaging have significantly increased the resolution to enable its use to observe and diagnose cerebral structural differences among fetuses. Therefore, ultrasound was chosen for this study since it is already routinely administered throughout pregnancy and is widely accepted by mothers to be safe and worthwhile to detect anomalies with their unborn baby.

2.1.4 Maternal PCOS and fetal brain development

Polycystic ovary syndrome (PCOS) is a set of symptoms due to elevated androgens, particularly testosterone. Increased testosterone in the mother who has PCOS has been shown to influence the overall development of her offspring. Recent research has demonstrated physical changes (e.g., lengthened anogenital distance (AGD) in female offspring (Barrett et al., 2018)) and cognitive developmental changes (e.g., falling behind peers in motor, personal-social and communication development in both males and females (Bell et al., 2018)) as well as increased likelihood of a later autism diagnosis in the offspring in both sexes (Cherskov et al., 2018; Kosidou et al., 2016). To date, brain development or size differences in the fetuses of mothers with a diagnosis of PCOS (compared to mothers without PCOS) has not been documented. However, recent research from animal models has suggested that PCOS begins in the brain of the affected individual. Caldwell et al. (2017) conducted a series of experiments in mice, observing the effects of removing androgen receptors. Researchers linked androgen receptors in two area to the development of PCOS, these were receptors found in the brain and ovaries. They found that when androgen receptors were removed from the mouse brain, the mice did not develop PCOS. Conversely, if the receptors were only removed from the ovaries, the mice were still able to develop PCOS[*]. This provides some evidence that PCOS may begin prenatally in the brain and a potential rationale for examining prenatal brain development in relation to PCOS and hirsutism.

[*] Testosterone and dihydrotestosterone are mediated via androgen receptors (Davey & Grossmann, 2016). Both hormones have been associated with the size and functionality of a number of brain areas (Celec et al., 2015; Filová et al., 2013).

2.1.5 Ultrasound and fetal brain development

During the prenatal period, the fetus' brain is small with many of the structures difficult to see (due to size) or not fully developed. The relatively low resolution of ultrasound technology limits visualisation of some of the structures of the brain, although the use of multiplanar visualisation and an overall increase in the resolution of ultrasound has allowed researchers to observe gross structural development during these early stages. However, there are still very few areas of the fetal brain that can be observed using ultrasound. This chapter will focus on several UK standard fetal biometry measurements (Transcerebellar diameter (TCD), HC and VA) that can be routinely measured using ultrasound and one novel measurement (middle cerebral artery (MCA)).

2.1.5.1 Fetal Cerebellar Development

The cerebellum is one of the earliest structures to develop in the fetal brain (*see figure* 4) (Garel et al., 2011) putting it at increased vulnerability to a range of developmental effects during prenatal development (Garel et al., 2011; Koning, Dudink, et al., 2017). Cerebellar growth trajectories across gestation show a positive linear pattern (Koning, Dudink, et al., 2017). Increasing in size as the fetus gets older and HC also increases in size. Atallah et al. (2019), identified that TCD in fetuses measuring below the 5th centile[*] is a relevant marker to detect associated anomalies such as a high rate of fetal malformations, chromosomal anomalies, severe *intrauterine* growth restriction (IUGR) and genetic disorders. Additionally, Garel et al. (2011) suggested that a reduced cerebellar diameter might be associated with increased fluid spaces in the posterior fossa or tentorium cerebelli, which could result in later developmental delays or disorders (e.g., reduced fetal cerebellar diameter has been implicated in atypical general movement in infants at 1 and 3 months (Spittle et al., 2010)[†] and poor motor and cognitive functions (Park et al., 2014)). Despite the potential importance of TCD as a prognostic factor for fetal growth anomalies and later development, it is not currently routine

[*] Parents are presented with charts containing curved lines plotted with their baby's measurements (see appendix 6 for example). The curved lines on these charts or referred to as 'centile' and usually depict the 5th, 25th, 50th, 75th and 95th centiles. They show the average for the taken measurement (e.g. head circumference) for babies and fetuses of that age (*Your Baby's Weight and Height*, 2017).

[†] General movements were assessed using Phrechtl's method of assessing spontaneous movements (e.g., writhing) (Spittle et al., 2010).

practice across the world to collect TCD measurements during routine ultrasounds. No studies have observed the relationship between TCD in the fetus and maternal or neurodevelopmental conditions such as PCOS, autism and hirsutism* using ultrasound. Further, there is no previous literature examining sex differences in TCD.

2.1.5.2 Head Circumference

HC is an accepted measure to be used as a proxy for brain size in pre- and postnatal (Joffe et al., 2005) research. Using 3D ultrasound imaging, researchers have found that fetal brain volume strongly correlates with HC and biparietal diameter (BPD) measurements (L. K. Endres & Cohen, 2001). As a result, research has focused on the relationship between fetal HC or maternal influences on postnatal development and later adult outcomes.

Differences in estimated fetal weight (EFW) between the sexes are well-documented. EFW is most commonly calculated using several fetal biometric measurements, including HC (*see figure* 2) (Hadlock et al., 1985). Since there is a difference in EFW between the sexes it would be expected for this to be apparent within individual biometric measurements. Limited research has found a marginal significant sex difference in HC (Joffe et al., 2005) which limits the clinical utility of reference charts separated according to sex of the fetus for this measure. Due to the lack of clinical significance or differentiation between the sexes during the fetal stages of development, most research examining influences on fetal development does not account for sex differences† (e.g., the effect of maternal smoking on fetal growth (Iñiguez et al., 2013)). More recently, new reference charts produced by the WHO (Kiserud et al., 2017) suggest the need to have different reference charts based on the sex of the fetus‡. Existing reference charts

*As discussed in the introduction (*section 1.3.1.2*) hirsutism is also associated with hyperandrogenism and will therefore be included as a separate sub-group to women with a PCOS diagnosis. To date there is no research observing the influence of hirsutism on fetal development.

† This is a problem that has been identified by DiPietro and Voegtline (2017) in a recent review. Whilst sex differences in development and vulnerability during the prenatal stages has been identified, the majority of research fails to report analysis on sex differences within the research conducted, in particular any interaction terms that might be apparent. Therefore, the influence fetal sex has on early development is still largely unknown.

‡ Kiserud et al. (2017) measured a significant (at the 5% level) difference in estimated fetal weight (EFW) between the sexes for all centiles. Demonstrating a natural variation in fetal growth influenced by sex, suggesting fetal sex should be included as a potential adjustment factor for local clinical use to improve diagnostic and predictive performance.

do not differentiate by fetal sex (further discussed in **Chapter 4**), nor do other reference charts currently under creation (e.g., National Institute of Health and Human Development (Buck Louis et al., 2015) or INTERGROWTH 21st study (Hirst et al., 2016), further discussed in **Chapter 4**) and the WHO reference chart is the only one that discusses having separate reference charts according to sex of the fetus. Measuring fetal biometry according to sex is still a debated topic among clinicians and researchers. To date, there is no strong scientific evidence for this separation to be implemented in research and clinical charts.

Research exploring the relationship between HC and autism has been contradictory. As discussed in **Chapter 1**, (*section 1.3.2.1*) several research groups have found no significant difference between autistic children or 'high risk' sibling* groups compared to a non-autistic control group (Unwin et al., 2016, 2016; Whitehouse et al., 2011) in HC measurements. Unwin et al. (2016) suggest that examining the growth of subregions of the fetal brain may reveal potential differences between fetuses who later receive an autism diagnosis compared to those who do not, rather than using a proxy measure of brain growth (e.g., HC). HC growth velocity has also been of interest to researchers when observing later developmental outcomes such as autism. Bonnet-Brilhault et al. (2018) observed an atypical prenatal head growth trajectory after 22 weeks' gestational age (GA) in children later diagnosed with autism. Similarly, Abel et al. (2013) also noted a difference in growth patterns in children later diagnosed with autism. Both studies observed overgrowth in prenatal HC during the second and third trimester (Abel et al., 2013; Bonnet-Brilhault et al., 2018).

Unlike in previous research exploring the link between autism and fetal development, whilst there is growing evidence to suggest maternal PCOS has an impact on fetal growth (preterm birth and reduced birth weight (BW)) and pregnancy outcomes (increased likelihood of unplanned caesarean section) (Palomba et al., 2015; Puttabyatappa et al., 2016; Qin et al., 2013).To date there is no research showing the influence of a maternal PCOS diagnosis or hirsutism in relation to individual fetal biometric measurements such as HC.

* 'High-risk sibling groups' is defined as individuals with a sibling (brother or sister) who already has an autism diagnosis.

2.1.5.3 Ventricles

Ventriculomegaly is defined as having excess fluid in the lateral ventricles of the developing fetal brain (Cardoza et al., 1988), with mild cases beginning from a 10 mm VA diameter (*see figure* 3). It is one of the most common central nervous system abnormalities, occurring in 1% of pregnancies (Salomon et al., 2007). Studies have shown that fetal ventriculomegaly is associated with later developmental delays (e.g., in fine motor and expressive language skills (Lyall et al., 2012)) and psychiatric diagnoses (autism and schizophrenia (Gilmore et al., 1998; Gilmore et al., 2001; Palmen et al., 2005)). Therefore, measurement of the VA has become common during the second trimester ultrasound scan. Similar to HC, research has found a statistically significant sex difference in VA width (Kramer et al., 1997). Kramer et al. (1997) within a sample of 8516 patients (mean GA: 26.7 weeks and 42% of the sample identified at time of scan as female), found a mean difference of 0.2mm between male (7.1 mm) and female (6.9mm) VA measurement. However, this is not deemed to be clinically significant as to warrant separate growth charts between the sexes. Overall it was argued for the use of a single growth chart (accounting for both sexes) to be enough to observe potential abnormalities and counsel patients (Kramer et al., 1997). Observations into fetal ventricular width throughout gestation have shown size to increase until circa 20 weeks GA and then remain stable, or steadily decline afterwards (Salomon et al., 2007). Therefore, we would expect to see either stability or decrease in VA size, between the 2nd and 3rd trimester scans.

To date, MRI studies have explored the relationship between autism and VA size and found larger volumes in children and adolescents with an autism diagnosis (ages 4-15 years') (Gilmore et al., 2001; Palmen et al., 2005) compared to neurotypical controls. However, research is yet to examine the influence of a maternal or older sibling autism diagnosis on fetal VA width or examine whether VA width measured prenatally is associated with later autistic traits. Further, research has not explored whether maternal PCOS or hirsutism influences the development of the VA.

2.1.5.4 Middle Cerebral Artery (MCA)

The MCA is the longest branch of the circle of Willis (Degani, 2009) (*see figure* 5). It is a critical artery and is the most common pathologically affected blood vessel in the brain (Callum & Barrett, 2007). Doppler ultrasonography measurement of the MCA provides information about the hemodynamic behaviour of the fetus: pulsatility index (PI), S/D ratio, resistance

index (RI), and peak systolic velocity (PS), are used to help detect potential risks to the fetus (e.g., anaemia (Mari, 2005) and IUGR (Harneet et al., 2009)). The MCA-PI plays an important role in enabling clinicians to assess and monitor fetal oxygenation of the brain (Akolekar et al., 2015). Research has focused on combining this value with umbilical artery (UA) PI to help predict and monitor pre-eclampsia (Simanaviciute & Gudmundsson, 2006), fetuses who may be small for gestational age (SGA) (Giancarlo Mari & Deter, 1992), prematurity and neonatal intraventricular haemorrhage (IVH) (Baschat et al., 2002). Akolekar et al. (2015) explored potential influences of maternal characteristics (e.g., ethnicity) and medical history (e.g., diabetes or hypertension) on MCA- and UA-PI, concluding that they both influence the PI of these arteries and should be taken into account when assessing 'normal' ranges in pregnancy. However, studies are yet to determine whether maternal endocrine conditions influence these measures, or whether there are sex differences in the PI in these arteries.

The following research questions were addressed: (1) whether there is sexual dimorphism in fetal brain measurements; (2) if there is an influence of maternal or a neurodevelopmental condition related to excess testosterone (PCOS, Hirsutism and autism) on fetal brain measurements; (3) whether there are sex differences in growth between time points (12-, 20- and between 26-30 weeks' gestation); (4) if there is a difference in growth between time points within mothers with a diagnosis of PCOS, autism or hirsutism.

2.2 Feasibility Pilot Study: An Overview

One mother with a singleton female fetus was recruited to assess the feasibility of the developed protocol. The aim was to observe and assess the following: (1) expected duration of the scan, (2) potential limitations of the developed procedure and (3) ability to measure AGD. Consent was given by the mother for me, two sonographers and an advisor to the project (Dr Gerald Hackett) to be present for the duration of the scan.

For the pilot the procedure detailed below was adopted (see *section 2.2.3*). The main finding from the pilot study was the need for 40-minute slots for scans compared to the standard 20-minute slots offered to parents at the 12- and 20-week scan time points. The 40-minute scans were to be inclusive of time needed to: (1) capture measurements for each fetal measurement (see *table 4* for list of measures), (2) capture several measures for AGD measurement, (3) move

the fetus into a better position (either through manually applying pressure to the mothers stomach, drinking water, consuming sugar or going for a quick walk), (4) give sufficient time to talk the parents through the scan and (5) give sufficient time to capture 3D images for the parents as a thank-you for taking part.

For this study, it was decided to include all the traditional 20-week ultrasound measurements (see *table 4*), along with the addition of AGD and MCA. The rationale behind including these common measurements (such as HC, TCD and FL) was that expected growth in these measurements are well documented therefore comparable. Furthermore, the taking of the measurement itself is standardised (with little to no variation between sonographers and training/experience). The addition of MCA came as a result of the pulsability index (PI) being identified as an important measure for physicians to assess and monitor fetal oxygenation of the brain (see *section 2.1.5.4*) (Akolekar et al., 2015). Whilst the addition of measuring other brain areas was discussed (e.g. amygdala) this was not deemed possible due to the limited resolution of ultrasound. In addition these areas of the brain are not easily visible when using an abdominal transducer probe. Transvaginal probes were not used within the study as they are deemed to be invasive, and unnecessary for a study of this nature. Transvaginal probes are only used in clinic to assess causes of problems such as subfertility, abnormal vaginal bleed and assessment of ovaries and/or cysts.

AGD is a novel measurement to be included within this study (rationale described in **Chapter 3,** *section 3.1*). The initial part of the scan was spent familiarising the sonographers with how this measurement was to be taken. The method used and detailed by Gilboa et al (2014) (see **Chapter 3**, *section 3.2.3* for details) was followed by both sonographers. The pilot demonstrated the need for: (1) the fetus to have their legs uncrossed, (2) the measurement to be taken several times for a mean length to be taken, (3) both measured and unmeasured images to be saved (for reliability measures to be taken at a later date, see **Chapter 3**, *section 3.2.3*) and (4) the fetus to be moved for the measurement to be taken, if view of genitalia was obscured by the mother's pelvis. The fetus having their legs uncrossed, movement of the fetus for a better view of the genitalia and taking of the measurement multiple times for a mean length to be taken, is common amongst AGD researchers.

Lastly, prior to the scan, the couple expressed their wishes not to know the sex of the fetus (changing their mind at the start of the scan). This allowed for the sonographers and I to develop

a method of taking the AGD measurement without the sex of the fetus being revealed. The following procedure was adopted: (1) the family would be notified that this measure was about to be taken, (2) the overhead monitor (if applicable) would be turned off and (3) I would draw their attention away from the sonographers screen and engage in conversation for a long as necessary. During the study we were able to successfully apply this to all families recruited into the study who wanted to keep the sex of their baby a surprise. This procedure was used for approximately half of my sample.

2.3 Methods

2.3.1 Participants

219 healthy fetuses, 104 males and 115 females, were recruited prospectively from the Rosie Maternity Hospital in Cambridge, UK (see *appendix 1* for participant information sheets). Eligibility inclusion criteria for the study were as follows: pregnant women who were willing to have an additional ultrasound scan between 26-30 weeks' gestation (average GA: 28 weeks, SD = 1.25), with (1) little/no consumption of alcohol during pregnancy, (2) no smoking or recreational drug use during pregnancy, (3) a singleton fetus whose measurements indicated their size to be appropriate for gestational age, (4) the absence of any major fetal anomalies and (5) fetus is not considered to have an *intrauterine* growth restriction (IUGR) or be large-for-gestational age (LGA). Eligibility criteria for including the fetus in the final data analysis was the birth of a clinically healthy baby (*see table* 2 for characteristics of the mothers and fetuses).

In this sample of 219 pregnancies, 17 mothers had a maternal diagnosis or a child with a diagnosis of autism (9 males, 8 female fetuses)[*], 26 had a diagnosis of PCOS (17 males, 9 female fetuses) and 65 indicated they had hirsutism during their adult years' (42 male, 23 female fetuses) with no diagnosis of PCOS (*see table* 3 for population details, split by groups)[†].

[*] 15 of these mothers were recruited using the Cambridge Autism Research Database (CARD), twitter via the Autism Research Centre (ARC) account and through word of mouth.

[†] Initial G*power estimates with an effect size of 0.5 and a power of 0.8 needed a sample size of; 64 in each group. Additionally, an effect size of 0.8 and a power of 0.8 needed a sample size of 26 in each group. However due to the rarity of these groups (autism and PCOS) an opportunistic approach was taken, attempting to recruit as many individuals into these sub-groups as possible.

Hirsutism was defined by excess hair growth in a number of areas. A definition of hirsutism was provided to participants and a self-report questionnaire (see *appendix 2* for pregnancy history questionnaire) asked 'during your adult years', have you found coarse, dark, hair growing in any of the following areas: upper lip, chin, breasts, back, belly, chest between breasts, upper thighs and/or upper arms?'. Mothers were included in this group if they answer yes to any of these.

Within the sample of 219 fetuses, one male fetus displayed mild ventriculomegaly (ventricular atrium measuring 10.1mm) at the time of the scan (approx. 27 weeks). The fetus was followed up subsequently by the sonographer (at 30 weeks) where this was no longer the case. Therefore, this fetus was included within the final sample. No other fetal or maternal based complications were observed during the 12-, 20- or research scans.

2.3.2 Recruitment

Out of the 219 pregnancies, 192 families were recruited directly from the Rosie Maternity Hospital, they were recruited either by being approached by a researcher (myself) at the time of their 12- or 20-week scan[*] with an information sheet, or via posters placed around the ultrasound clinic. Ten families were recruited from antenatal yoga classes where I was given permission to briefly talk to families about the study prior to their class and leave behind my contact information. Parents were asked to contact myself (see *appendix 1* for participant information sheet) to register their interest. When registering their interest in taking part parents would be sent a short questionnaire to check that they would be eligible to take part (see eligibility criteria above *section 2.2.1*). If eligible, parents would then be sent several options of dates (between 26-30 weeks' gestation) and times for them to choose from. Mothers would then be booked in for this additional scan (see *appendix 3*) and sent details of the location of the scan, questionnaires for the study (could be completed prior to, or at the time of the scan) (see *appendix 4*) and a consent form (to be completed at the time of the scan) (see *appendix 5*).

[*] At the time of the study, a number of different studies were also recruiting using this method. CUSP was allowed to recruit in person from the ultrasound unit every Wednesday morning between 8am – 11am. Every parent within the clinic waiting area was approached by myself to avoid selection bias from the researcher.

Expectant mothers would be encouraged the bring partners, friends or family along with them to the scan (if they would like to). Due to the nature of the study (offering of an additional scan and 3D images) there were no issues in recruiting participants. Any family who was eligible for the study was recruited and scheduled in for a scan*.

A number of families expressed interest in taking part form as early as 12-weeks' gestation. These families were asked if they were happy to provide the date of their scheduled 20-week scan and with researchers (myself) being in touch after this appointment to see if they would still be interested in taking part. Two families (not included within the 219) lost the fetus before the 20-week scan.

'High risk' families recruited using CARD, twitter or through word of mouth from outside of the local Cambridgeshire area, would be offered overnight accommodation for themselves and their family. Several families who initially expressed interest within the study withdrew (pre consent stage) as they were unable to travel to Cambridge (e.g., for needing to travel via plane or unable to travel). These families were not included within the final sample.

2.3.3 Ethics

Ethical permission for the study was granted 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. All mothers gave written informed consent (see *appendix 5* for consent form).

2.3.4 Procedure

Ultrasound scans were performed using a GE Voluson 8 Expert ultrasound system, with a (4-8 MHz curvilinear abdominal transducer)†. All women had completed a normal 12- and 20-

* Between the months of December 2017 and February 2018 the study stopped recruiting due to the popularity and the inability to keep up with demand.

† Research scan slots were offered to CUSP initially Wednesday mornings only, 7am – 12pm, then moving to Monday afternoons, 1pm – 7pm. Families were booked in during this allocated time. However, where there was a 'high risk' family travelling from outside of Cambridge, the clinic offered flexibility in the date and time I was able to offer (no weekend scans were offered). The time of day did not influence the data collected, this study

week anomaly scan and were made aware that this additional scan (between 26-30 weeks gestation) was for research purposes and was not a routine medical scan[*]. During various stages of fetal development, standard ultrasound measurements were taken (*see table* 4). Fetal brain measures included HC, VA, TCD and MCA. These measurements were taken by trained sonographers using standard ultrasound planes specific to each fetal measurement. As only physical (no behavioural) measurements were taken, the fetus did not need to be awake for the scan. HC was measured as standard by obtaining a cross-sectional view of the fetal head at the level of the ventricles (*see figure* 2) and measuring around the outer edge of the skull. At this view, the VA was also measured (*see figure* 3). For the TCD measurement, the back of the fetal head was visualised keeping in view the septum pellucidum. To measure the diameter, electronic callipers were placed on the outer, lateral edges of the cerebellum (*see figure* 4). Lastly MCA flow was measured on the axial section of the brain, where the thalami and the sphenoid bone wings can be visualised (*see figure* 5). The research team were given access to the mothers' previous 12- and 20-week routine medical ultrasound scans to access previous measures of those described above[†].

2.3.5 Challenges of Ultrasound

If any of the described measurements above were not obtainable due to fetal positioning, the mother was given cold water, asked to walk around for several minutes or asked to come for a second appointment[‡]. In instances where the fetus was breech in presentation, the examination bed was tilted to move the fetus out of the pelvis and remove shadowing whilst the measurement was being taken. Failure to obtain any measurement could have been due to fetal

used still images for measurements and did not use 4D ultrasound to observe general motor movements or behaviours of the fetus.

[*] The additional research scan (between 26-30 weeks gestation) lasted approximately 40 minutes. During the scan, general fetal biometric measurements were also taken (e.g., HC, AC (abdominal circumference) and femur FL, including dopplers), to observe current fetal growth and check that the fetus had not become IUGR, LGA or developed any abnormalities since the last routine scan.

[†] Consent was obtained from the mothers for previous medical scans (12- and 20-weeks' gestation) as well as birth records.

[‡] An additional appointment was offered to mothers during the 12- and 20- weeks anomaly scans only. It was not offered during the research scan due to time and financial constraints or limitations.

positioning and/or high maternal BMI [*]. For the 12- and 20-week scans all listed measurements were taken (see *table 4*). If measures were not obtainable, mothers would be invited back for a second scan (within a few days to a week) of the initial scan[†], however due to budget and time constraints this was not possible for the research scan.

For both the 12- and 20-week ultrasound scan the Rosie Maternity Hospital allocates 20-25 minutes per visit. Increasing each visit to 40 minutes gave the sonographer and the researcher (myself) the additional time to get each measurement, even when unsuccessful in the first incidence. The 40-minute scan enabled us to give the mother time to consume water, sugar or have a quick walk before attempting the measurement again. Additionally, the use of standardised ultrasound measurement (e.g. HC and MCA) increased the overall chances of achieving a measurement each time. Overall there was between a 1.9% to 12.3% failure to obtain a measurement (see *table 5*). In particular during the research scan visit, five fetal HC measurements were missing. If the expectant mother had a scan within the past month, general growth measurements (i.e. HC, AC and FL) would not be taken again; as per hospital procedure. Challenges in obtaining the novel AGD measurement are discussed in **Chapter 3**, *section 3.2.4*.

[*] This method will be consistent throughout the thesis when obtaining ultrasound measurements. No fetal anomalies were observed during any of the scans in the included sample.

[†] Whilst all measurements were obtained from the mothers during the 12- and 20-week scans, only measures obtained during the initial visit were accessible and used by the researchers for analysis.

	Females (n =115)	**Males (n = 104)**	**All (n = 219)**
	N (%)	N (%)	N (%)
Ethnicity			
White	94 (81.7)	82 (78.8)	176 (80.4)
Black	1 (0.9)	2 (1.9)	3 (1.4)
East Asian	2 (1.7)	4 (3.8)	6 (2.7)
South Asian	2 (1.7)	3 (2.9)	5 (2.3)
Not Disclosed	16 (13.9)	13 (12.5)	29 (13.2)
Fetal position			
Breech	30 (26.1)	31 (29.8)	61 (27.8)
Cephalic	79 (68.7)	64 (61.5)	143 (65.3)
Transverse	2 (1.7)	2 (1.9)	4 (1.8)
Variable	2 (1.7)	2 (1.9)	3 (1.4)
Not Available	2 (1.7)	4 (3.8)	6 (2.7)
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Maternal Age	32.2 (4.3)	32.7 (4.8)	32.4 (4.5)
Gestational age (GA) at time of scan	27.9 (1.25)	28.0 (1.25)	28.0 (1.25)
EFW[†]	1196.1 (228.3)	1238.6 (232.36)	1216.5 (230.7)
Birth Information[‡]	**Females (n = 99)**	**Males (n = 92)**	**All (n = 191)**
	N (%)	N (%)	N (%)
Delivery type			
SVD	63 (63.6)	46 (50)	109 (57)
C-Section	27 (27.3)	30 (32.6)	57 (30)
Assisted Vaginal	9 (9.1)	15 (16.3)	24 (12. 5)
Vaginal breech	0 (0)	1 (1.1)	1 (0.5)
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Birth Weight (g)	3382.0 (540.4)	3460.9 (481.7)	3420.0 (513.16)
Gestation at birth (weeks)	39.6 (1.6)	39.6 (1.5)	39.6 (1.56)

[†] EFW data was not obtainable for 4 fetuses (1 male, 3 females) due to fetal positioning.

[‡] Birth data was not obtainable for 25 infants, as they gave birth at home or in another country.

[*]Percentages may not add up to 100 due to rounding.

Table 1: Characteristics of the mothers and fetuses in the study.

	All (n = 219)	Maternal autism or sibling diagnosis (n = 17)	Maternal PCOS diagnosis (n = 26)	Hirsutism but no PCOS diagnosis (n = 65)	No maternal conditions, (n = 108)
			Mean (SD)		
Maternal Age	32.4 (4.5)	34 (4.5)	31.8 (4.2)	32.7 (4.7)	32.1 (4.5)
GA at time of scan (weeks)	28.0 (1.3)	30.4 (1.2)	27.5 (1.2)	28.2 (1.2)	28.0 (1.3)
EFW (g)	1216.5 (230.7)	1242.3 (240)	1118.7 (189.6)	1251.4 (230.5)	1215.2 (234.7)

Table 2: Characteristics of the mothers and fetuses in the study, sorted into maternal condition groups.

	1st Trimester Scan	2nd Trimester Scan	Research Scan
Fetal Biometry Measurements			
Crown-rump length	X		
Nuchal Translucency	X		
Head circumference	X	X	X
Abdominal circumference		X	X
Femur length		X	X
Transcerebellar diameter	X*	X	X
HC/AC		X	X
Ventricular atrium		X	X
Amniotic fluid	X	X	X
Umbilical cord artery		X	X
Middle-cerebral artery			X
Estimated fetal weight		X	X

*TCD at 1st Trimester Scan: This measurement was taken at the discretion of the sonographer which resulted in a very small number of women having a record of fetal TCD at this time point.

Table 3: List of fetal biometry measurements taken during each ultrasound scan.

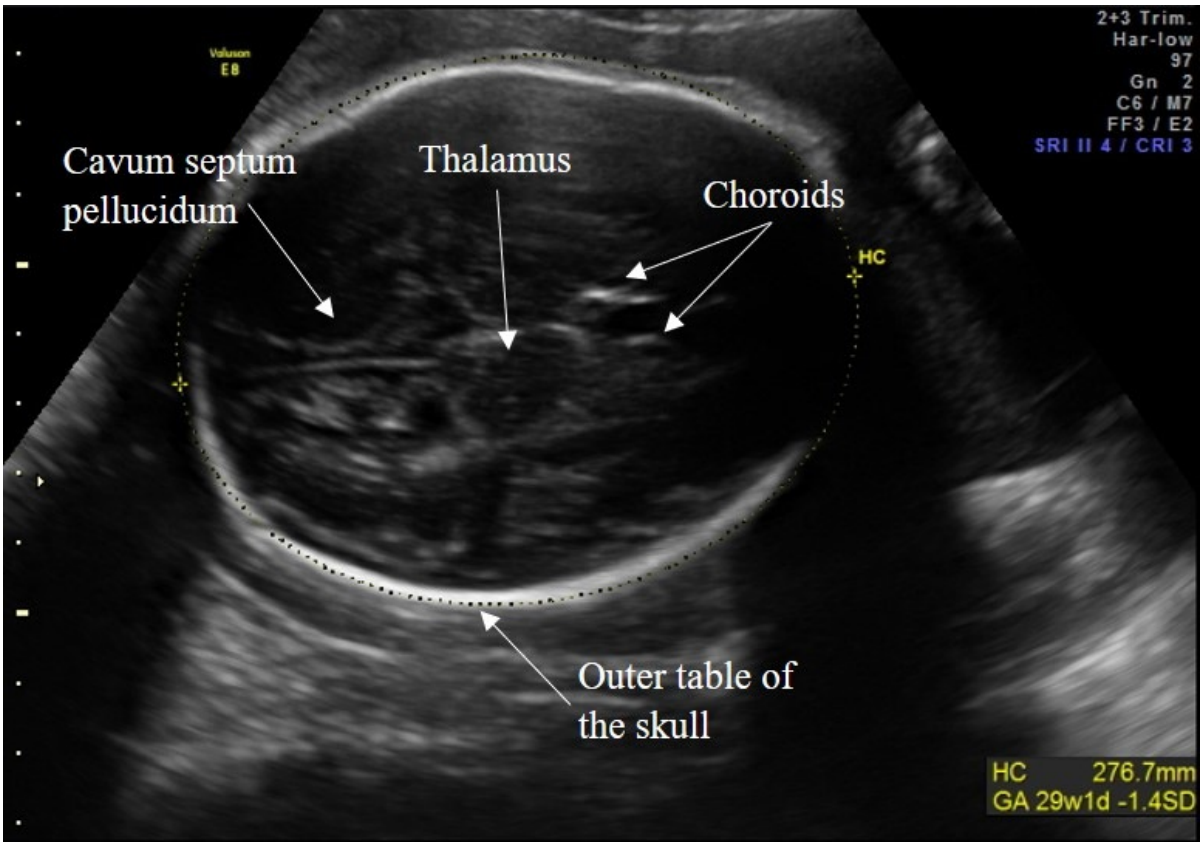

Figure 2: Example of the HC in a 29-week and 1-day old fetus female fetus.

HC was measured at a plane where the cavum septum pellucidum, thalamus and choroid plexus in the atrium of the lateral ventricles was visible. Electronic callipers were placed around the outer table of the skull (highlighted with a yellow dotted line). The shadowing observed at either side of the head was caused by the mother's pelvic bones

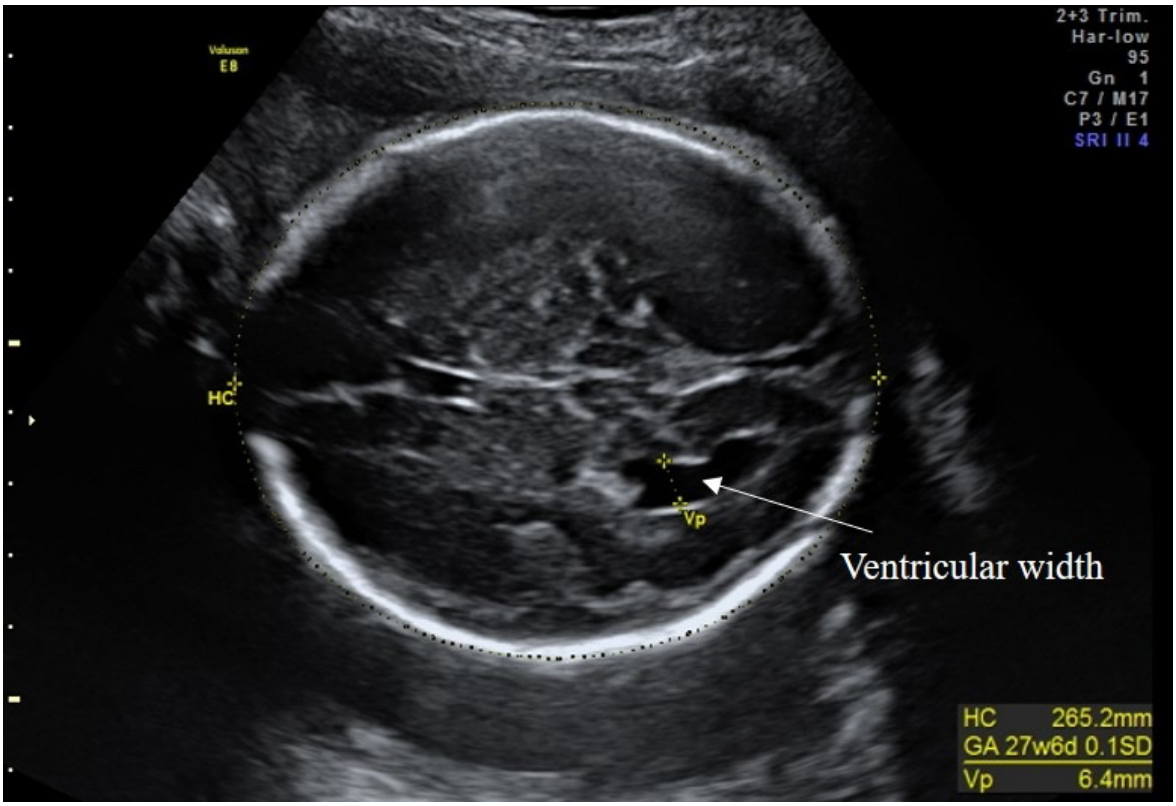

Figure 3: Example of a VA measurement in a 27-week and 6-day old female fetus.

VA was measured slightly above the level of the thalami in the axial plane of the fetal brain. Electronic callipers were placed perpendicular to the long axis of the ventricle (highlighted by the yellow dotted line).

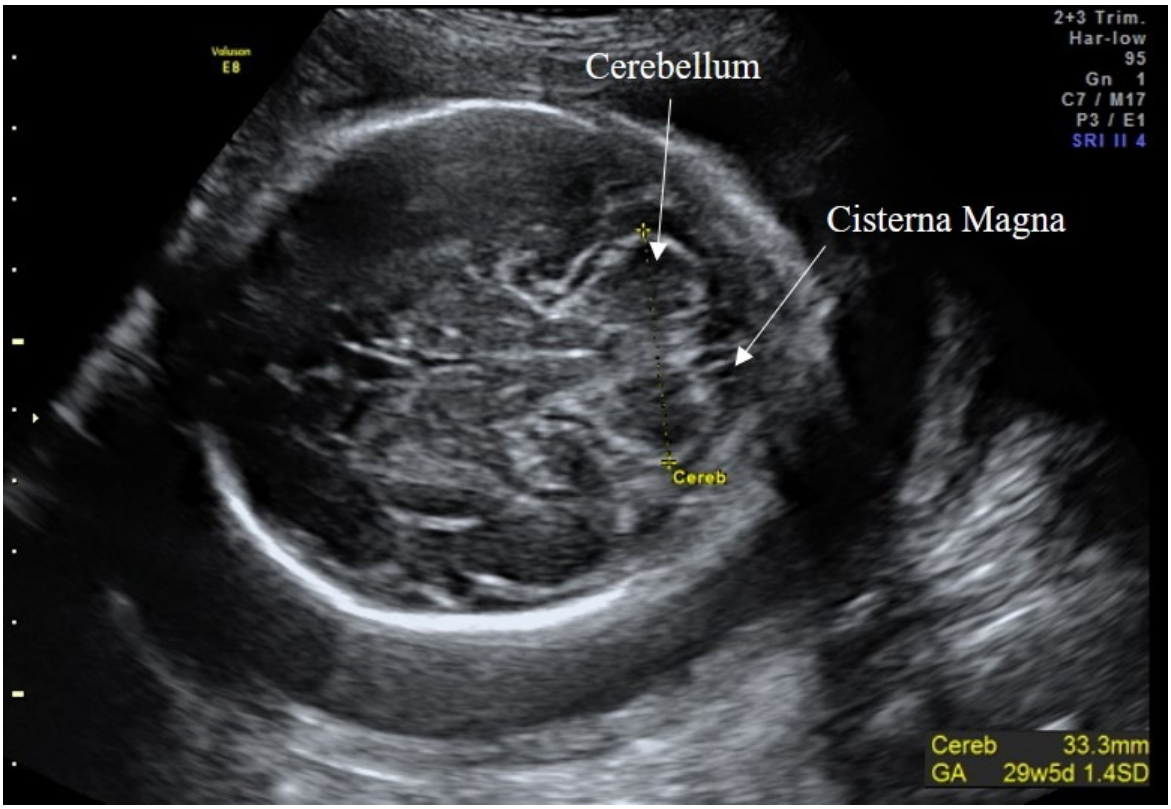

Figure 4: Example of the TCD measurement in a 27-week and 6-day old female fetus.

For TCD measurements, the transducer was slightly rotated from the thalamic plane (used to measure HC) until the posterior fossa was visible. TCD was measured using the 'outer-to-outer' method. TCD taken during the research scan always showed LGA. This is due to the fact that the internal growth charts on the GE Voluson 8 Expert ultrasound scanner (Hill et al., 1990) did not cover this GA. However, all TCD were within expected 5-95 centile ranges.

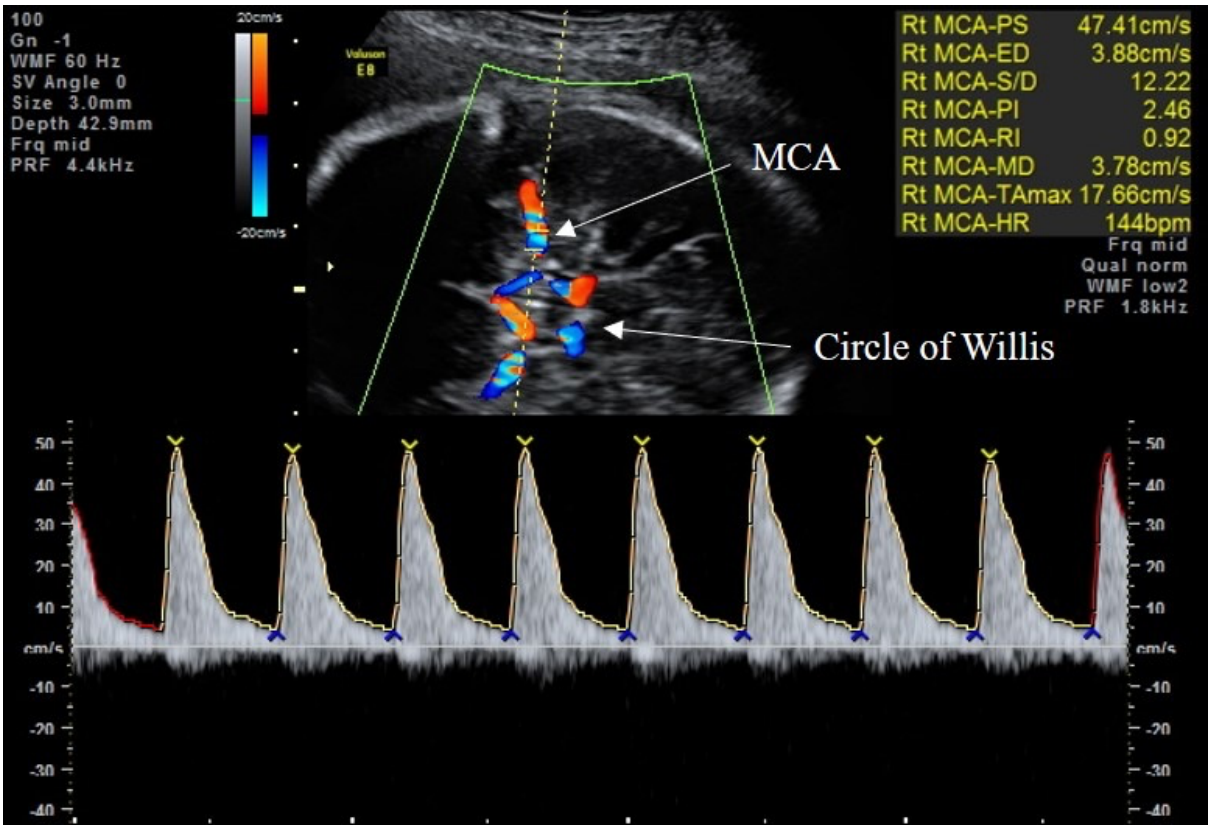

Figure 5: Example of the MCA in a 27 week and 6-day old female fetus.

MCA was measured on the axial section of the brain, where the thalami and the sphenoid bone wings can be visualised. Colour flow mapping was used to identify the circle of Willis before a measure of flow could be taken.

	Females	Males	All
		Mean ± SD (n)	
1st Trimester Scan			
GA at time of scan	12.6 ± 0.7 (104)	12.6 ± 0.8 (94)	12.7 ± 0.7 (198)
Fetal Biometric measures			
HC	79.9 ± 10 (88)	82.2 ± 10.9 (82)	81.0 ± 10.5 (170)
2nd Trimester Scan			
GA at time of Scan	20.2 ± 0.5 (108)	20.4 ± 0.4 (95)	20.3 ± 0.4 (203)
EFW†	345.0 ± 33.4 (90)	356.5 ± 38.2 (86)	350.6 ± 36.2 (176)
Fetal Biometric measures			
HC	174.5 ± 8.0 (112)	179.0 ± 6.5 (95)	176.5 ± 7.7 (207)
TCD	20.4 ± 0.9 (105)	20.7 ± 0.8 (94)	20.6 ± 0.8 (199)
VA	6.7 ± 1.0 (102)	6.8 ± 0.9 (94)	6.7 ± 0.9 (196)
Research Scan			
GA at time of scan	28.0 ± 1.2 (115)	28.0 ± 1.3 (104)	28 ± 1.3 (219)
EFW	1196.1 ± 228.3 (112)	1238.6 ± 232.4 (103)	1216.5 ± 230.7 (215)
Fetal Biometric measures			
HC	262.6 ± 14.4 (111)	266.3 ± 14.0 (103)	264.4 ± 14.3 (214)
TCD	33.2 ± 2.5 (106)	33.4 ± 2.5 (91)	33.3 ± 2.5 (197)
VA	4.9 ± 1.4 (107)	5.4 ± 1.5 (88)	5.1 ± 1.5 (195)
MCA	2.1 ± 0.3 (112)	2.1 ± 0.3 (103)	2.1 ± 0.3 (215)

Table 4: Characteristics of fetal biometrics in the study.

	Females	Males	All
	Mean ± SD (n)		
Fetal Biometric measures			
HC[1]	0.00 ± 0.34 (88)	-0.00 ± 1.40 (82)	12.59 ± 0.65 (169)
HC[2]	0.36 ± 0.99 (104)	-0.04 ± 1.02 (94)	11.42 ± 0.78 (198)
TCD	0.01 ± 1.06 (96)	-0.01 ± 0.93 (84)	1.65 ± 0.16 (180)
VA	-0.12 ± 0.91 (93)	0.15 ± 1.09 (79)	-0.21 ± 0.22 (172)

HC[1]: Growth velocity between 12- and 20-weeks' gestation.
HC[2]: Growth velocity between 20 weeks' gestation and research scan.

Table 5: Growth velocity of fetuses in the study between trimesters.

	Maternal autism or sibling diagnosis	Maternal PCOS diagnosis	Hirsutism but no PCOS diagnosis	No maternal conditions or risk
		Mean ± SD (n)		
Fetal Biometric measures				
HC[1]	0.85 ± 0.33 (6)	0.04 ± 0.37 (21)	0.66 ± 0.35 (54)	-0.06 ± 1.34 (89)
HC[2]	0.53 ± 0.73 (8)	-0.05 ± 1.21 (25)	-0.22 ± 1.07 (64)	-0.01 ± 0.92 (101)
TCD	0.24 ± 1.38 (6)	0.23 ± 1.22 (23)	-0.03 ± 0.98 (61)	-0.05 ± 0.93 (90)
VA	-0.22 ± 0.56 (5)	0.46 ± 1.16 (24)	-0.09 ± 0.93 (57)	-0.06 ± 1.00 (86)

HC[1]: Growth velocity between 12- and 20-weeks' gestation.
HC[2]: Growth velocity between 20 weeks' gestation and research scan.

Table 6: Growth velocity between fetal biometry separated by maternal risk groups.

2.4 Statistics

2.4.1 Analysis

The following research questions are addressed: (1) whether there is sexual dimorphism in fetal brain measurements; (2) if there is an influence of maternal or a neurodevelopmental condition related to excess testosterone (PCOS, Hirsutism and autism) on fetal brain measurements; (3) whether there are sex differences in growth between timepoints (12-, 20- and between 26-30 weeks' gestation); (4) if there is a difference in growth between time points within mothers with a diagnosis of PCOS, autism or hirsutism.

Research questions 1 and 3 were answered using independent samples t-tests. Independent samples t-test were used to compare the differences between the mean measurement for each fetal brain structure (HC, VA and TCD) and sex (female and males) for each gestation separately in for question 1. The growth velocity of these fetal brain structures (HC, VA and TCD) and sex for question 3. To examine questions 2 and 4 a one-way ANOVA was used to compare the mean structural measurements (HC, VA and TCD) between the 4 maternal groups (no maternal condition or diagnosis, PCOS, hirsutism and autism[*]).

Calculating growth velocity: The difference in growth between two time points was examined using the following growth velocity formula[60] (for HC the difference between time points 1 and 2, and points 2 and 3 was examined separately *see table*s 6 and 7);

$$\Delta FB = \frac{(FB_{t2} - FB_{t1})}{(GA_{t2} - GA_{t1})}$$

(Key: Δ – velocity; FB – fetal biometry; GA – gestational age; t – time point)

A z-score was created for analysis accounting for GA at time of scan[†]. A single measurement was taken using the standardised procedure. Therefore, an intra-observer and inter-observer were not necessary.

2.4.2 Results

Due to varying orientation of the fetus during ultrasound, it was not possible to obtain all biometric measurements from every fetus (*see table* 5 above for fetal biometry frequencies). There were no significant differences in growth velocity in fetuses of mothers with no maternal condition, PCOS, Hirsutism or autistic mothers or mothers with an older autistic child. Nor was a difference observed when split by sex. Therefore, statistical analysis for research questions 3 and 4 will not be reported below.

[*] A one-way ANOVA was used to explore the difference in fetal growth between the four maternal groups (PCOS, hirsutism, autism and no condition). The main issue with using a one-way ANOVA in unequal group sizes is that it can affect the homogeneity of the variance assumption, however an ANOVA (Welch's) is still applicable to use providing the other normal assumptions are still met (e.g. the DV is a continuous variable and there were no significant outliers).

[†] All analysis using fetal biometric measures will use a z-score created accounting for gestational age.

	Maternal autism or sibling diagnosis	Maternal PCOS diagnosis	Hirsutism but no PCOS diagnosis	No maternal conditions or risk
		Mean ± SD (N)		
First Trimester				
HC	82.9 ± 9.5 (6)	86.2 ± 12.2 (21)	79.3 ± 10 (54)	80.6 ± 10.2 (89)
Second Trimester				
HC	173.2 ± 12.9 (12)	179 ± 6.2 (25)	176.6 ± 7.5 (64)	176.3 ± 7.2 (106)
TCD	20.3 ± 1.2 (7)	20.6 ± 0.7 (25)	20.5 ± 1 (64)	20.6 ± 0.8 (103)
VA	6.6 ± 0.7 (7)	6.5 ± 1 (25)	6.8 ± 1 (62)	6.8 ± 0.9 (102)
Research Scan				
HC	265.8 ± 17.7 (16)	260 ± 12.4 (26)	266.1 ± 14.8 (65)	264.2 ± 13.9 107)
TCD	33.5 ± 2.5 (14)	32.6 ± 2.6 (24)	33.5 ± 2.3 (62)	33.2 ± 2.6 (97)
VA	4.7 ± 1.2 (15)	5.7 ± 1.6 (25)	5.1 ± 1.4 (60)	5.1 ± 1.5 (95)
MCA	2.1 ± 0.4 (17)	2.1 ± 0.2 (25)	2.1 ± 0.3 (63)	2.1 ± 0.3 (110)

Table 7: Characteristics of fetal biometry in the study split by maternal conditions.

2.4.2.1 Head Circumference

There were no significant differences between male and female HC during the first trimester (p = .796), second trimester (p = .333) or the additional research scan (p = .970). Additionally, there were no significant differences between maternal conditions (no condition, PCOS, hirsutism and autism) in HC at any of the three time points. First trimester HC; F(3, 166) = 2.094, p = .103, second trimester HC; F(3,198) = 1.589, p = .193, additional research scan; F(3,210) = 1.603, p = .190 (*see figure* 6).

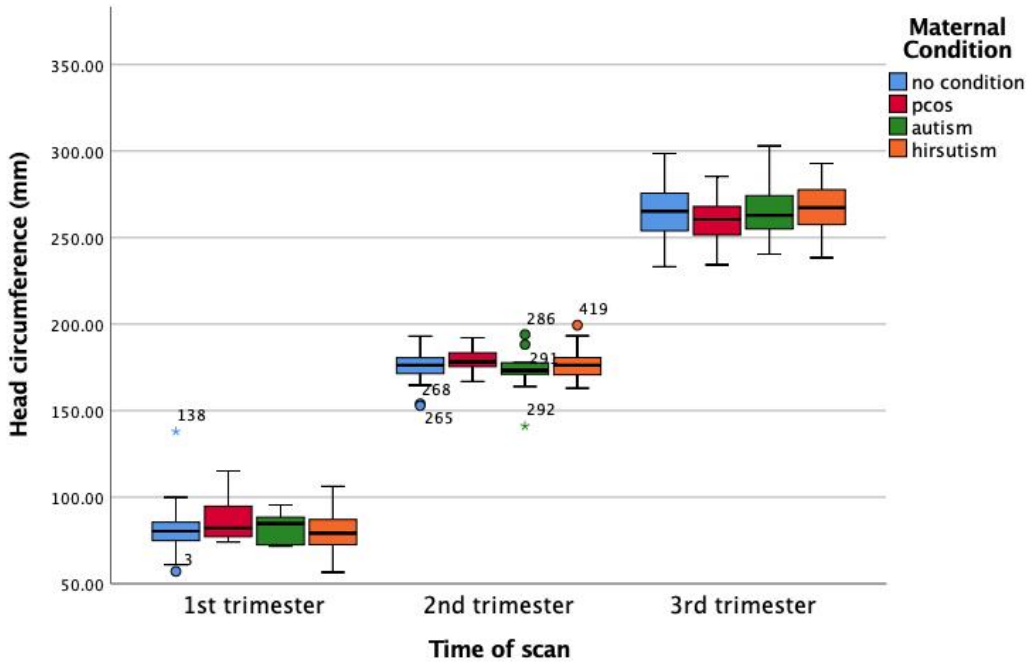

Figure 6: A box plot of raw head circumferences measurements split by maternal conditions; no condition (blue), PCOS (red), autism (green) and hirsutism (orange)

2.4.2.2 Transcerebellar Diameter

There were no significant differences between male and female TCD during the second trimester (p = .426) or the additional research scan (p = .692). Furthermore, there were no significant differences between maternal conditions in TCD at either of the two time points. Second trimester TCD; F(3,194) = .339, p = .797, additional research scan; F(3,193) = 1.474, p = .223 (*see figure* 7).

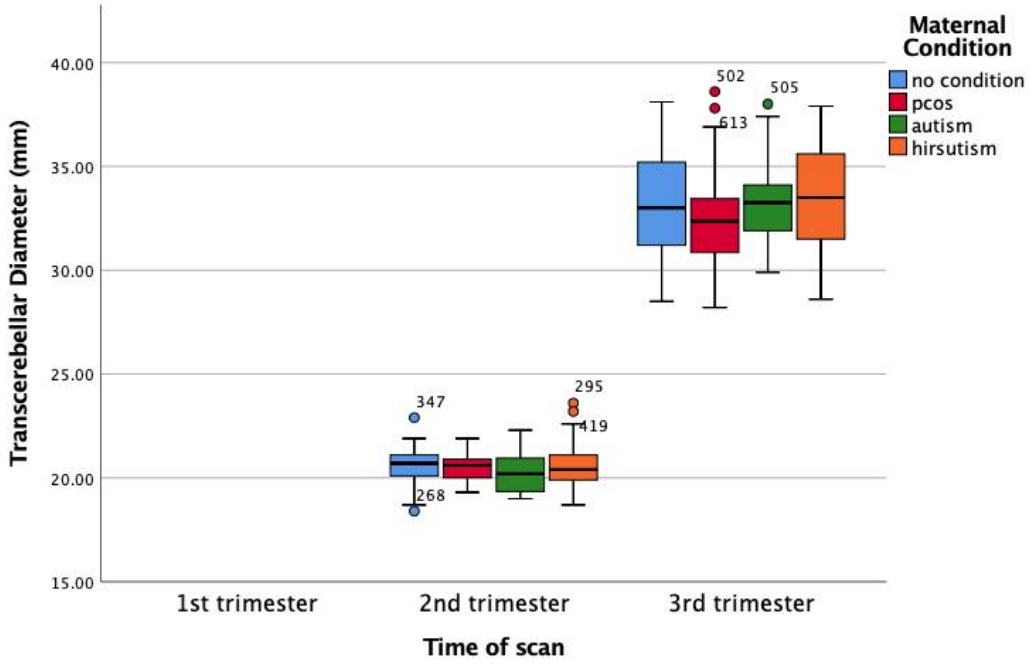

Figure 7: A box plot of raw transcerebellar measurements split by maternal conditions; no condition (blue), PCOS (red), autism (green) and hirsutism (orange)

2.4.2.3 Ventricular Atrium

There were no significant differences between male and female VA during the second trimester ($p = .470$) or the additional research scan ($p = .799$). There were no significant differences in VA width between maternal condition groups at either of the two time points. Second trimester VA; $F(3,191) = .097$, $p = .962$, additional research scan; $F(3,191) = .219$, $p = .883$ (*see figure* 8).

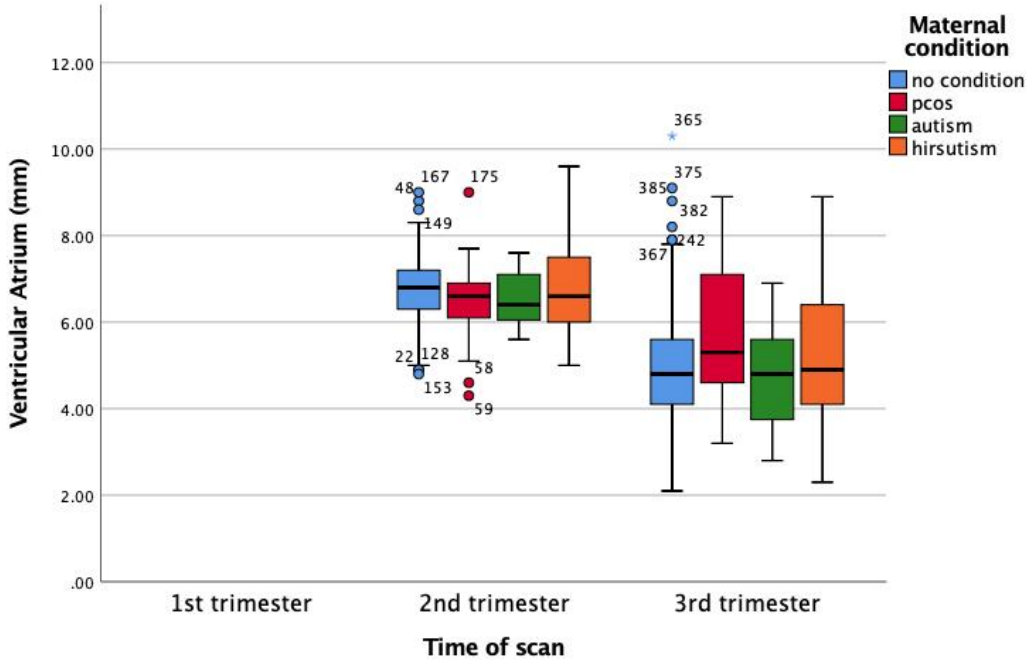

Figure 8: A box plot of raw ventricular atrium measurements split by maternal conditions; no condition (blue), PCOS (red), autism (green) and hirsutism (orange)

2.4.2.4 Middle Cerebral Artery

Lastly, no significant differences were observed between male and female MCA during the additional research scan (p = .791) and there were no significant differences in MCA between maternal conditions at the additional research scan; F(3,211) = .783, p = .254 (*see figure* 9).

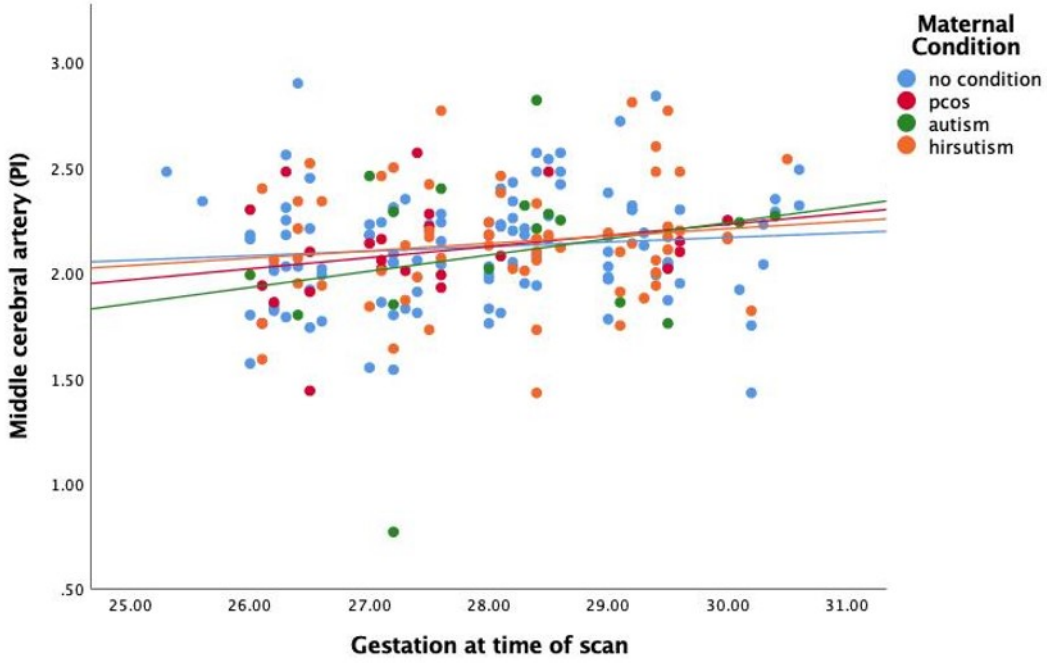

Figure 9: A scatter plot of raw middle cerebral artery PI split by maternal conditions; no condition (R2 linear = 0.011, blue), PCOS (R2 linear = 0.069, red), autism (R2 linear = 0.047, green) and hirsutism (R2 linear = 0.022, orange).

2.5 Discussion

This is the first study to explore the influence of maternal endocrine (PCOS, hirsutism) and neurodevelopmental (autism) conditions on gross fetal brain development. The results from this study show no significant sex differences in fetal biometry (HC, TCD, VA or MCA) measures. Further, no significant sex difference was found in growth velocity. There were no differences in fetal biometry measures according to whether the mother has autism (or whether the fetus had an older autistic sibling) or PCOS diagnosis or potential hirsutism. These results suggest that during these stages of development (12-30 weeks' gestation) there is no effect of these conditions on the developing fetus brain and no observable sex differences.

Conversely, Joffe et al. (2005) found that in human fetuses, males displayed larger HC than females (2.2% at 16-28 weeks' GA and 1.3% at 28-37 weeks' GA) in a sample of 1,427 pregnancies. However, it is unclear whether EFW or GA were accounted for when observing HC (as is standard when observing fetal biometrics), instead only using femur length (FL) to create z-scores for the measures. Therefore, these findings should be interpreted with caution as FL alone is not a good measure of fetal size or GA (Stirnemann et al., 2017). As discussed, new growth charts created by WHO (Kiserud et al., 2017) suggest accounting for sex differences in fetal biometry. This is based on research showing an influence of sex on EFW. However, observations of individual fetal biometric measures (e.g., HC) and sex differences was not reported within this report (Kiserud et al., 2017). Sex-based differences in biometry is mixed and results are inconsistent (Broere-Brown et al., 2016; Joffe et al., 2005; Melamed et al., 2013; Smulian et al., 1995). Within this study there were no sex differences in HC. Additionally, the previous sex-based differences measured within HC whilst statistically significant, are not varied enough to warrant separate sex-based growth charts (Smulian et al., 1995) [*], therefore the lack of observed sex differences on HC measurement may not be unfounded.

Two studies have observed sex differences in VA, with male fetuses having statistically significantly larger VA width; 0.29 mm (Salomon et al., 2007) and 0.6 mm (Patel, Goldstein, Tung, & Filly, 1995) compared to females. Similarly to HC, whilst studies have shown there to be a statistically significant difference, this has been deemed clinically irrelevant (Salomon et al., 2007) mainly due to the inconsistency in observed sex differences across studies (Patel et al., 1995; Salomon et al., 2007). The current study was not able to replicate these findings. Clinically, the 10 mm cut off value[†] has been demonstrated as a stronger marker for atypical development (e.g., later language delay) (Mercier et al., 2001; Sadan et al., 2007). None of the fetuses in this study measured above the 10 mm cut-off in the VA. The results did not demonstrate any differences between the maternal condition groups in VA. However, the lack of difference between the groups could reflect the small sample size in each.

[*] Smulian et al. (1995) examined differences in fetal biometry between males and females in a sample of 539 fetuses (288 male and 251 females). Concluding for small but not clinically significant differences to be apparent between the sexes. The largest sex difference was observed in femur length of 1.7mm (males, mean: 22.1mm ± 3.3mm, females, mean: 21.8mm ± 3.7mm). HC differences between the sexes was not reported.

[†] Ventriculomegaly (defined as the VA measuring >10mm)

Whilst TCD measurement is not uncommon in the UK it is still considered a novel fetal biometry measurement as it is not standard across the world. Similarly to the combination of AC, FL and HC, current research has demonstrated that fetal TCD gives a reliable estimation of GA in the fetus (Reddy, 2017). TCD is therefore a clinically useful measurement of fetal growth. However, there is current disagreement between clinicians as to whether TCD is affected by growth restrictions (e.g., IUGR) or being LGA(Agrawal et al., 2016; Nagaraju, 2015; Reddy, 2017). The susceptibility of this measurement to fetal growth disturbances suggests it may also be affected by other influences such as maternal conditions. This is a novel measure and there has been no research observing sex differences or the influence of maternal conditions on the cerebellum to date. The current study did not demonstrate any group differences in TCD measurements according to group (autism, PCOS or hirsutism) or in growth velocity.

Previous research observing brain volume in autism has found there to be significant differences in numerous areas between individuals with and without a diagnosis (e.g., cerebellum and total brain volume (Hardan, Minshew, Mallikarjuhn, et al., 2001)). Conversely, research observing early brain growth in infants subsequently diagnosed with autism has found two trends: (1) hyper-expansion of the cortical surface area between 6-12 months' of age; (2) brain overgrowth at 12-24 months' of age (Piven et al., 2017). No differences have been found between typically developing and individuals with autism in HC measurements at birth (Hazlett et al., 2005). Similar to these previous findings exploring prenatal development (Hazlett et al., 2005; Unwin et al., 2016), the current study found no differences in HC between typically developing and the 'high risk' of autism group. Research implicates these differences in brain growth and volume begin postnatally, suggesting the prenatal influence on brain development to be mainly on the circuitry and via the organisational effects of sex steroids. The lack of differences observed between the groups, may support previous suggestions that differences observed between typically developing individuals and individuals with autism begin postnatally. **Chapter** 5 will provide longitudinal data of the cohort up to 18-20 months in the postnatal period to see if there is a relationship between prenatal HC and early autistic traits (measured via the Q-CHAT), early behavioural development (measured via the CDI and ITSQ) at 18-20 months' of age.

In contrast to autism, maternal PCOS has not been associated with any brain development or size differences in infants of mothers with a diagnosis. Previous research observing the

influence of PCOS on fetal development has linked fetal IUGR and low birth weight (LBW) (Stirnemann et al., 2017) with later onset of PCOS and fetal SGA with maternal PCOS (Sir-Petermann et al., 2005), which suggests there is an influence of PCOS on fetal biometry. As discussed in *section 2.1.4*, recent animal research has suggested that PCOS originates within androgen receptors found in the brain (Caldwell et al., 2017). However, this idea is still in its infancy and is yet to be observed in humans (Caldwell et al., 2017; Silva et al., 2018). To date, no research has observed the influence of maternal conditions such as PCOS or hirsutism on individual fetal biometry. The current study failed to show any gross anatomical or growth velocity differences between the maternal groups, further supporting the suggestion that gross anatomical volumes and growth differences may only be observed postnatally.

There is ongoing debate about the effectiveness of current prenatal growth charts. Based on the existing standardised growth charts, we can use fetal biometry as a valid marker for potential early growth and later developmental abnormalities. One of the main identifiers used by clinicians is abnormal growth *in utero*. Clinicians are currently exploring the identification of LGA, SGA and IUGR (all identified using fetal biometry measurements taken via ultrasound) and if the definitions should be updated to fit in with today's changing population, as it is suggested fetuses are being misidentified (Ben-Haroush et al., 2004). Until this debate is resolved, this queries the potential for novel measures such as TCD (or any fetal biometric measure) to be used as a valid marker for early growth and developmental abnormalities, which may in turn influence observed differences (or lack thereof) in fetal growth trajectories and patterns. However, based on the existing growth charts, the current study was unable to demonstrate any differences in fetal brain growth between the sexes or show an influence of maternal condition on the growth of the fetus.

There are some limitations to this study, including the limited sample size and size of the maternal conditions' groups. These limitations (which apply to all the studies reported in this thesis) will be addressed in the general discussion in **Chapter** 7.

In summary, results show there to be no influence of maternal condition or a neurodevelopmental condition (PCOS, hirsutism or autism) on fetal brain development. Nor for there to be an influence of fetal sex on these measurements (HC, TCD or VA). This cohort will be followed longitudinally with assessments of language and early autistic traits between 18-20 months' of age. In addition, the influence of maternal PCOS, hirsutism and autism

likelihood on physical and cognitive development will be explored. Future studies should focus on measuring fetal brain development in a larger cross-section (inclusive of fetal complications), with a larger number of pregnancies within each maternal condition.

3 Fetal AGD: Sexual differentiation and maternal influences

3.1 Introduction

3.1.1 Defining Anogenital Distance (AGD)

Anogenital distance (AGD) refers to the length of the perineal area between the anus and genitals (Gilboa et al., 2014; Thankamony, Pasterski, Ong, Acerini, & Hughes, 2016; Thankamony, Ong, Dunger, Acerini, & Hughes, 2009). AGD has been implicated as a predictor of androgen-related outcomes in later life (Jain & Singal, 2013) including reproductive (Barrett et al., 2018; Freire et al., 2018) outcomes. Additionally, there is growing interest in whether AGD may also predict neurodevelopmental outcomes associated with elevated prenatal androgen steroids, such as autism (Baron-Cohen et al., 2015; Baron-Cohen, Knickmeyer, & Belmonte, 2005).

3.1.2 Anogenital Distance and Hormone Exposure

In rodents, AGD length has been experimentally shown to be influenced by prenatal androgen exposure (Thankamony et al., 2016) during the prenatal 'masculinisation programming window' (MPW) (MacLeod et al., 2010; Welsh et al., 2008). AGD is highly sexually dimorphic in both animal (Welsh et al., 2008) and human (Arfi et al., 2016; Gilboa et al., 2014; Salazar-Martinez et al., 2004; Ajay Thankamony et al., 2009) studies. In humans, sexual dimorphism can be observed from as early as 11-13 weeks gestation (Arfi et al., 2016; Thankamony et al., 2016). AGD continues to increase after birth, is correlated with birth weight (BW) (Papadopoulou et al., 2013; Salazar-Martinez et al., 2004); and sexual dimorphism observed prenatally in AGD are maintained across an individual's lifespan (Thankamony et al., 2016). Unlike other proxy measures of early hormonal exposure such as 2D:4D (Dean & Sharpe,

2013) or penile length (Pasterski, Acerini, et al., 2015), postnatal hormonal exposure has not been found to further influence AGD (Papadopoulou et al., 2013) in humans. This supports the theory that prenatal androgen exposure acts as the driver for AGD length. In adults, AGD correlates with circulating serum testosterone (Eisenberg et al., 2012), as well as with the aromatisation ratio (of circulating testosterone to estradiol) (Zhou et al., 2016). This suggests AGD reflects different aspects of the masculinisation pathways in development. Furthermore, there is a relative balance between testosterone and estrogens in the masculinisation pathways, rather than testosterone being the sole driver. Taken together, AGD may be a suitable proxy measure to estimate prenatal androgen exposure.

3.1.3 Anogenital Distance and Endocrine Disruption

In animal studies, AGD is routinely used to observe relationship between early development and *in utero* exposures to endocrine disrupting chemicals (Earl Gray et al., 2006; Foster, 2006). Human research has demonstrated a relationship between: AGD, various endocrine conditions, and environmental factors, in both males and females (Hsieh et al., 2008; Sánchez-Ferrer et al., 2017). Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women and is associated with elevated levels of androgens (e.g., testosterone) (Franks & Berga, 2012). Although the aetiology is not wholly understood, research findings suggest that prenatal androgen exposure plays an important role in its later diagnosis (Franks, 2002; Xita & Tsatsoulis, 2010). A positive association has been found between a diagnosis of PCOS, their testosterone levels, and AGD length in women (Mendiola et al., 2012; Mira-Escolano et al., 2014; Y. Wu et al., 2017). This association is also observed in infants of a mother with a PCOS diagnosis (Barrett et al., 2018). Similarly, increased AGD has also been observed in girls with congenital adrenal hyperplasia (CAH) (Callegari et al., 1987); a disorder also characterised by an over-production of androgens, in particular testosterone (White & Speiser, 2000). Females exposed to higher prenatal androgen levels due to CAH have been observed to display a more masculinised gender identity and behaviour in later childhood[*] (Pasterski et al., 2011;

[*] Pasterski et al. (2011, 2015) observed potential differences in gender identity and behaviour in females with CAH. Researchers used a variety of methodologies, such as parent-report measures child-self report interviews to assess gender identity and behaviour. Measures assessed a child's cognitive and emotional understanding of their gender and desire to be the other sex, as well as observing preferences in gender-related play and playmate preferences. Researchers found for females with CAH show increased cross-gender behaviour (e.g., want to participate in stereotypical games of the other sex) (Pasterski et al., 2011; Pasterski, Zucker, et al., 2015) and identification (e.g., giving more ambiguous responses to questions such as "Do you ever feel more like a boy that a girl?")(Pasterski, Zucker, et al., 2015).

Pasterski, Zucker, et al., 2015). Similarly, AGD has also been positively associated with early observed masculine behaviours in males (Pasterski, Acerini, et al., 2015). Pasterski et al. (2015) found for children with increased AGD to display preferences towards male-typical activities (e.g., 'likes to play with cars, trains or airplanes.') in the preschool activities inventory. In males, shorter AGD has been implicated as a measure of later reproductive (Dean & Sharpe, 2013) and genital abnormalities (MacLeod et al., 2010; Swan et al., 2005). Shorter AGD has also been implicated in issues such as infertility (Eisenberg et al., 2012; Eisenberg & Lipshultz, 2015) and reduced penile length (MacLeod et al., 2010; Pasterski, Acerini, et al., 2015).

Animal models have demonstrated a positive relationship between prenatal androgen exposure (e.g., fetal testosterone (fT)) and AGD (Hotchkiss et al., 2006; MacLeod et al., 2010). Whilst research is yet to directly measure fT in relation to AGD in humans, researchers are in agreeance that knowledge from animal models, combined with evidence from endocrine disruption in humans supports the suggestion that AGD may be a suitable proxy to estimate prenatal androgen exposure, in particular fT.

3.1.4 Using Ultrasound to Measure Anogenital Distance

Measuring AGD in 2D ultrasound scans has been shown to be feasible and reliable (Arfi et al., 2016; Gilboa et al., 2014, 2017) and has the potential to improve early identification and understanding of the pathogenesis of genital development (Arfi et al., 2016), PCOS (Wu et al., 2017); and anorecta (Jain & Singal, 2013; Liu et al., 2009), and male genital (Gilboa et al., 2017) malformation. However, little is known about the influence of maternal conditions (e.g., PCOS[*]) on fetal AGD development. In this chapter, I will confirm the sexually dimorphic nature of this measure and then explore whether two maternal endocrine (PCOS and hirsutism) and a neurodevelopmental (autism) conditions influence AGD development since both have been associated with increased testosterone levels; the link between PCOS, autism in the mother and AGD in the fetus remain relatively unexplored.

[*] Previous research examining the influence of maternal PCOS on AGD has measured AGD length in women with a diagnosis (Mendiola et al., 2012; Mira-Escolano et al., 2014; Sánchez-Ferrer et al., 2017; Wu et al., 2017) and in neonates born to women with a PCOS diagnosis (Barrett et al., 2018).

3.1.5 The Influence of Maternal Conditions on Prenatal Anogenital Distance

In the last decade, research examining fetal development has begun to observe the influence of maternal psychological wellbeing (Diego et al., 2009; Rondó et al., 2003; Weinstock, 2005) and medical conditions (including obesity) (Leddy et al., 2008; Patterson, 2002) on the developing fetus. For example, high levels of stress or anxiety during pregnancy has been implicated as a factor increasing risk of preterm birth and growth malformations (Patel, Rahman, Jacob, & Hughes, 2004; Staneva et al., 2015); suggesting that elevated levels of steroidal hormones *in utero* influence physical fetal development.

3.1.5.1 Polycystic Ovaries (PCOS)

Difficulties in conception and spontaneous loss of the fetus during pregnancy has been well documented in women with a PCOS diagnosis (Homburg, 2006; Teede et al., 2010). Spontaneous loss is reported to occur in 40% of these women, which has been associated with hormonal imbalances caused by the disorder (Lizneva et al., 2016). Clinicians and researchers have identified the importance of early diagnosis of androgen dysfunction which may lead to a later diagnosis of PCOS in adulthood (Franks, 2002). Early identification of PCOS may lead to the prevention of some of the associated co-morbidities and long-term health implications (Gilbert et al., 2018). However, these studies also indicate the need for continued lifestyle intervention, behavioural and hormone therapy (Fitzgerald et al., 2018) when PCOS is identified post menarche.

Several studies have explored the clinical use of AGD as a potential biomarker for prenatal androgen exposure (Barrett et al., 2013; Swan et al., 2015; Swan et al., 2005) and as an early marker for later PCOS diagnosis (Hernández-Peñalver et al., 2018; Wu et al., 2017). Increased AGD has been found to predict the PCOS in adult women (Wu et al., 2017) and to potentially improve the diagnosis between different phenotypes (Hernández-Peñalver et al., 2018)[*]. More recently, researchers have explored the effect of a maternal PCOS diagnosis on their new-born child's AGD. Barrett et al. (2018) found that mothers with a diagnosis of PCOS or hirsutism gave birth to daughters with longer AGDs. Authors suggest that an increase in AGD may be

[*] PCOS can be defined into several phenotypical subtypes, each with differing symptomology. According to the NIH (2012) (*Evidence-Based Methodology Workshop on Polycystic Ovary Syndrome (PCOS),* n.d.) these subtypes are: (1) Androgen excess (including both hyperandrogenemia and hyperandrogenism), (2) Ovulatory disfunction (Oligo-anovulation) and (3) Polycystic ovarian morphology (polycystic ovaries).

due to female fetuses being exposed to elevated levels of prenatal testosterone *in utero*. Barrett et al. (2018) initial findings suggest AGD may be a candidate as a newborn biomarker for androgen exposure and later PCOS diagnosis. However, this study will continue to follow this cohort to see the influence (if any) of longer AGD on later development and potential PCOS diagnosis.

3.1.5.2 Autism Spectrum Conditions ('Autism')

Similar to PCOS, autism spectrum conditions (autism) is also implicated in elevated levels of *in utero* androgen or estrogen exposure. There is a greater prevalence of autism in males than females (Lai et al., 2015; Werling & Geschwind, 2013). Higher levels of fT have been shown to be positively correlated with autistic traits in children, measured by the child Autism Spectrum Quotient (AQ-C) and the Childhood Autism Spectrum Test (CAST) (Auyeung et al., 2009). This observed sexual dimorphism, combined with atypical behavioural patterns in autistic individuals, may provide a clue into the underlying aetiology of autism (Baron-Cohen, 2005). These findings support the 'extreme male brain' (EMB) (Baron-Cohen, 2002) theory, which proposes that the gender bias in autism and the difference seen in systemising and empathising may, in part, be the result of elevated testosterone exposure *in utero* (Knickmeyer et al., 2006). Exposure to elevated levels of fetal testosterone (fT) during pregnancy is associated with atypical brain development (Grimshaw et al., 1995) that may later manifest itself in the atypical behaviours seen in autism. To our knowledge, no research has explored AGD length in autistic individuals or in siblings of autistic individuals.

The potentially sexually dimorphic nature of AGD lends itself as a potential predictor for later diagnoses such as PCOS and autism, triggered by prenatal hormonal imbalances such as increased exposure to testosterone. Initial research exploring the utility of AGD as a predictor of PCOS diagnosis concluded AGD to be a reliable and reproducible measure in adults, that has the potential to improve the diagnosis of PCOS and its phenotypic subtypes (Hernández-Peñalver et al., 2018). The aim of the study described below will examine the feasibility of measuring AGD *in utero*, in pregnancies where there is a maternal diagnosis of PCOS or autism, or in pregnancies where an older sibling of the fetus has an autism diagnosis.

The following research questions are addressed: (1) whether the use of 2D ultrasound during second- and third-trimester to measure AGD can be reliably replicated*; (2) whether similar observations of sexual dimorphism during second- and third-trimesters can be seen in a UK-based population; (3) whether there is an influence of estimated fetal weight (EFW) on AGD length *in utero*†; (4) whether there is a difference in AGD length in fetuses of mothers with and without an autism diagnosis; (5) whether there is a difference in AGD length in fetuses of mothers with and without a diagnosis of PCOS; (6) whether there is a difference in AGD length in fetuses of mothers with and without hirsutism; Finally, (7) whether there is a sex difference in AGD length between the maternal conditions.

3.2 Methods

3.2.1 Participants

See **Chapter 2**, (*section 2.2.1)* for details of study population (*section 2.2, table* 2 for characteristics of population). The same inclusion and exclusion criteria within this study were the same to those used in the Chaim Sheba Medical Centre study (Gilboa et al., 2014).

3.2.2 Ethics

See **Chapter 2**, (*section 2.2.2),* for details on ethics obtained for the study. Gilboa et al. (2014) states the 'study protocol was approved by the local institutional ethics committee.'

3.2.3 Procedure

For details of UK sample see **Chapter 2** (*section 2.2.1),* and for details of the procedure see **Chapter 2** (*section 2.2.3*). AGD measurements were taken on the tangential section of the fetal perineum, where the anal sphincter would first be observed. AGD was measured from the centre of the anus to the base of the scrotum in males, and to the posterior convergence of the

* Previous work by Gilboa et al. (2014) demonstrated the feasibility and reliability of measuring AGD during the second- and third trimesters of development. Additionally, results showed a significant difference in AGD length between the sexes. In this chapter I will aim to replicate findings in a UK population using the same methodology.

† There is a clear relationship between general fetal biometry (HC, AC and FL) and EFW (Hadlock, Harrist, Sharman, Deter, & Park, 1985). To date no research has looked at whether this relationship is also present between AGD and EFW (e.g., the higher the EFW the larger the AGD).

fourchette in females using electronic callipers, following the same procedure used in previous research measuring fetal AGD (Arfi et al., 2016; Gilboa et al., 2014) (*Figures 10 and 11*). This is also referred to as AGD-AF*. For this measurement to be taken, the fetus' legs must be apart to accurately visualise the scrotum (in males) and fourchette (in females). If the legs were not separated when this measure was attempted the mother was given cold water or asked to walk around for several minutes. In instances where the fetus was breech in presentation the examination bed was tilted to move the fetus out of the pelvis and remove shadowing whilst the measurement was being taken. Failure to obtain any AGD measurement was only seen in 4.1% (9 fetuses, 3 male and 6 females; fetal positioning, 4 breech, 4 cephalic and 1 not available) of the total sample (*see table* 2 for population details). Due to the low failure rate of obtaining this measure, more invasive techniques such as transvaginal ultrasound and external cephalic version were not considered.

3.2.4 Considerations for AGD Measurement

Whilst this is a relatively new and novel measurement, the guidelines or techniques used do not differ to other anatomical structures routinely measured during an ultrasound scan. Comparable to other measurements a number of anatomical landmarks were used to assist the sonographer in placement of the electronic calipers, for taking of the measurement (see figures 10 and 11). As there was slight variability in AGD measurement due to the presentation of the fetus, several measurements (range 1-3 freeze frame measurements) were taken and averaged for inter-observer reliability purposes. Ideally researchers aimed to obtain 3 measurements from each fetus, however this was not always possible due to the potential shift of the fetus' position during scanning. Failure to obtain all three measurements was only seen in 14.55% (15 males and 17 females), similarly, failure to obtain two measurements occurred in 8.18% of scans (11 male and 7 females) out of the final sample of 210. Maternal placement for the measure is discussed in *section 3.2.3*.

As highlighted during the feasibility pilot study (see **Chapter 2**, *section 2.2*) for purposes of inter-observer reliability, both measured and unmeasured images were saved. Unmeasured images did not contain an electronic caliper measurement, demonstrated by the yellow

* AGD-AF (anofourchette) is the distance from the centre of the anus to the posterior convergence of the fourchette.

markings and distance in mm; seen below in *figures 10* and *11*. These unmeasured images were then re-measured by the sonographer without reference to the original measurement. Unmeasured images were saved for all scans, and this method was adopted for 20% of the scans chosen at random (reliability for this measure is further discussed in *section 3.3.1 below).*

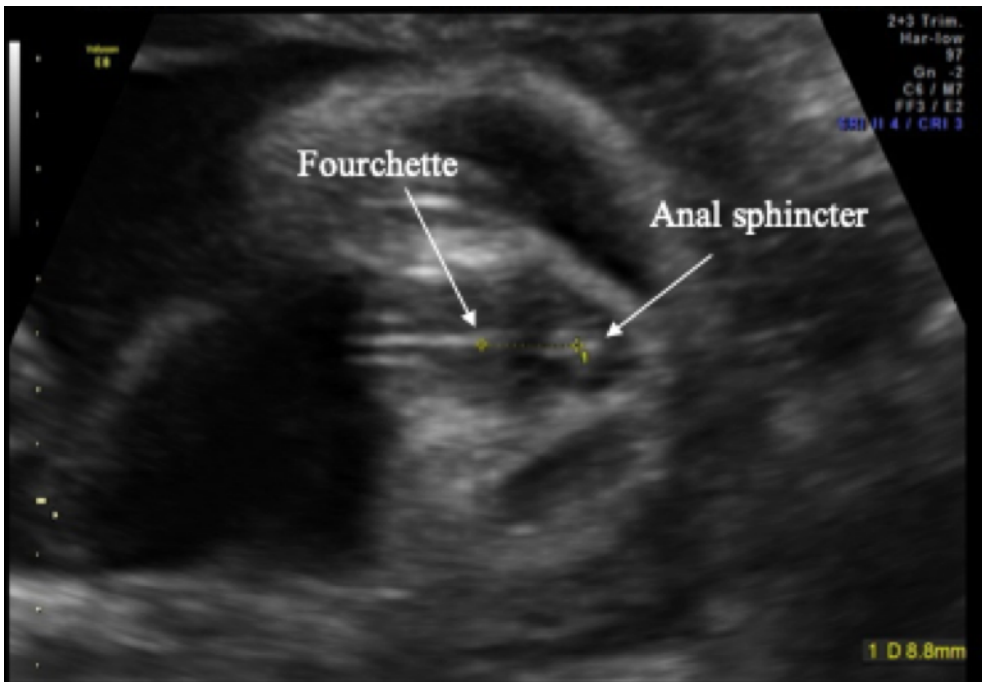

Figure 10: Example of the perineum in a female fetus demonstrating the anogenital distance measurement.

AGD was measured from the centre of the anus to the posterior convergence of the fourchette. The posterior convergence of the fourchette was identified by the visibility of three white lines.

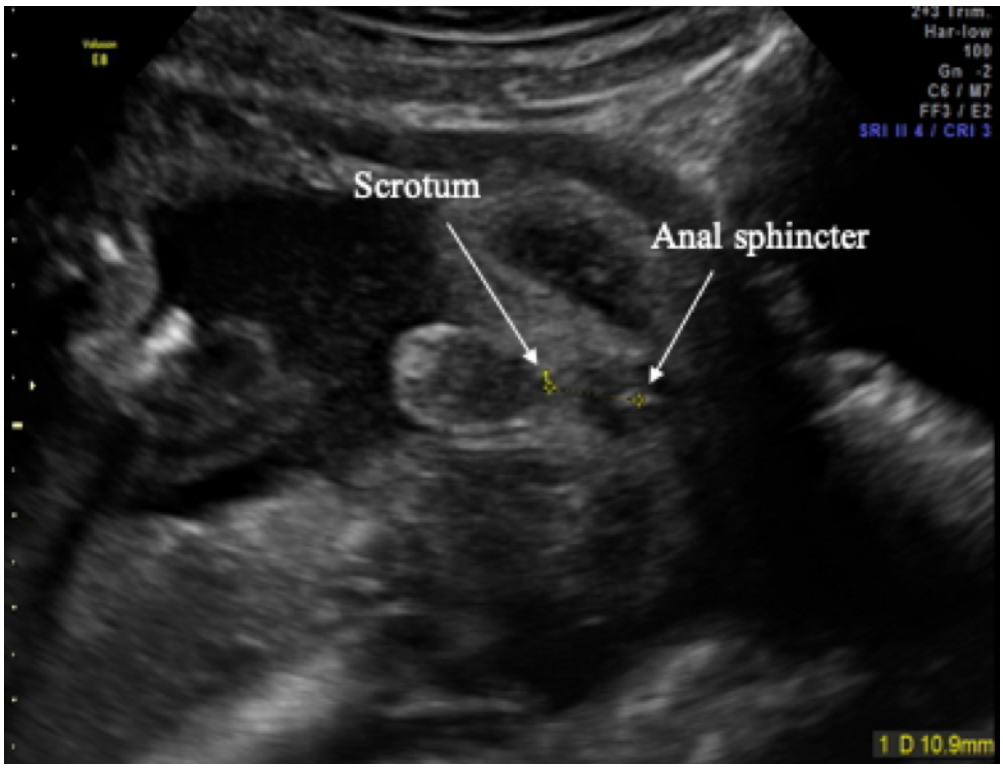

Figure 11: Example of the perineum in a male fetus demonstrating the anogenital distance measurement.

AGD was measured from the centre of the anus to the base of the scrotum. The scrotum was identified by the visibility of the scrotal sack.

Sex assignment was performed by observation of sonographic landmarks such as labial lines or fetal scrotum, in line with the established gold standard (Arfi et al., 2016; Odeh et al., 2009), and was further confirmed at birth.

	All	**Males**	**Females**
		Mean (SD)	
UK AGD	12.15 (3.35)	14.85 (2.18)	9.61 (1.98)

Table 8: Mean fetal AGD measurements (between 26-30 weeks gestation), sorted by sex.

	All (n = 219)	Maternal autism or sibling diagnosis (n = 17)	Maternal PCOS diagnosis (n = 26)	Hirsutism but no PCOS diagnosis (n = 65)	No maternal conditions, (n = 108)
			Mean (SD)		
Genital measurements					
Male fetuses	14.9 (2.2)	14.7 (2.5)	14.8 (2.0)	14.7 (2.8)	15.1 (2.0)
Female fetuses	9.7 (2.1)	9.4 (1.6)	9.5 (1.9)	9.3 (1.7)	10.2 (2.4)

Table 9: Mean fetal AGD measurements (between 26-30 weeks gestation), sorted into maternal condition groups.

3.3 Statistics

3.3.1 Analysis

SPSS statistical version 25 was used. Data were examined using a t-test and a paired samples t-test. As several freeze frame measurements were taken for AGD, intra-observer variability was assessed. The difference between the raw measures is presented in a Bland-Altman plot (*Figure* 13). Linear regression showed no proportional bias (p = 0.753). Inter-observer variability was assessed by comparing the mean measurements of two sonographers on 20% of the sample. Each sonographer was blind to the other's AGD measurements. The difference between the two mean measures is presented in a Bland-Altman plot (*Figure 14*). Linear regression showed no proportional bias (p = 0.911). The feasibility of obtaining an AGD measurement according to fetal position was assessed using a Pearson's chi-squared test.

Associations of AGD with maternal and fetal characteristics were assessed with univariate Pearson's linear regression (*see table* 11) separately for each sex, to investigate potential covariates. Maternal body mass index (BMI) was calculated on weight and height measurements, as reported by the participants at the time of this additional scan*. Nominally significant variables were then consecutively introduced to multivariate linear regression models, according to their level of significance, with AGD as the dependent variable and nominally significant characteristics as the independent variables (*see table* 11).

* Maternal BMI was calculated using pre-pregnancy height and weight of the mothers.

Maternal influence on AGD was examined using an ETA correlation* and a one-way ANOVA. To test for differences, z-scores were created for AGD and EFW, these scores were then transformed into a composite score†. EFW is calculated using the Hadlock formula (Hadlock et al., 1985), taking into account HC, AC and FL.

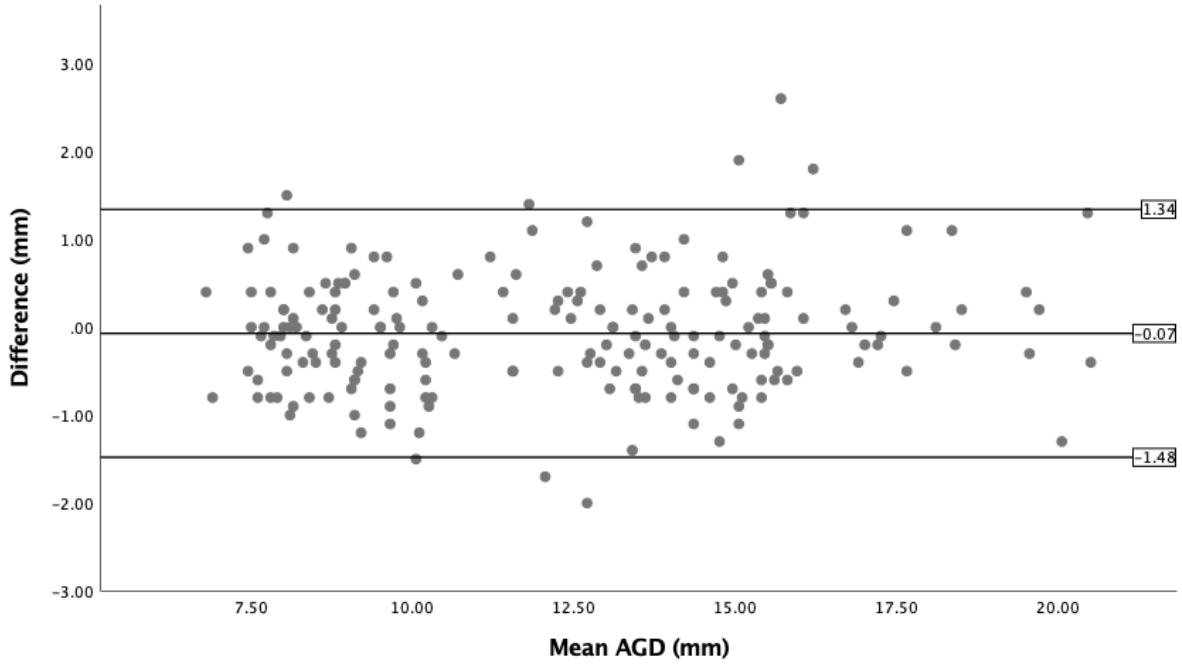

Figure 12: Bland-Altman plot observing intra-observer variability in taken AGD measurements.

*An ETA correlation was used to look for an association between an interval (AGD length) and categorical (maternal endocrine and neurodevelopmental condition) variables.

† A composite score was created taking into account EFW of the fetus as these two variables are known to be, and have shown to be highly related to each other. Whilst gestational age (GA) was also found to be significantly correlated with AGD, statistically the combination of EFW, GA and AGD into a composite score had no influence on the statistical outcome. It was therefore not used.

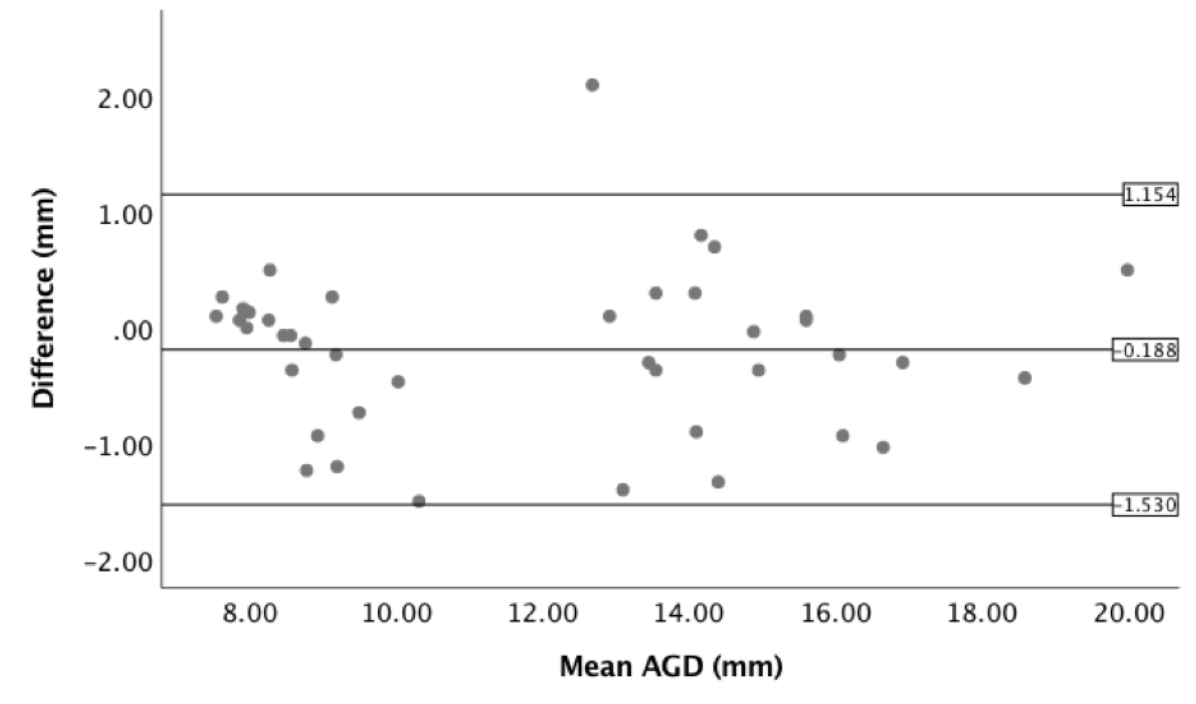

Figure 13: Bland-Altman plot observing inter-observer variability in taken AGD measurements.

3.3.2 Results

The study included 210 fetuses (101 male and 109 female). 9 fetuses (3 male and 6 female) were excluded from the analysis as adequate AGD measures could not be obtained due to fetal orientation during the ultrasound. There was no significant association between any of the specific fetal positions (e.g., breech, *see table* 10 for frequencies) and whether an adequate AGD measure could be obtained (Pearson's Chi-squared test: $\chi^2 = 6.278$, $p = 0.393$).

There was a significant difference in AGD between males (range: 9.80-20.40mm, mean: 14.90mm, SD: 2.18) and females (range: 6.00-15.30mm, mean: 9.72mm, SD: 1.97), t-test: $t = 17.406$, [95% confidence interval (CI): 4.602 to 5.775] $df = 204.45$, $p < 0.0001$. The raw AGD measurements split by sex is plotted in *Figure 15*.

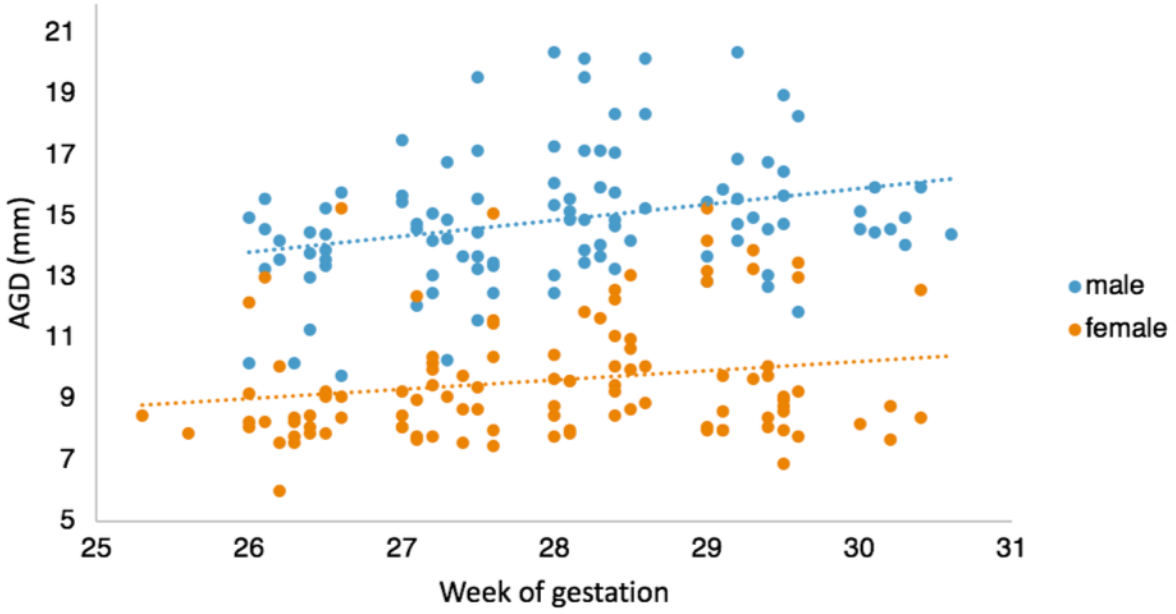

Figure 14: A scatterplot of raw AGD measures split by sex; male fetuses (blue) and female fetuses (orange) between 25-31-weeks' gestation. Mean linear regression lines have been plotted by sex.

Results from the univariate logistic regression analysis indicated that maternal BMI was significantly positively associated with AGD in male fetuses, but not in females; while other maternal characteristics were not associated with AGD. The estimated fetal weight of the fetus, as well as GA and birth weight were all significantly associated with AGD in male but not in female fetuses.

	Females		Males	
	Coefficient	*p-value*	*Coefficient*	*p-value*
Maternal characteristics				
Age	-0.09	0.38	0.13	0.32
BMI	-0.02	0.86	**0.25**	**0.03***
Parity	-0.10	0.30	-0.05	0.60
Ethnicity	0.13	0.20	-0.01	0.93
PCOS	-0.03	0.73	0.02	0.83
IVF	0.11	0.27	-0.04	0.69
Fetal characteristics				
GA†	0.18	0.06	**0.25**	**0.01***
EFW	0.15	0.13	**0.27**	**0.01***
Birth weight	0.06	0.54	**0.25**	**0.02***
Fetal position	0.19	0.054	0.17	0.09

†GA: gestational age at the time of AGD measurement

Table 10: Maternal and fetal characteristics and their effect on AGD in male and female fetuses, as assessed by Pearson's linear regression. Asterisk denotes nominal association.

There was a partial significant correlation between EFW and AGD (r =.193, p = .005), however when the analysis was split by sex (females (r = .135, p = .162), males (r = .272, p = .006)), this correlation only remained significant in males.

Variables that were associated with AGD (*table* 12) were included in multivariate linear regression models. The maximum variance that could be explained by all predictors was 64.5% (adjusted R^2: 0.645, p <0.0001, with fetal sex being the most significant predictor of AGD, across all models (*table* 12).

	Coefficient	*Standard Error*	*p-value*
Model 1			
Sex	5.19	0.30	<0.0001*
	R^2: 0.60		<0.0001
Model 2			
Sex	5.05	0.30	<0.0001*
EFW	0.00	0.00	0.00*
	R^2: 0.60		<0.0001
Model 3			
Sex	5.20	0.31	<0.0001*
EFW	0.00	0.00	0.00*
Birth weight	0.00	0.00	0.14
	R^2: 0.62		**<0.0001**
Model 4			
Sex	5.05	0.35	<0.0001*
EFW	0.00	0.00	0.00*
Birth Weight	0.00	0.00	0.05*
Maternal BMI	0.06	0.039	0.14
	R^2: 0.64		**<0.0001**
Model 4			
Sex	5.10	0.35	<0.0001*
EFW	-0.00	0.00	0.48
Birth Weight	0.00		0.01*
Maternal BMI	0.06	0.04	0.10
GA[†]	0.76	0.41	0.09
	R^2: 0.65		**<0.0001***

[†]GA: gestational age at the time of AGD measurement

Table 11: Multivariate linear regression models, with AGD as the dependent variable.

Data from 207 fetuses (100 male and 107 female) were analysed to examine the association between maternal endocrine (PCOS and hirsutism) and neurodevelopmental (autism) conditions and AGD. 3 fetuses (1 male and 2 female) were excluded from the analysis[*]. There was no association between AGD and maternal endocrine or neurodevelopmental conditions, between the groups: $F(3,203) = .734$, $p = .533$ and $ETA = .104$, $ETA^2 = .0011$ (*see table* 9 for means and SD of raw measurements). Additionally, there was no significant difference

[*] 3 fetuses were excluded as 2 mothers had a diagnosis of both autism and PCOS, one mother had a diagnosis of PCOS and satisfied the criteria to be included in the hirsutism group.

between maternal conditions and AGD in males, F (3, 96) = 1.507, p = .218 or females, F (3, 103) = .393, p = .758 (*see table* 10 for means and SD of raw measurements).

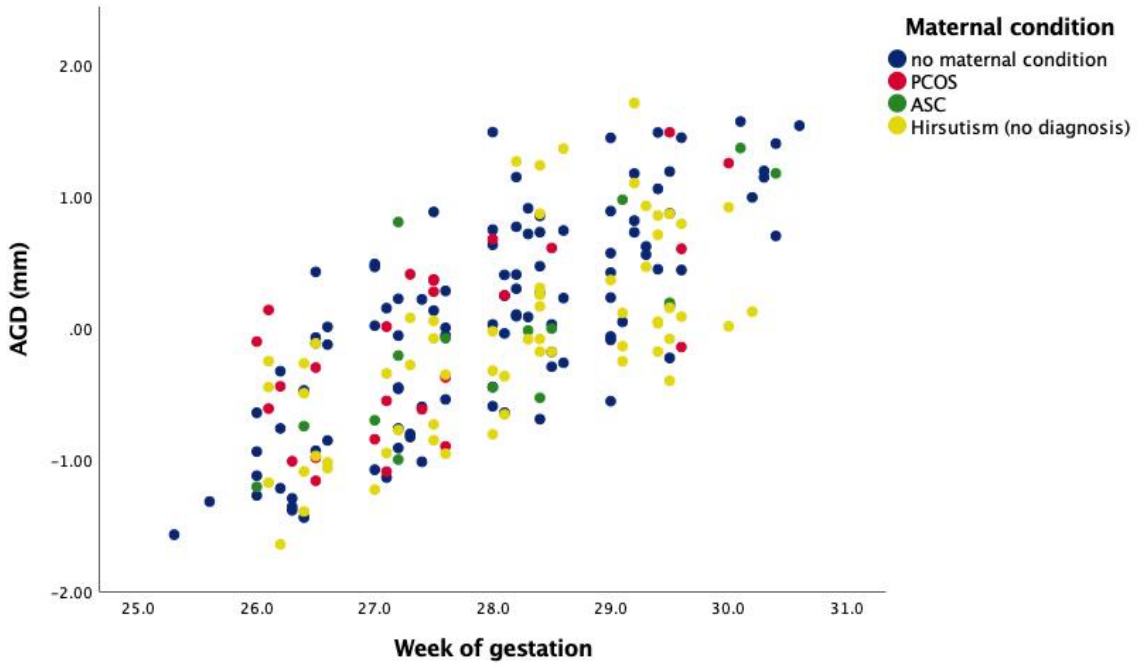

Figure 15: A scatterplot of raw AGD measures split by maternal condition groups; no maternal conditions (blue), PCOS (red), autism (green) and Hirsutism (yellow) between 25-31 weeks' gestation.

3.4 Discussion

Results confirm AGD measurement *in utero* using ultrasound is feasible for the majority of pregnancies and that AGD can be reliably measured *in utero* during the second and third trimesters of pregnancy. In addition, there are strong sex differences (male AGD was significantly larger than female AGD), consistent with previous results suggesting that AGD is influenced by prenatal androgen exposure. Additionally, there was no significant difference between groups of mothers who have androgen related conditions (autism, PCOS and hirsutism) in fetal AGD. This is the first study to explore an association between fetal AGD and maternal androgen related conditions, and therefore further exploration is needed.

The minimal variability in AGD measurement between independent raters suggests that AGD can be measured reliably by sonographers and researchers. In addition, previous results showing fetal AGD is strongly sexually dimorphic from the late second trimester of pregnancy was replicated (Gilboa et al., 2014).

Previous research has demonstrated that measuring AGD from 21 weeks' gestation is feasible and reliable (Gilboa et al., 2014). Research examining newborn (Salazar-Martinez et al., 2004; Thankamony et al., 2009), infant (Sathyanarayana, Beard, Zhou, & Grady, 2010; Thankamony et al., 2009), and adult (Castaño-Vinyals et al., 2012; R. T. Mitchell et al., 2015) AGD has linked this measure to a range of reproductive and developmental outcomes in later life. As a result, researchers have suggested the usefulness of measuring AGD to help assess later developmental outcomes. Early identification of predispositions to later diagnoses (e.g., PCOS) or postnatal outcomes (i.e. genital anomalies) could help inform both early pharmacological and psychological treatment. The ability to reliably measure AGD *in utero* shows this measure has potential to be introduced as part of a routine ultrasound scan. However further longitudinal research, in larger samples is warranted to assess the usefulness of this measure in early identification.

Neonatal and animal research shows a weak correlation between an individual's weight and AGD measurement (Gallavan et al., 1999; Salazar-Martinez et al., 2004). We found a similar significant correlation to EFW and birth weight with AGD (*see table 11*) in male but not in female fetuses. This could be due to sexual dimorphism in the regulation of growth (Toro-Ramos et al., 2016) or it may be because the range of AGD is greater in males (due to levels of testosterone), making it easier to detect the correlation. In addition, we noted a modest association of maternal BMI at the time of the ultrasound scan (mean GA: 28 weeks) with AGD in males. This may be attributed to fetal weight as well, since the effect was not detected after combining maternal BMI with measures of fetal weight (see *table* 12).

Current research observing the relationship between maternal PCOS and AGD has identified AGD to be a potential marker of prenatal androgen exposure (Barrett et al., 2018; Mendiola et al., 2012; Mira-Escolano et al., 2014; Mira-Escolano et al., 2014). However, there is discrepancy between the use of AGD-AC* and AGD-AF with several studies finding a

* AGD-AC (anoclitoral) is the measurement of AGD from the centre of the anus to the tip of the clitoral hood

correlation between PCOS and both AGD measurements (Barrett et al., 2018; Mendiola et al., 2012; Wu et al., 2017) and others finding a correlation with one measurement and not the other (Mira-Escolano et al., 2014; Mira-Escolano et al., 2014; Sánchez-Ferrer et al., 2017). Measuring only AGD-AF, this finding was not replicated in the fetal stages of development, nor was there a relationship between AGD and familial (both maternal and sibling) autism diagnosis. There was no difference in AGD between the four groups. PCOS and hirsutism are categorised as conditions resulting from increased levels of hormones; such as testosterone, luteinising hormone and prolactin (*Polycystic Ovary Syndrome - Diagnosis*, 2017) within the body. However, the severity of the condition may vary dependent upon the level of imbalance within the body, placing this condition on a spectrum (Goodarzi et al., 2011). The negative findings from both this chapter may be a result of mothers within these groups having higher than average levels of hormonal imbalance (enough to receive or suspect a diagnosis) whilst not having a high enough imbalance to influence the fetus' development. Further research with maternal serum and analysis of hormone levels is required to assess the influence of maternal endocrine conditions (such as PCOS) on fetal development. It could be assumed that severity of these conditions could influence fetal development in different ways. In addition, the self-report questions used by researchers to define hirsutism could have led to the reported lack of effect (further discussed in **Chapter 7**, *section 7.2*). Hirsutism can be observed through excess hair growth in a 'male-like' pattern. The Ferriman-Gallwey scale for hirsutism is used to assess the severity of the condition giving an individual a score out of 15 (Bode et al., 2012; Sachdeva, 2010). Inclusion of this assessment in future research may help clinically quantify the level of hirsutism within the sample, if serum options are not available. Similarly, requesting information from the participants in regard to their PCOS diagnosis[*] could enable researchers to categorise severity, if serum analysis is not available.

Furthermore, in addition to small samples sizes, we suggest failure to observe a relationship may be due to possible inaccuracy in EFW. BW and weight (in neonates and babies), height (in infants to adult age), and BMI (in adults) must be taken into consideration, whilst observing any physical measure. Unlike previous findings, using neonates and adults, a direct measurement of height and/or weight is not possible during fetal development; instead EFW is calculated using 2-3 biometric measurements (HC, AC and FL) (Hadlock et al., 1985).

[*] In the UK a PCOS diagnosis is usually made if 2 out of 3 of the following criteria are met: 1. Irregular periods or infrequent periods, 2. Blood tests showing high levels of testosterone, 3. Scans showing polycystic ovaries.

Current research into the accuracy of EFW and its ability to predict actual BW has been mixed (Francis, Tonks, & Gardosi, 2014; Goetzinger, Odibo, Shanks, Roehl, & Cahill, 2014; Milner & Arezina, 2018). Whilst the most commonly used Hadlock A formula (Hadlock et al., 1985) using all three biometric measures is considered to be the most accurate and reliable, it was developed over two decades ago, and it may no longer be relevant to the current fetal growth and population (Milner & Arezina, 2018), potentially influencing these results.

There are some limitations to this study, including the size of the sample, size of maternal conditions groups, and the narrow GA range of 26-30 weeks' gestation. It was not possible to coax the fetus into a more optimal position for AGD measurement for n = 9 (4.1%), using non-invasive techniques (i.e. cold water). Due to time and resource constraints, mothers were not invited back for a second attempt at taking the measurement. Whilst the failure rate of obtained a measurement was low (4.1%), future studies may wish to consider the use of more invasive techniques including transvaginal ultrasound or external cephalic version to obtain this measure in fetuses where non-invasive methods are unsuccessful. However, based on the small failure rate of obtaining an AGD measurement, using more invasive techniques is not necessary.

In sum, the introduction of reliable and reproducible ultrasound measures such as AGD will help further our understanding of the role of the *intrauterine* environment, fetal reproductive programming and its influences on later adult outcomes through non-invasive studies. Whilst direct clinically relevant outcomes remain to be thoroughly explored and growth charts remain to be created, as presented in this chapter, the sexually dimorphic nature and feasibility of obtaining prenatal AGD measurement demonstrated the potential to assist in early diagnosis for a number of outcomes (e.g., male genital malformation, PCOS and other sexually dimorphic developmental conditions such as autism). To our knowledge this is the first time the influence of maternal endocrine (PCOS and hirsutism) and neurodevelopmental (autism) conditions on fetal AGD has been explored. However further longitudinal exploration of fetal AGD and later development and outcomes is needed before the utility of this measure can be accurately assessed. This cohort will be followed longitudinally and the potential relationship between AGD and later infant development (sensory and language) as well as autistic traits will be discussed in **Chapter 6**.

4 Population Based Differences in Fetal Biometry.

4.1 Introduction

In previous chapters (**2** and **3**), the influence of fetal sex and three maternal conditions on novel fetal biometric measurements have been explored. In this chapter, the applicability of findings from these previous chapters (focusing on anogenital distance (AGD)) to an independent population, outside of the UK will be assessed.

The creation and use of standardised growth charts[*] to assess fetal growth has undoubtedly positively impacted pre-, post-natal mortality and morbidity rates, as well as reducing risks during pregnancy for the mother (e.g., *intrauterine* growth restriction (IUGR) (Sharma et al., 2016)). The growing importance placed on fetal growth charts has come from the understanding that prematurity and abnormal fetal growth is linked to common non-communicable diseases (e.g., cancer and diabetes (the Born Too Soon Preterm Birth Action Group et al., 2013)) and mental health conditions (e.g., depression (Pettersson et al., 2019) and bipolar affective disorder (Nosarti et al., 2012)) in later life. However, in recent years there has been some controversy and disagreement amongst practitioners about the accuracy and

[*] Within this chapter I will discuss the use of three different types of fetal growth charts; (1) standardised growth charts; (2) population-based growth charts; (3) customised growth charts. **Population-based growth charts** are defined as charts created using 'norms derived from either heterogeneous or highly selected patient cohorts that do not account for individual variability (Chiossi et al., 2017)' (e.g., Hadlock et al, 1985) The terms 'population-based' and 'standardised' are used interchangeably to refer to the same type of growth chart. **Customised growth charts** are 'norms created by accounting for individuals variables that are known to affect growth, as they allow measurement of deviations from an ideal fetal growth potential, rather than deviations from an expected norm for the population (Chiossi et al., 2017)' (e.g., the Gestation Related Optimal Weight 'GROW' model (Gardosi et al., 2018))

applicability of these growth charts for today's populations (Buck Louis et al., 2015; Drooger et al., 2005; Grobman et al., 2018; Kiserud et al., 2017; Ro et al., 2019).

Research has documented the influence of BW on later health and psychosocial wellbeing (Gale & Martyn, 2004; Hack, 2006; Johnson & Marlow, 2014), but with the increase in awareness of the potential negative implications of fetal growth, research has shifted into investigating the importance of estimated fetal weight (EFW). EFW has been linked to pregnancy complications (Henriksen, 2008), delivery methods (increase in assisted delivery methods such as forceps) (Froehlich et al., 2016) and later health (e.g., obesity (Zhang et al., 2018)). As mentioned previously, EFW is calculated using a number and combination of fetal growth parameters and is used as a potential predictor for later developmental abnormalities[*]. Additionally, the importance of assessing individual growth measurements during development has also been observed (Stirnemann et al., 2017), such as head circumference (HC) for a risk of microcephaly (Leibovitz et al., 2016) and abdominal circumference (AC) for IUGR (Hadlock, Deter, Harrist, Roecker, & Park, 1983). However, in order for potential risks to be correctly identified, reliable reference charts need to be available.

In the last two decades, researchers and clinicians alike have begun to examine maternal influences (e.g., socioeconomic status) as well as potential population influences on fetal growth, such as ethnicity, race and diet. Currently, the gold standard for fetal biometric charts (including EFW) are those introduced by Chitty et al. (1994a, 1994b), and Hadlock et al. (1985) in the late 1980's to early 1990's based on predominately white, middle class populations in the UK and ultrasound. These references charts are the most commonly used fetal growth charts by practitioners worldwide. However, they are now considered to be outdated, and require updating to be in line with the changing characteristics of the maternal population. For example, the National Institute of Child Health and Human Development (NICHD) fetal growth studies have observed many of today's mothers are older, heavier and countries are more ethnically diverse (Buck Louis et al., 2015). Identification of growth restrictions, abnormal growth trajectories and patterns are vital when observing the health of the developing

[**] However, as mentioned previously, the accuracy and applicability of these growth charts (e.g., Hadlock et al., 1985) to calculate EFW for today's population is under scrutiny. The use of these outdated charts are leading to Fetuses being incorrectly identified as IUGR, SGA or LGA, in turn leading to unnecessary interventions (e.g., early induction) (Ben-Haroush et al., 2004).

fetus and predicting later outcomes. Based on existing standardised growth charts, a portion of fetuses are being misidentified as being small-for-gestational age (SGA), large-for-gestational age (LGA) or IUGR*, and unnecessary obstetric interventions are being implemented (e.g., elective early induction and caesarean delivery) (Ben-Haroush et al., 2004).

The existence of population-based growth charts has been around since the late 1990's. Hirst et al. (2016) noted over 100 population-based customised fetal growth charts are currently in use†. The increased number of population-based charts has led to discrepancies in definitions and cut-offs of potential abnormalities, such as SGA. The creation of these charts has largely been flawed or limited by inappropriate sample size, lack of a detailed protocol (e.g., how the ultrasound measurement was taken, what anatomical landmarks were used), and limited to one hospital or location. Conversely, efforts to create population-based fetal growth charts have shed light on the differences between populations in the rates of fetal growth and expected birth weight (BW). This has furthered understanding of the influence of maternal characteristics and environment on early fetal growth. Fetal growth has been shown to be influenced by maternal weight, height, age, nutrition, parity, and fetal sex (Gardosi et al., 2018; Premru-Srsen et al., 2019). However, all of the observed differences between populations cannot be wholly attributed to these variables alone, suggesting an additional interplay with ethnicity and race (Drooger et al., 2005; Grobman et al., 2018; Ro et al., 2019). Hirst et al. (2016) suggest for ethnicity‡ to account for 3% of the variance observed in fetal growth.

The need for updated fetal growth charts has led to several global initiatives to create charts in a reliable and detailed manner (Buck Louis et al., 2015; Hirst et al., 2016; Kiserud et al., 2017, 2018). However, within these several large cohort studies there have been disagreements about

* Previous research has suggested for birth weight (BW) and EFW to be incorrectly predicted in between 12.4-25% pregnancies (Ben-Haroush et al., 2004; Divon et al., 1984; O'Reilly-Green & Divon, 1997). Regardless ultrasound based EFW accuracy has been found to decrease with increasing BW (Ben-Haroush et al., 2004).

†Whilst Chitty et al (1994a, 1994b) and Hadlock et al (1985) are the most commonly used fetal growth charts worldwide, 100 population-based customised fetal growth charts are currently in use (Hirst et al., 2016). From discussions with sonographers and health care professionals it has become apparent that throughout the world clinics and hospitals may opt to use different growth charts. Within a countries several different growth charts may be in use.

‡ Ethnicity in respect to fetal growth has been defined to include factors such as, social inheritance and nutritional traditions. Encasing both genetic differences as well as factors that influence epigenetic settings (Kiserud et al., 2018).

whether customisation of these charts should be population-based or only partially customized from a 'one-size-fits-all' approach (Hirst et al., 2016). All large cohort studies, INTERGROWTH-21st Project (Hirst et al., 2016), NICHD fetal growth studies (Buck Louis et al., 2015), and a recent study sponsored by the World Health Organisation (WHO) (Kiserud et al., 2017) observed fetal growth in multi-ethnic populations. These three studies recommended adopting discrepant growth standards approaches: (1) 'one-size-fits all' (Drooger et al., 2005; Grobman et al., 2018; Ro et al., 2019); (2) only standardising according to fetal sex (Kiserud et al., 2017); (3) only standardising population-based charts to different ethnic-racial groups (Buck Louis et al., 2015). Whilst two studies suggested that the created charts may need to be adjusted for local use if they are to be used for diagnostic and predictive purposes (Hirst et al., 2016; Kiserud et al., 2017), NICHD fetal growth studies have suggested that observed racial and ethnic population differences should be included along with the effect of maternal characteristics (e.g., height and age) on fetal growth (Buck Louis et al., 2015). Therefore, suggesting that it is not necessary to adjust fetal growth charts for local use based on maternal characteristics. Regardless of the multi-ethnic or multi-site nature of these studies, the standards created were based on women who were educated, had adequate nutrition and were clinically healthy, and have therefore come under scrutiny as to their applicability across different populations (Leibovitz et al., 2016).

In addition to population-based fetal growth charts, there is an ongoing debate about the utility of customised charts. Several studies have demonstrated a benefit in customised fetal growth charts when identifying adverse prenatal outcomes (e.g., still birth) (Clausson et al., 2001; Ego et al., 2006; McCowan et al., 2005). However, the positive effects of these charts has been attributed to potential artefacts as a result of the calculation method used when creating reference values, rather than actual predictive ability of potential abnormalities in fetal growth (Hutcheon, Zhang, Cnattingius, Kramer, & Platt, 2008; Zhang, Mikolajczyk, Grewal, Neta, & Klebanoff, 2011; Zhang, Platt, Cnattingius, Joseph, & Kramer, 2007). The study reported below will focus on potential differences in fetal growth between different populations. Whilst it is acknowledged that customised fetal growth charts may help to identify fetal growth abnormalities and potential developmental risks in the future, the interplay between maternal characteristics, ethnicity, race, and fetal growth is still relatively unknown. Last year, Gardosi et al. (2018) introduced 'Gestation Related Optimal Weight' (GROW) charts, allowing for practitioners to adjust for all maternal characteristics (height, weight, parity, and ethnic group). Whilst studies are still ongoing to assess the feasibility and reliability of these charts, initial

studies using these charts have demonstrated increased accurate identification of SGA (Fay et al., 2019; Premru-Srsen et al., 2019) and still birth risk (Andre Francis et al., 2018) in multi-ethnic groups.

Based on previous research, and the observed need for population-based fetal growth charts for the standard growth biometrics (HC, AC and femur length (FL)), it is important to explore potential population-based differences in other novel fetal growth measures, such as AGD. The study reported below will explore these potential differences in AGD across populations and discuss the impact population-based differences may have if they are to be used to inform research or used clinically. To our knowledge, Israel is the only country to have observed and produced fetal AGD reference ranges in both males and females (Gilboa et al., 2014). No other reference ranges for this measure have been made available. Therefore, for the purposes of this chapter, populational differences in AGD will be observed between these two countries.

The following research question was addressed: (1) whether AGD requires population-based charts (similar to other fetal biometric measures), if it is to be used to predict fetal and potentially, postnatal developmental outcomes.

4.2 Methods

4.2.1 Participants

See **Chapter** 2, (*section 2.2.1)* for details of the UK population (*section 2.2.3* for characteristics of UK population*). To observe differences in reference ranges between a UK and Israeli, normal modelled AGD charts created from an Israeli population was used. The Israeli sample consisted of 424 healthy fetuses, (218 female and 206 male) between 20-35 weeks' gestation. However, from this overall sample, a subsample of 118 fetuses (59 female and 59 male) between the same gestational age as the UK sample (26-30 weeks) was used from Gilboa et al. (2014) for this comparison.

* Characteristics for the Israel population were not provided by Gilboa et al. (2014).

4.2.2 Ethics

See **Chapter 2** (*section 2.2.2)* for details on ethics obtained for the study. Gilboa et al. (2014) states the 'study protocol was approved by the local institutional ethics committee.'

4.2.3 Procedure

For details of UK sample see **Chapter 2** (*section 2.2.3)* and for details of the procedure see **Chapter 2** (*section 2.2.3)*

4.3 Statistics

4.3.1 Analysis

SPSS version 25 was used. To test for population differences (*see table* 13 for raw AGD measurements of both populations), statistical analysis was performed using bilinear interpolation* of the observed data from the UK set against an average fetal AGD biometry chart using the normal modelled AGD charts created from an Israeli population(Gilboa et al., 2014). This used gestation and centiles to provide average expected AGD from a different population sample that were then gestation- and centile-matched to this UK population sample and analysed using a Paired samples t-test.

4.3.2 Results

A comparison of AGD between 26-30 weeks' gestation in an Israeli sample and a UK sample showed a significant difference (t =17.214, [95% CI = 2.606 to 3.280], p = .000, d = 1.21). When split by sex, there was a significant difference in both males and females (after controlling for GA (weeks and days)); females (t = 9.489, [95% CI = 1.596 to 2.393] p < .0001, d = .93), males (t = 16.80, [95% CI = 3.461 to 4.389] p < .0001, d = 1.70). This combined sample included 202 fetuses (98 male and 104 female). *See figure 17.* Two males and five

* Bilinear interpolation allows for the finding of a value between two points on a line or curve. Gilboa et al. (2014) created a reference charts for AGD separated by weeks and centiles. Bilinear interpolation was used to extrapolate for example what a fetus of 26.4 weeks gestational age measuring on the 16th centile would measure.

females from the UK sample were not included in this analysis as centile measurements from their ultrasound were not obtained. *See table* 14 for means and SD of the two populations.

Week of gestation	**AGD male fetuses mean ± SD (n)**	**AGD female fetuses mean ± SD (n)**	**AGD male fetuses mean ± SD (n)**	**AGD female fetuses mean ± SD (n)**
	Israel		*UK*	
26	14.1 ± 2.5 (16)	9.3 ± 2.0 (14)	13.4 ± 1.8 (19)	9.0 ± 2.0 (23)
27	14.5 ± 1.9 (12)	9.8 ± 1.6 (13)	14.5 ± 2.1 (26)	9.4 ± 1.8 (25)
28	16.3 ± 2.3 (10)	11.6 ± 2.0 (10)	16.2 ± 2.3 (26)	10.4 ± 1.9 (28)
29	18.5 ± 1.5 (11)	11.0 ± 1.3 (12)	15.1 ± 2.2 (21)	10.0 ± 2.5 (26)
30	17.9 ± 2.0 (10)	12.2 ± 2.3 (10)	14.9 ± 0.7 (9)	9.1 ± 2.0 (5)

Table 12: An overview of raw AGD (mm) measurements in Israel and UK cohorts.

Data has been split by gestational week and separated by sex for both the Israel and UK samples. Israel population data has been taken from Gilboa et al. (2014).

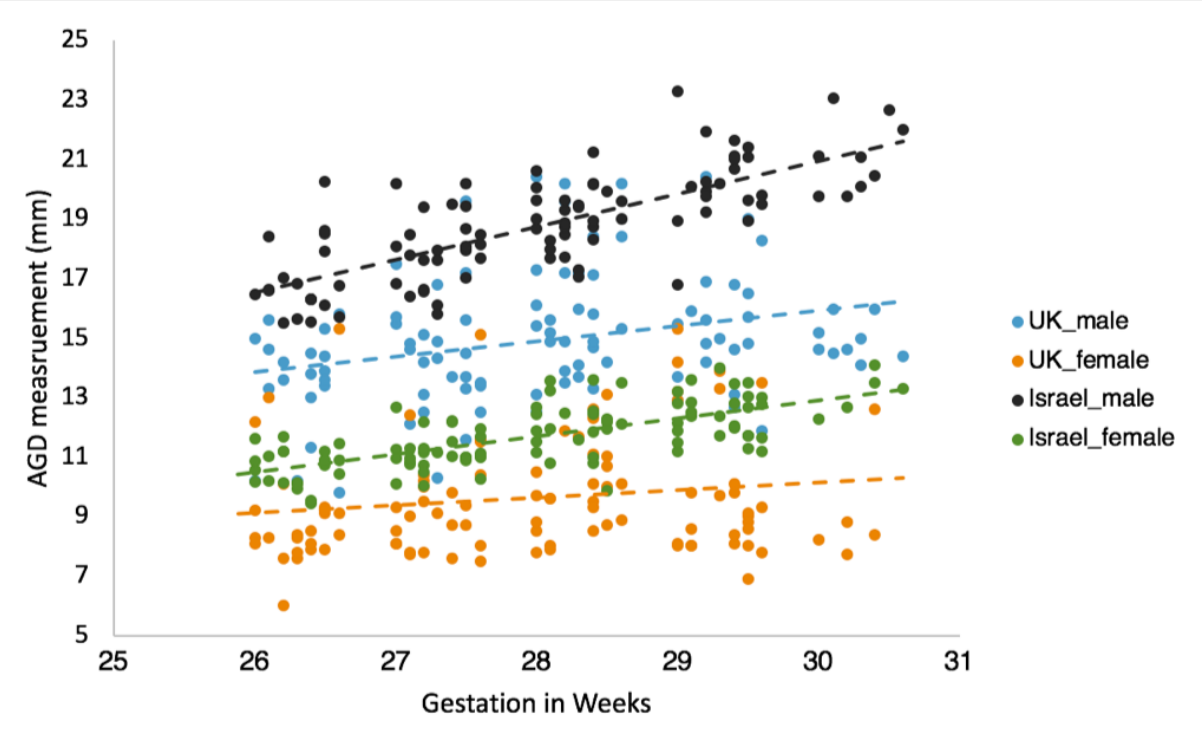

Figure 16: A scatterplot of matched raw AGD measures taken in both UK and Israel populations by sex between 26- and 31-weeks' gestation. Mean linear regression lines have been plotted by sex.

	All	Males	Females
		Mean (SD)	
Israel AGD	15.09 (3.86)	18.78 (1.75)	11.63 (1.06)
UK AGD	12.15 (3.35)	14.85 (2.18)	9.61 (1.98)

Table 13: Mean and SD of raw AGD measurements (mm) from both Israel and UK samples. Presented data have been combined across gestational weeks.

4.4 Discussion

The gold standard of growth charts (Chitty et al., 1994a, 1994b; Chitty & Altman, 2002; F. P. Hadlock et al., 1985) are currently under debate by the medical community as they are deemed outdated and cause misidentification of IUGR, SGA, and LGA, leading to unnecessary inventions (e.g., caesarean delivery (Ben-Haroush et al., 2004)). In addition to replicating the sexually dimorphic nature of this measure in the previous chapter, in line with current literature and clinical views, this study demonstrated that similarly to other fetal growth measure, there are significant differences in AGD between Israeli and UK populations, potentially attributed to ethnicity. This supports the need for population based or customisable growth charts if this

measure is to be used clinically. However further research is warranted to assess the clinical usefulness of this measure.

Previous research has suggested potential differences in AGD means and ranges across ethnic backgrounds (Gilboa et al., 2014). The influence of biological and environmental factors on prenatal growth has been well documented (Buck Louis et al., 2015; Cheng et al., 2016; Gardosi et al., 2018). This study found significant variation between the UK sample and gestational growth charts from a sample in Israel. This demonstrates that similar to fetal biometric charts (such as FL and HC), there is population-specific variability in AGD. The observed variation in AGD length between the UK sample and the Israel sample could be related to differences in fetal positioning during the ultrasound. As the same anthropometric protocol was implemented (e.g., initial identification of the anal sphincter) to guide the measurement and use of the same ultrasound plane, this should result in little to no variability in the measurement taken between the two sites. Alternatively, differences in genetic predispositions within populations (e.g., to congenital adrenal hyperplasia (CAH) in Israel) could account for the observed variation in measured distances (Paperna et al., 2005). Causes of this variability would need to be investigated, but given our knowledge of its existence, population-based charts need to be created if this measure is to be used clinically or for research purposes.

In **Chapter 3**, the sexually dimorphic nature of AGD along with its potential clinical uses were discussed (e.g., polycystic ovary syndrome (PCOS) risk and CAH identification). It is therefore apparent that AGD has the potential to be a clinically useful measurement in early diagnosis and identification in testosterone related disorders (to be further explored in **Chapter 6**). However, in this chapter, it was reiterated that the significant differences observed between populations is an important consideration for further research into means and ranges between populations and the sex differences within these populations, in order to accurately assess risk and not to intervene unnecessarily in induction and/or caesarean deliveries.

Within this study, it was not possible to explore how population-based differences influence the other novel biometric measurements discussed in this thesis (e.g., transcerebellar diameter (TCD), **Chapter 2** or the differences of maternal conditions on AGD between populations). To date, no researchers have created population-based fetal growth charts for these biometrics. Lastly, this study involved teams from Israel and the UK. Inter-observer variability between

the two centres could not be measured, this is therefore acknowledged as a potential limitation of the study. Similarly to Gilboa et al. (2014), future research may wish to create UK-based AGD growth charts if this measure is to be used for clinical or research purposes within a UK sample. Additionally, any potential population-based size differences in these novel fetal biometrics (e.g., TCD), should be explored.

A limitation of this study was the lack of maternal demographic information (such as education levels and SES) made available within the publication, for comparison. The only maternal information provided was mothers included within the study have no 'maternal disease.' However, the classification of 'maternal disease' is not provided and could encompass a range of conditions and disorders from gestational diabetes to cancer (*see **Chapter 1**, section 1.3 for discussion on maternal influences*). In regard to commonly collected demographic information (e.g. SES) previous research has shown associations between finances, SES, fetal development and pregnancy outcomes. For example financial strain during pregnancy is associated with reduced birth weight (Mitchell & Christian, 2017) and maternal nutrition (linked to SES) has been associated with stunted fetal growth and IUGR (Osrin & Anthony, 2000; G. Wu et al., 2004). Therefore, without insight into the maternal population used to create these reference charts, findings and conclusions drawn from this chapter must be interpreted with caution.

5 Fetal Brain Measurements and Early Infant Development.

5.1 Introduction

Research indicates, that structural differences exist in the brains of individuals who later display developmental disorders (further discussed in **Chapters 1** and **2**) (e.g., autism (Brambilla, 2003; Courchesne, 1997; Hardan, Minshew, Mallikarjuhn, et al., 2001)). To date only a handful of studies have explored the relationship between fetal brain growth and later infant outcomes (e.g., fetal ventriculomegaly* and its association to later developmental difficulties such as ADHD (Ball et al., 2013)). In particular several studies have examined the relationship between standard fetal biometrics such as head circumference (HC), biparietal diameter (BPD), femur length (FL) and later autism diagnosis (Abel et al., 2013; Unwin et al., 2016). The findings have been inconsistent and unable to suggest any underlying mechanisms for potential differences in size and growth of these fetal measures (Abel et al., 2013) in relation to a later autism diagnosis. This chapter will explore the potential association between sub regions of the developing fetal brain (HC, transcerebellar diameter (TCD) and ventricular atrium (VA)) and later infant outcomes (sensory, language and early autistic traits) at 18-20 months' of age. Whilst initially the intention of this study was to observe the potential early emergence of autism from the fetal stages of life, it is also important to observe typical development. Therefore, the additional measures of sensory and language development were added to observe infants' development in their ability to process, interact and effectively communicate and with world around them.

* Ventriculomegaly is defined as the ventricular atrium (VA) measuring >10mm.

5.1.1 Early Sensory Development

An individual's response to the world around them is partly defined by their ability to take sensory input from their environment and body, to provide information to the brain. The brain then uses this information to help the individual understand experiences and organise their response (Dunn, 2007). Alterations in sensory processing ability have been shown to interfere with everyday life, for example sensation avoiding behaviours may result in an individual to withdraw from situations very quickly; conversely individuals with sensory sensitivity have shown to be reactive in situations (e.g. irritable or constantly fidgeting). Previous research has identified for various conditions to display alternation in sensory processing ability, such as autism, schizophrenia, ADHD and Fragile X syndrome.

For the most part, the majority of fetal neurosensory development occurs after 20 weeks' gestation (Graven & Browne, 2008). Previous research has identified that preterm birth and low birth weight (LBW) are associated with increased risk for atypical sensory processing, such as displaying stronger sensation seeking, sensory sensitivity and sensation avoiding behaviours (Eeles et al., 2013). Lickliter (2011), suggested these differences in processing occur as a result of time spent in a neonatal intensive care unit (NICU) shortly after preterm birth. Spending time in NICU can have an over-stimulating influence on the developing auditory and visual systems, and an under-stimulating influence on the tactile and vestibular system (Lickliter, 2011). Neonates born preterm display an accelerated rate of physical maturation such as in their cardiovascular function; however this rate of acceleration is not observed in their neurological processes (Graven & Browne, 2008). Rahkonen et al. (2015) found that infants born before 28 weeks' gestation showed white and/or grey matter abnormalities which were associated with increased sensation seeking in the Infant/Toddler Sensory Profile (ITSP). Both studies suggested sensory development can be influenced postnatally.

Previous research comparing available sensory processing tools for infants has identified several questionnaires appropriate for infants up to the age of three (Eeles et al., 2013); the Sensory Rating Scale, ITSP and the Test of Sensory Function in Infants. Authors highlighted the efficiency of both the ITSP and Sensory Rating scale as they can be completed online. During initial development of the ITSP, Dunn and Daniels (2002) reported low to adequate internal consistency (using crochbach's alpha) on all sensory processing sections for 7-36

months' of age. Visual, vestibular and oral sensory processing sections demonstrated poor internal consistency (0.55, 0.42 and 0.55 respectively). Adequate internal consistency was reported for general, auditory and tactile processing (0.63, 0.70 and 0.71 respectively). Comparatively the Sensory Rating Scale reported low internal consistency for vision and taste/smell (0.54 and 0.52 respectively), adequate consistency for touch, movement and hearing (0.75, 0.75 and 0.65 respectively) with high consistency for temperament/general sensitivity and total score (0.82 and 0.90). Whilst it could be argued the Sensory Rating Scale displays a higher level of internal consistency across the sensory processing sections, the ITSP has undergone more rigorous evaluation[*]. Therefore, for the purposes of this thesis, the ITSP will be used to explore infant sensory preferences.

The ITSP measures how a child processes sensory events and evaluates how those responses affect their functional performance (e.g., a child's ability to affectively interact with the environment around then) (Eeles et al., 2013). The caregiver reports frequency (e.g., almost always, frequently, occasionally, seldom and almost never) of child engagement in certain behaviours (e.g., 'My child is unaware of people coming in and going out of the room' and 'My child enjoys making sounds with his/her mouth') (Daniels & Dunn, 2002). Atypical sensory processing can lead to behaviours associated with hyper- or hyposensitivity to stimuli (e.g., intensely reacting to bright lights) (Gourley et al., 2013).

Atypical sensory processing has been associated with difficulties in emotional and attention regulation (Miller et al., 2004), and temperament (Fox & Polak, 2004). Sensory hyper- or hyposensitivity has also been associated with developmental outcomes such as autism (Thye et al., 2018). One study of 197 autistic children used the ITSP and identified 86.8% to have at least one atypical sensory domain[†] (Dellapiazza et al., 2019). Research has utilised the ITSP

[*] For example, the initial assessment of reliability for Sensory Rating Scale was conducted using 117 infants. Whilst ITSP used a sample of 401 infants aged 0-36 months. Furthermore, the ITSP has also been developed using the author's previous experience in observing patterns of sensory processing. Using studies of more than 1000 children without and 100 children with conditions, such as ADHD (Eeles et al., 2013). Comparatively the Sensory Rating Scale was developed based on the author's review of literature on behaviours associated with sensory sensitivity (Eeles et al., 2013).

[†] Dellapiazza et al. (2019) identified for 86.8% of children diagnosed with autism to show atypical sensory processing in at least one of the four quadrants measures by the ITSP (low registration, sensation seeking, sensory sensitively and sensation avoiding). These children's mean scores were significantly lower on all quadrants when compare to the typically developing group.

when observing potential early likelihood for autism. Philpott-Robinson et al. (2016) used the ITSP to observe sensory behaviours in infants (aged 12-24 months') with an increased likelihood for the development of autism*. Results showed that high autism likelihood infants had 'distinct pre-diagnostic sensory subtypes.' Differences were found between the autism likelihood and typically developing groups in the sensory reactive and adaptive subtypes (hypersensitivity in the auditory and vestibular domains). In addition, the 'high risk' infants showed less maturity in the oral sensory domain. Whilst atypical sensory behavioural differences are a ubiquitous feature of autism, it is by no means a behavioural characteristic only attributed to autism. Sensory Processing Disorders (SPD) are observed in a number of other conditions such as ADHD and fragile X syndrome (Dunn & Bennett, 2002).

The use of the ITSP in pre- and postnatal development has mainly been used to explore the impact of preterm birth on sensory development (Beranova et al., 2017; Crozier et al., 2016; Rahkonen et al., 2015). To date no research has explored fetal growth from *utero* in relation to later sensory development.

5.1.2 Early Language Development.

Language development is a critical part of an infant's development. It allows the infant to be able to communicate, express and understand emotions, whilst also supporting their ability to think, problem-solve, develop and maintain relationships. Delayed language development has been associated with a number of behavioural problems (e.g. ADHD), social difficulties and learning disabilities. Whilst research has shown there to be a parental influence on an infant's language development and acquisition (Bornstein et al., 2000; Kuhl, 2004), studies have also shown a relationship between fetal growth and language.

To date, a small body of research has measured the potential relationship between HC and language development (Davidovitch et al., 1996; Deutsch & Joseph, 2003; Sacco et al., 2007; Whitehouse et al., 2012), with inconsistent results. Two studies concluded there is no association between infant or fetal HC and language (Davidovitch et al., 1996; Deutsch & Joseph, 2003). Conversely, Whitehouse et al. (2012) identified that there were no differences

* Philpott-Robinson *et al.* (2016) defined 'high risk' infants to be those with either (1) a sibling with a diagnosis or (2) born prematurely before 37 weeks' gestation.

in HC during the second trimester, although there was a significant association between specific language impairment and small HC at birth. Similarly, in a sample of autistic children, Sacco et al. (2007) found that children (aged 3 to 16 years' of age) whose verbal language was within the typical range had a larger median HC. The inconsistency in these results may stem from factors such as age range of sample and small sample sizes[*]. To date, Whitehouse et al. (2012) is the only study to have observed fetal biometrics in relation to language development.

Unlike HC, the cerebellum has been widely examined (in adults) in relation to speech production, control (e.g., speed, emphasis and pitch) (Spencer & Slocomb, 2007) and reading (Salman & Tsai, 2016), with more robust results. Research has linked differences in the cerebellum volume to problems with motor control and language processing in children aged 6 to 13 years' (Hodge et al., 2010). However, the majority of these studies differences have been found in the structure and function of the cerebellum (e.g., a larger posterior-lateral cerebellar lobule VIIIA in children with specific language impairment) (Hodge et al., 2010). This level of detailed observation is not possible using ultrasound methodology, nor during the fetal stages of development. Allin et al. (2001) explored the association between cerebellum size, cognitive and motor functions of the individuals in children born preterm (before 33 weeks' gestation). This was observed using a longitudinal design; behavioural assessments at 1, 4 and 8 years and an MRI at 14-15 years of age. Associations were found between cerebellum size and cognitive measures such as intelligence and reading ability. Within this study a reduced cerebellum size was associated with poorer cognitive ability (e.g., scoring lower on intelligence and sequential processing). Authors suggest the reduced cerebellum size might be reflective of a loss of cell populations or prenatal atypical development which in turn influences later cognitive ability (Allin et al., 2001). In contrast, Hodge et al. (2010) did not find an association between cerebellum size and language impairment in children aged 6-13 years. To

[*] Deutsch and Joseph (2003) had a total sample of 63 children aged between 4-14 years (54 males and 9 females). Davidovitch et al. (1996) included 148 children, mean ages 51.2 months (for group 1: HC measuring on or above the 98th centile) and 46.9 months (for group 2: HC measuring below the 98th centile) with 123 males and 25 females. Both studies found no association with later language development and HC measurement. Conversely, Sacco et al. (2007) included 241 children aged 3-16 years (205 males and 36 females) and Whitehouse et al. (2012) had a sample of 60 children (30 with specific language impairment, 30 without) samples were matched 12 males and 18 females. Both studies found a negative association between HC and language development, children with smaller HC demonstrated poorer language skills and development.

date, research examining the relationship between cerebellum volume and later language outcomes has been conducted postnatally (from childhood onwards).

To our knowledge, for infants between the ages of 18-20 months there are currently four norm-referenced instruments available for assessing language ability. These are; MacArthur-Bates Communication Inventories (CDI) (Fenson & others, 2007), Preschool Language Scale (PLS) (Zimmerman et al., 1997), Receptive-Expressive Emergent Language Test (REEL) (Bzoch & League, 1991) and the Vineland Adaptive Behaviour Scales (Sparrow et al., 1984). The PLS is the most popular tool used to assess infants and young children's language skills, however it requires in-person administration. Previously, researchers have found for parents to be a reliable indicator of their child's vocabulary development (Larry Fenson et al., 1993; Larry Fenson & others, 2007). From the above measures, the most widely used standardised parental report and observation for language abilities is the CDI. The developed short form of the CDI (used within this thesis) demonstrated a high reliability of 0.97 (cronbach's alpha) and high validity on vocabulary production (personal correlation: 0.74) (Larry Fenson & others, 2007). This chapter will explore whether there is a relationship between fetal cerebellum size and infant language development using the communication development inventory (CDI).

The CDI assesses a child's communicative skills, including vocabulary comprehension, production, gestures and grammar, completed by caregivers. It is one of the most widely used early language development tools by clinicians and researchers (Fenson et al., 2007). Language starts to emerge at around 6 months' of age, when infants begin to babble, developing the ability to form sentences by the age of 3 years (Kuhl, 2004). Furthermore, brain growth (size) has been linked to first (native) language acquisition in the first 15 years of life (from birth to 15 years) (Sakai, 2005), with brain growth reaching peak size at around 6 years of age.

Thus far research into prenatal development and language development has mainly focused on prenatal language exposure and how *in utero* exposure shapes postnatal response to speech and language (e.g., infants are born showing familiarity for their native language) (May et al., 2011). In the developing fetus, the auditory system is functional from 25 weeks' gestation (Graven & Browne, 2008). Kisilevsky et al. (2003) demonstrated that a fetus[*] can hear and

[*] Mean age of fetuses in the study were 38.4 weeks gestational age. All were low risk pregnancies with a singleton fetus.

remember sounds *in utero* showing the ability to discriminate between familiar (mothers') and unfamiliar (strangers') voices. This was measured through differences in heart rate and general fetal movement (e.g., movements in the head or torso). The influence our *in utero* exposure has on language and sound has been further supported in studies by DeCasper et al. (1980, 1986), showing new-born ability to discriminate and preference for their mother's voice. Research is yet to explore the relationship between fetal growth and later language development and acquisition.

5.1.3 Early Emergence of Autistic Traits.

As discussed in the introduction to the thesis (**Chapter 1**, *section 1.3.2.1*) early detection and identification of autism or autistic traits is now possible from 12 months' (e.g., using expressive, receptive language ability (Eyler et al., 2012) and the 1-year well-baby check-up(Pierce et al., 2011)) and 18 months' of age (e.g., using the Quantitative Checklist for Autism in Toddlers (Q-CHAT) (Allison et al., 2008)). Studies observing the early emergence of autism have helped further our understanding of the condition. Retrospective studies examining children who were later diagnosed with autism found that these children display differences in social and communication development and play within the first 12 months of life (Kraemer, 2000). Furthermore Bolton et al. (2012) in a study observing precursors and early signs of autism also found early differences hearing and vision[*]. Parents of children later diagnosed with autism reported more concerns relating to their child's vision and hearing during the first year of life. For example, parents reported concerns about responses to certain noises (for hearing) and a tendency towards poor eye contact (for vision). Previous research has demonstrated differences in the early behaviours in infants later diagnosed with autism. Highlighting, in infants the potential early emergence of autism through observation of behaviour and physical development. The table below provides information on commonly used measures to assess the early emergence of autism between 18-20 months' of age (see *table 15*).

[*] Bolton et al. (2012) do not specify what measures were used to assess hearing and vision, instead stating they 'selected measures of correlated features concerned with vision, hearing, and motor development' from the Avon Longitudinal Study of Parents and Children (ALSPAC) study dataset.

Screening Tool	Age range	Completion time	Test performance	Additional Information
Checklist for Autism in Toddlers (CHAT)	18-42 months	5-10 minutes	Se: 18% Sp: 100% PPV: 75% NPV: 99.7%	Whilst the specificity of this tool was excellent, the sensitivity was too low to warrant the use of this questionnaire as an exclusive tool for identifying the early emergence of autistic traits.
Modified Checklist for Autism in Toddlers	16-20 months	5-10 minutes	Se: 85% Sp: 91-99% PPV: 48% for Autism NPV: 95% for a developmental delay.	Is a two-stage assessment: a 20-item questionnaire with a follow-up interview. Due to the nature of the follow-up at 18-20 months being online-only. This tool was not deemed appropriate.
Qualitative Checklist for Autism in Toddlers	18-24 months	5-10 minutes	Se: 91% Sp: 89% PPV: n/a NPV: n/a	The QCHAT and ITC (below) have similar sensitivity and specific scores, the Q-CHAT was chosen to be used within the study for the main reason that researchers within the project have greater experience with the use of this specific tool. Additionally, the original validation of the QCHAT took place in Cambridgeshire.

Infant Toddler Checklist (ITC)	8-24 months	5-10 minutes	Se: 93% Sp: 83% PPV: 71-79% NPV: 88-99%	Due to the reasoning above, this questionnaire was not considered.
Rapid interactive Screening Test for autism in Toddlers (RITA_T)	18-36 months	10 minutes	Se: 100% Sp: 84% PPV: 88% NPV: n/a	This screening tool involves additional training and a two level assessment (one of which is in-person). The in-person assessment is carried out after an initial screening via the use of a questionnaire such as the M-CHAT.

Se; Sensitivity, Sp; Specificity, PPV; Positive predictive value, NPV; Negative predictive value.

Table 14: Commonly used tools to assess the early emergence of autism.

The Q-CHAT questionnaire is used to assess early autistic traits in infants between the ages of 18-30 months. It is a revision of the earlier Checklist for Autism in Toddlers (CHAT), aiming to increase the sensitivity in identification of toddlers at risk for autism. The Q-CHAT developed on the existing CHAT by increasing the range of available responses from 'yes' and 'no' to include frequencies (i.e., always, usually, sometimes, rarely and never) and combining both health professional and parental report sections into parent-report only (Allison et al., 2008). The Q-CHAT is a 25-question parental report questionnaire that asks caregiver to report on the frequency of behaviours that may be indicative of autism during the toddler period. The questionnaire covers 5 domains; joint attention, pretend play, repetitive behaviours, language and sensory development and additional social communication behaviours.

Previously research has used the Q-CHAT to observe the relationship between prenatal development and autistic traits in infancy. Auyeung et al. (2010, 2012), demonstrated an association between prenatal testosterone levels and autistic traits. Fetal testosterone (fT) in

amniotic fluid (taken as part of routine care in high risk pregnancies) was measured and compared to autistic traits between 18 – 20 months' of age (mean ages: 19.25 (Auyeung et al., 2010) and 19.34 months (Auyeung et al., 2012)) using the Q-CHAT. Findings showed a positive correlation between prenatal fT level and infant Q-CHAT scores. However, this correlation was not observed in postnatal testosterone levels (Auyeung et al., 2012)[*], suggesting prenatal testosterone to have a greater influence on later autistic traits. Additionally, as discussed in **Chapter 2**, there is a plethora of research suggesting there are differences in brain growth in children later diagnosed with autism. However, similar to the other measures (ITSP and CDI), research exploring the relationship between fetal brain measurements and early autistic traits is yet to be explored. The developmental measures described in the section above will be used in the study described below to assess infant development; as well as exploring how these outcome measures may be associated with fetal biometry measures (VA, HC and TCD).

It would be misguided to suggest that the chosen questionnaires used within this chapter; ITSP, CDI and Q-CHAT, did not overlap in developmental outcomes measures (i.e. different ways to observe autism). However, considering the majority of the population observed within the study are not high risk, it was important to also include questionnaires to explore different aspects of general development. In **Chapter 1**, *section 1.3.2* autism is defined as a disorder with "persistent difficulties in social communication and interaction, as well as restricted and repetitive patterns of behaviour's activities or interests (including sensory behaviours)" (Mottron & Bzdok, 2020). Atypical sensory and/or language development is observed in individuals with an autism diagnosis. However, this is not true of all cases of autism. As discussed throughout the introduction to this chapter, sensory and language difficulties are also a presentation in other disorders and general delays in infant development (e.g. ADHD (Dunn & Bennett, 2002)). A recent study by Beranova et al (2017) has explored the use of ITSP as a screening for autism. Researchers concluded ITSP to be useful in identification of some cases of autism when used in adjunct with other screening measures such as the M-CHAT (Beranova et al., 2017). Therefore, whilst the combination of these measure can be used to create a clearer picture of the early emergence of autism, they can also be explored as individual measures of development. In addition to the data collected on early autistic traits, language and sensory

[*] Postnatal testosterone levels were measured via a saliva sample taken from infants when they were three months' of age.

development, the parents were also asked to complete an Ages and Stages questionnaire observing communication, gross motor, fine motor, problem solving and personal-social development. However, due to collection errors (i.e. parents not being displayed an age appropriate version of the ages and stages) this data was deemed unusable.

The following research questions are addressed: (1) whether there is a relationship between structural brain measurements (VA, TCD and HC) and early autistic traits (using the Q-CHAT); (2) whether there is an association between structural brain measurements (VA, TCD and HC) and early language development (using the MB-CDI); (3) whether there is an association between structural brain measurements (VA, TCD and HC) and early sensory development (using the ITSP); (4) if there are sex differences in any of the above three associations.

5.2 Methods

5.2.1 Participants

See **Chapter 2** (*section 2.2.1)* for details of the study population (*section 2.2.3, table* 2 for characteristics of population).

5.2.2 Ethics

See **Chapter 2** (*section 2.2.2),* for details on ethics obtained for the study.

5.2.3 Procedure

For details of UK sample see **Chapter 2** (*section 2.2.3)* and for details of the procedure see **Chapter 2** (*section 2.2.3)*.

18 month-follow up

When the infants were between 18-20 months (mean age; 18 months and 22 days), parents were sent an online version of Quantitative Checklist for Autism in Toddlers (Q-CHAT), Infant/Toddler Sensory Profile (ITSP) and the MacArthur Communication Development Inventory (MB-CDI) short form. 177 parents completed this online follow-up; 92 females, 85 males (*see table* 16 for mean questionnaire scores).

	Males	**Females**	**All**
	Mean ± SD (n)		
Q-CHAT	30.0 ± *7.8 (85)*	29.6 ± *7.6 (92)*	29.8 ± *7.7 (177)*
MB-CDI (short form)	22.6 ± *18.2 (84)*	25.6 ± *20.4 (92)*	24.1 ± *19.4 (176)*
ITSP			
a. General Processing	12.9 ± *2.3 (74)*	12.5 ± *2.2 (84)*	12.7 ± *2.2 (158)*
b. Auditory Processing	36.9 ± *5.9 (74)*	36.1 ± *8.2 (84)*	36.5 ± *7.2 (158)*
c. Visual Processing	21.3 ± *4.7 (74)*	20.4 ± *5.3 (84)*	20.8 ± *5.0 (158)*
d. Tactile Processing	49.2 ± *11.2 (74)*	46.6 ± *11.9 (83)*	47.8 ± *11.6 (157)*
e. Vestibular Processing	17.1 ± *3.8 (73)*	16.3 ± 3.9 (83)	16.7 ± *3.9 (156)*
f. Oral Processing	27.9 ± *3.4 (19)*	24.9 ± *5.6 (26)*	26.2 ± *5.0 (45)*

[a] General Processing: no raw total score is calculated for this section.

[b] Auditory Processing: Typical performance total score is between 35-43

[c] Visual Processing: Typical performance total score is between 20-27

[d] Tactile Processing: Typical performance total score is between 48-61

[e] Vestibular Processing: Typical performance total score is between 18-23

[f] Oral Processing: Typical performance total score is between 23-31 (13-18 month olds) and 24-32 (19-24 month olds)

Table 15: Mean scores from the 18-20 month follow-up questionnaires, split by sex.

		Males	Females	All
		Mean ± SD (n)		
Head Circumference				
12 weeks	Low likelihood*	82.1 ± 12.3 (55)	79.2 ± 10.2 (59)	80.6 ± 11.3 (114)
	High likelihood	85.1 ± 6.9 (14)	81.3 ± 9.5 (9)	83.6 ± 8.0 (23)
20 weeks	Low likelihood	178.5 ± 5.6 (67)	174.9 ± 7.8 (77)	176.6 ± 7.0 (144)
	High Likelihood	179.7 ± 9.7 (14)	175.7 ± 8.4 (12)	177.8 ± 9.2 (26)
Research Scan	Low likelihood	265.7 ± 13.4 (69)	262.4 ± 14.0 (80)	263.9 ± 13.8 (149)
	High likelihood	269.7 ± 13.2 (15)	271.8 ± 10.1 (10)	270.5 ± 11.9 (25)
Transcerebellar Diameter				
20 weeks	Low likelihood	20.6 ± 0.7 (66)	20.4 ± 0.9 (73)	20.5 ± 0.8 (139)
	High likelihood	20.9 ± 1.0 (14)	20.8 ± 1.1 (10)	20.9 ± 1.0 (24)
Research Scan	Low likelihood	33.2 ± 2.3 (61)	33.2 ± 2.5 (78)	33.2 ± 2.4 (139)
	High likelihood	34.2 ± 2.8 (14)	34.4 ± 1.2 (10)	34.3 ± 2.2 (24)
Ventricular Atrium				
20 weeks	Low likelihood	6.8 ± 0.9 (66)	6.5 ± 1.0 (70)	6.7 ± 0.9 (136)
	High likelihood	6.9 ± 0.9 (14)	7.2 ± 0.6 (10)	7.0 ± 0.8 (24)
Research Scan	Low likelihood	5.6 ± 1.6 (57)	4.9 ± 1.4 (77)	5.2 ± 1.5 (134)
	High likelihood	4.8 ± 0.7 (13)	4.6 ± 1.7 (11)	4.7 ± 1.2 (24)

Table 16: Mean fetal brain measurements split by sex and autistic traits (Q-CHAT score).

5.3 Statistics

5.3.1 Analysis

Data were examined using a Pearson's correlation for questions 1-3 to observe potential associations between fetal brain measurements (HC, VA and TCD) later language, sensory and autistic traits. Total (raw) scores for the Q-CHAT, ITSP and MB-CDI were used.

Potential sex-differences in the scores on developmental questionnaires were also assessed. There were significant differences between male and female scores on the oral processing domain assessed in the ITSP, $p = .018$, however the small sample size (45 total) with unbalanced group sizes (26 females and 19 males) could call into question the significance of this difference. No other significant differences were observed between the male and female

* High and low likelihood measured by the Q-CHAT. High likelihood defined as a score of 0-38) and low likelihood defined as a score of +39.

scores on; Q-CHAT (p = .168) CDI (p =.729) or ITSP domains (auditory (p = .059), visual (p = .363), tactile (p = .409) and vestibular (p = .651) domains).

It is important to note, previous research has used both (M)ANOVA (Germani et al., 2014; Rahkonen et al., 2015) and Pearson's correlation (Bart et al., 2011) to observe the potential differences in development using the ITSP. A Pearson's correlation was used to examine the sensory profile scale scores as continuous variable in relation to fetal brain measurements (also as a continuous variable). Research opting to use ANOVA's have separated individuals into groups (less that others probably difference, less than others definite difference, typical performance, more than others probable difference and more than others definite difference) based on sensory processing domains and summary grid, provided by the questionnaire (see *appendix 6* for grid and summary of scores). Within this study the distribution of the sample between the group was unbalanced, calling into question their power (see *appendix 7*, sample sizes across sensory processing domains). Therefore, this statistical analysis was not used.

All analyses were also split by sex (as a secondary step) to observe sex differences. For all fetal biometry measurements, a z-score was created for analysis accounting for gestational age (GA) at the time of the scan. Furthermore, as few planned comparisons were being made, multiple comparisons within the analysis were not corrected for.

Whilst the use of the ITSP manual suggests the use of z-score instead of raw scores (Daniels & Dunn, 2002), there was no difference in the p-values, therefore raw scores were used in the analysis reported below. For the CDI and Q-CHAT no such recommendations have been made, however analysis was also run using the standardised z-scores; similarly to ITSP there was no difference in the observed p-values. Therefore, for all three developmental questionnaires the raw scores were used for analysis.

Results from the linear regression analysis indicated that GA at time of birth was not significantly associated with any of the follow-up measures (Q-CHAT or CDI). Within the ITSP, only tactile processing was significantly associated with gestational age at time of birth. However, as it only accounted for 3.2% of the variance (R^2 = .032) gestational age at time of birth was not controlled for within the following analyses.

	Total		
	Coefficient	*p-value*	R^2
Q-CHAT	-.572	.150	.013
CDI	1.342	.193	.011
ITSP			
Auditory processing	.533	.184	.013
Visual processing	.457	.272	.022
Vestibular processing	.279	.186	.013
Tactile processing	**1.358**	**.037***	**.032**
Oral processing	.310	.580	.008

Table 17: The associations between the follow-up measures used within the study gestation at time of birth, as assessed by a linear regression. Asterisk denotes nominal association.

Lastly, it is important to note, the mean Q-CHAT scores (*see table* 16) observed in this sample are significantly higher (p = .014) then those reported by Allison et al. (2008) (mean Q-CHAT scores for males = 27.5, females = 25.8 and total = 26.7).

5.3.2 Results

The study included 177 fetuses (92 females and 85 males). 1 fetus (male) was excluded from the analyses as an outlier.

5.3.2.1 Infant/Toddler Sensory Profile (ITSP)

There were no significant differences between infant sensory processing sections (auditory, visual, tactile, vestibular or oral) and fetal brain measurements (HC, VA and TCD) at any of the three gestational time points (*see table* 18 for p-values)

5.3.2.2 Communication Development Inventory (CDI)

There were no associations between language (number of words) and fetal brain measurements (HC, VA and TCD) at any gestational time point (*see table* 18 for p-values).

	Auditory Processing	Visual Processing	Tactile Processing	Vestibular Processing	Oral Processing
Head Circumference					
12 weeks	p = .934	p = .771	p = .937	p = .920	p = .524
20 weeks	p = .215	p = .696	p = .528	p = .728	p = .620
Research Scan	p = .312	p = .362	p = .284	p = .108	p = .868
Transcerebellar Diameter					
20 weeks	p = .122	p = .363	p = .209	p = .695	p = .663
Research Scan	p = .083	p = .913	p = .111	p = .133	p = .883
Ventricular Atrium					
20 weeks	p = .530	p = .833	p = .646	p = .770	p = .819
Research Scan	p = .826	p = .646	p = .207	p = .089	p = .672

Table 18: p-values from the Pearson's correlation observing the relationship between fetal brain measurements (HC, TCD and VA) and ITSP score on the 5 sensory processing domains.

5.3.2.3 Quantitative Checklist for Autism in Toddlers (Q-CHAT)

There was a positive correlation of TCD at 20 weeks' gestation only (range: 18.40 - 23.60mm, mean: 20.6mm) and Q-CHAT scores (range: 14.00 – 53.00), Pearson's correlation: r(160) = .247, p = .002. This significance remained only in females when stratified by sex, r(80) = .282, p = .010; males, r(78) = .216, p = .054,. Standardised TCD measurements by gestational age and raw Q-CHAT scores are plotted by sex in *Figure 17.*

There was a significant difference in VA at 20 weeks gestation only (range: 4.80 – 9.00mm, mean: 6.7mm) and Q-CHAT scores (range: 14.00 – 53.00), Pearson's correlation: r(157) = .185, p = .020. This significance was also observed when split by sex, but only in females; males r(78) = .145, p = .199 and females, r(77) = .234, p = .038. Standardised VA measurements by gestational age and raw Q-CHAT scores are plotted by sex in *Figure 18.*

No significant associations were seen at 12- or between 26-30 weeks' gestation of TCD or VA with Q-CHAT score. There were no correlations of HC and Q-CHAT at any of the time points (*see table* 19 for p-values and *table* 17 for mean fetal brain structure measurements split by sex and Q-CHAT high/low scores).

	Q-CHAT	***CDI***
Head Circumference		
12 weeks	*p = .938*	*p = .470*
20 weeks	*p = .0.61*	*p = .630*
Research Scan	*p = .246*	*p = .402*
Transcerebellar Diameter		
20 weeks	*p = .002***	*p = .275*
Research Scan	*p = .207*	*p = .742*
Ventricular Atrium		
20 weeks	*p = .020**	*p = .602*
Research Scan	*p = .813*	*p = .296*

**denotes significance at the 0.05 level (2-tailed)*
***denotes significance at the 0.01 level (2-tailed)*

Table 19: p-values from Pearson's correlations.

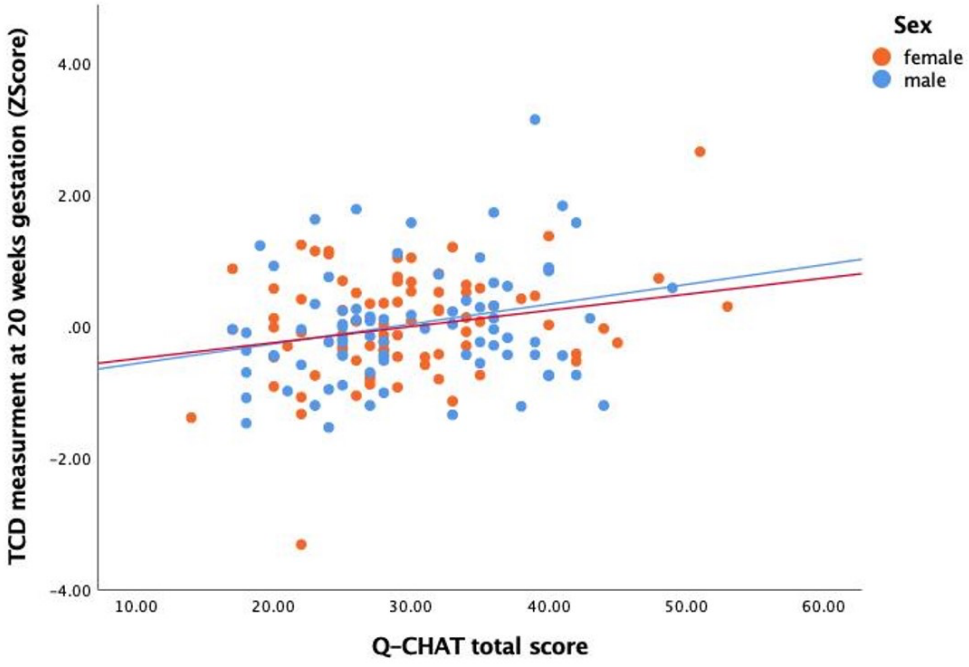

Figure 17: A scatterplot of TCD z-scores and raw Q-CHAT scores, split by male (R2 Linear = .047, blue) and female (R2 Linear = .079, orange) infants between 18-20 months' of age. Mean linear regression lines have been plotted by sex.

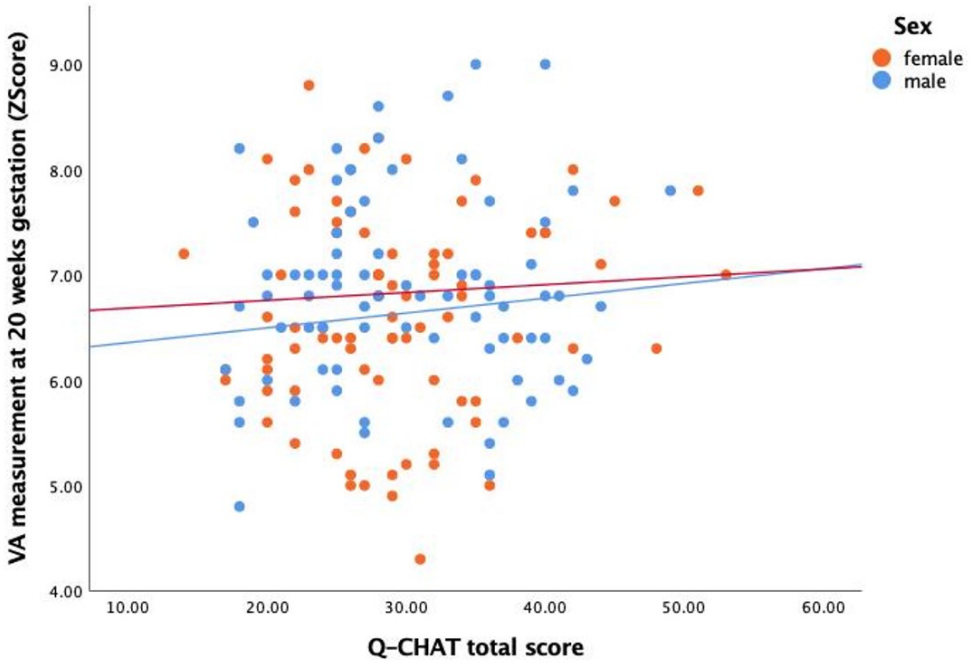

Figure 18: A scatterplot of VA z-scores and raw Q-CHAT scores, split by males (R2 Linear = .004, blue) and female R2 Linear = .013) infants between 18-20 months' of age. Mean linear regression lines have been plotted by sex.

5.4 Discussion

This chapter examined whether a relationship exists between brain measurements (HC, TCD and VA) taken during prenatal development and later sensory and language development in infancy. In addition, the relationship between these brain measurements and infant autistic traits was also explored. Results showed a positive correlation between VA and TCD size at 20 weeks' gestation and autistic traits. These results suggest that larger TCD and VA is associated with greater autistic traits in infants. This correlation remained significant only in females when stratified by sex. Previous studies by Abel et al. (2013) and Bonnet-Brilhault et al. (2018) associated prenatal brain overgrowth with a later diagnosis of autism. However, these studies (Abel et al., 2013; Bonnet-Brilhault et al., 2018) observed atypical growth in HC, using this measurement as a proxy for brain development. The current study was unable to replicate this finding (using HC); however, an association was observed between autistic traits and the brain growth in sub-regions (TCD and VA).

As discussed in the introduction to this thesis (*section 1.5.2*), the observed sex difference in neurodevelopmental disorders (Kraemer, 2000) and obstetric complications (Di Renzo et al., 2007) has been attributed to a female fetus' potential to effectively adapt to early adversity (e.g., during periods of famine mothers are more like to give birth to daughters (Song, 2012)) *in utero* (Sandman et al., 2013). There is a growing body of research around the idea of a 'protective female model' (Dupont et al., 2018; Jacquemont et al., 2014). Based on current research, females demonstrate the ability to adapt to early adversity *in utero* (Sandman et al., 2013). Suggesting female may be better at 'programming' against morbidity and later developmental impairments (e.g., autism (Jacquemont et al., 2014)) *in utero*. Based on the 'protective female model' compared to males, females may require stronger experienced adversity *in utero* and during fetal development to exhibit disorders such as autism (Jacquemont et al., 2014). This theory that female fetuses require a larger threshold of genetic modifications before exhibiting traits of conditions such as autism could explain the findings from this study. When split by sex only females displayed a positive correlation between levels of autistic traits at 18-20 months' and VA width and TCD size. In line with the 'protective female model' this could suggest that this larger mutational burden may result in physical changes to brain structure, such as larger VA width and TCD size at 20 weeks' gestation. Interestingly this difference in size was only observed during the second trimester anomaly scan (between 18-22 weeks' gestation), reasoning behind this could be based on placental barrier activity. As discussed in **Chapter 1** and **7** (*section 1.1 and section 7.2*) animal models have suggested that that placental barrier becomes less active between approximately 19-26 weeks' gestation (Seckl, 2006), resulting in a period of vulnerability for the fetus. Which in turn, is why a positive correlation between these brain structures (VA and TCD) and Q-CHAT scores were found only during this gestational time point.

Currently, no research has examined whether fetal brain measures influence the development of atypical sensory experiences. In this study no differences in fetal brain measures (HC, VA or TCD) were observed in relation to later sensory development. Previous research examining early sensory development has shown that postnatal experiences (NICU) influence an infants' sensory development. Two studies (Lickliter, 2011; Rahkonen et al., 2015) have shown sensory development to be influenced by preterm birth which has an impact on the neonates' level of

maturation* and early environmental exposure (e.g., increase in the amount of unfiltered auditory stimulation) (Graven & Browne, 2008). Combined with the findings from this study, this may suggest that a greater influence on an infants' sensory development occurs postnatally within the developing brain, as opposed to during the prenatal stages of development influencing structural growth.

Research examining the relationship between language development and gross physical measurements (e.g., HC) has been inconsistent. In this study no association between fetal HC (at 12-, 20- or between 26-30 weeks' gestation) and language development was observed. This supports findings from previous research by Whitehouse et al. (2012) and suggests language development may be related to postnatal as opposed to prenatal HC growth. However further longitudinal studies examining HC (from prenatal to infant age) in relation to language development are warranted. This will be explored using longitudinal data from this cohort (collected during at optional in person visit at 18-20 months), however the physical infant data collected during this follow-up will not be included within this thesis.

Currently, the use of fetal TCD and VA as a marker to assess early risk for later neurodevelopmental health is being explored (Ball et al., 2013; Koning, Dudink, et al., 2017). In this study, no association between these brain structures (TCD and VA) and language development was found. To date, this is the first study observing the relationship between TCD, VA and later language development. However, the lack of association could be due to the methodology used (ultrasound and MB-CDI). For example, whilst over the later few decades ultrasound resolution has increased to be able to detect subtle anatomical details (Prayer et al., 2006) it is still considered inferior to MRI. Further research is needed to explore subtle anatomical changes in the cerebellum during prenatal development in relationship to later language development, potentially considering the use of MRI methodology.

Overall limitations of the thesis will be summarised within the discussion in **Chapter 7**. One limitation of this study is the use of the CDI. For the follow-up portion of the study the MB-CDI (short form) was used, however this questionnaire was created using American-English words (e.g., applesauce) as opposed to British-English words. A UK version of the MB-CDI

* As discussed in the introduction to this chapter, unlike physical maturation (i.e. the development of their cardiovascular function) neonates born preterm do not experience an accelerated rate of maturation in their neurological processes (Graven & Browne, 2008) (which encompass sensory development).

has been created, the Oxford Communication Development Inventory (OCDI), however, it was not used. Research over the years has adapted the CDI to more accurately assess language development accounting for linguistic and cultural difference between languages (Frota et al., 2016). Using the MB-CDI (without adaptation) would have led to the inclusion of words British-English speaking infants have not been exposed to, therefore inaccurately measuring their language acquisition. Additionally, the correlations within this study were relatively small, therefore replication using larger samples is needed to truly observe whether an association between fetal brain measurements (VA and TCD) and autistic traits exist. Future studies may wish to observe sensory development in a larger sample and language development using a more UK appropriate measurement, such as the OCDI.

In addition, within the ITSP only sensory processing across the 5 domains were observed (e.g., tactile, auditory), an infant's reaction to sensory experience assessed via 4 quadrants (i.e., sensation seeking, sensory sensitive, low registration and sensation avoiding) could not be explored. During the follow-up phase only 45 of the 177 parents who responded to the questionnaire completed the 'oral sensory processing' section of the questionnaire (e.g., 'my child refuses to try new foods'). In order to calculate quadrant scores responses must be given to all sensory processing domains of the questionnaire. Due to the low number of completed ITSP's, quadrant scores were not calculated or explored within this sample.

It is also important to acknowledge, there was no significant sex differences in outcome measure scores (CDI, ITSP and Q-CHAT). This lack of difference may reduce the theoretical basis for stratifying the analysis in this chapter (and **Chapter 6**) by sex and potentially contribute to the lack of differences found between brain development and later outcomes. Lastly, the Q-CHAT scores within the cohort were significantly higher than those reported by Allison et al. (2008) (see *section 5.3.1*). This may in part be explained by the way the sample was chosen (i.e., opportunistic sampling) but also related to the catchment area of the hospital (i.e., Cambridgeshire). Cultural influences related to place of residence have been suggested to affect autism prevalence (Lauritsen et al., 2014), which may explain the higher mean Q-CHAT scores of infants within this study.

In summary, this chapter found a significant relationship between fetal brain measurements (VA and TCD) at 20 weeks' gestation and infant autistic traits (at 18 months postnatally). However, when stratified for sex, this association was only maintained in females, suggesting

an influence of fetal sex development and timing of exposures (to potential adversities experiences *in utero*). No relationship was observed between other fetal brain measurements and later language or sensory development at any of the time points.

6 Fetal Anogenital Distance and Infant Behavioural Development

6.1 Introduction

Previous research (discussed in **Chapter 5**) has associated fetal growth (e.g., head circumference (HC)) with later behavioural development (sensory and language) as well as early autistic traits (Davidovitch et al., 1996; Deutsch & Joseph, 2003; Rahkonen et al., 2015; Sacco et al., 2007; Whitehouse et al., 2012). In **Chapter 5**, results demonstrated a positive relationship between both fetal ventricular atrium (VA) and transcerebellar diameter (TCD) at 20 weeks' gestation with Q-CHAT scores between 18-20 months' of age. These results indicate a potential relationship between specific fetal growth measures (TCD and VA) and later autistic traits in toddlers. This chapter will explore the potential relationship between a novel fetal biometric: anogenital distance (AGD) (previously described in **Chapter 3**) and early infant development. Early development will be inclusive of language, sensory (e.g., visual and oral domains) as well as early autistic traits.

Due to the sexually dimorphic nature of AGD, this measure has been explored as a clinical indicator of reproductive health (Caballero-Campo et al., 2018; Eisenberg et al., 2012; Mendiola, Stahlhut, Jørgensen, Liu, & Swan, 2011; Sathyanarayana et al., 2015; Zhou et al., 2016); first trimester determination of fetal sex (between 11- and 14-weeks' gestation) (Arfi et al., 2016); and potential indictor of endocrine disruption (Barrett et al., 2018; Mendiola et al., 2012; Mira-Escolano et al., 2014; Mira-Escolano et al., 2014; Wu et al., 2017). Additionally, AGD has been implicated as a potential marker of early androgen exposure in humans (Barrett et al., 2018; Hernández-Peñalver et al., 2018; Mendiola et al., 2012; Mira-Escolano et al., 2014; Wu et al., 2017). It has therefore been hypothesised that AGD may be a useful predictor of

later development (e.g., precursor for PCOS diagnosis in females (Hernández-Peñalver et al., 2018) and reproductive health in males (Eisenberg et al., 2012; Freire et al., 2018)) and a potential predictor for later neurodevelopmental outcomes (Baron-Cohen et al., 2015; Baron-Cohen et al., 2005).

The introduction of this thesis (*section 1.5.2*) outlined the importance of acknowledging biological sex differences and how early exposure to sex hormones have potential long-term influences on development. Physiological (as a result of sex hormones) sex difference becomes apparent from as early as 8 weeks' gestation when the fetal testes become active (only in males) (Hines, 2008). Research has demonstrated gonadal hormones, in particular the major steroid hormones (i.e., estradiol, progesterone and testosterone), have an influence on the neural and behavioural development of humans (Hines, 2008). This has been linked to later cognitive (e.g., spatial awareness (Hausmann et al., 2000)), language (Schaadt et al., 2015; Whitehouse et al., 2010) and sensory development (e.g., auditory development (McFadden, 2002)). Whilst essential ethical constraints have precluded experimental manipulation of prenatal hormones, the use of animal models and observations in humans, via clinical syndromes related to hormonal abnormalities during life (e.g., congenital adrenal hyperplasia (CAH)), has enabled us to further our understanding (e.g., of the influence of steroid hormones on physiological development). Through this research we have been able to explore the influence of steroid hormones such as testosterone on the developing brain and later behavioural outcomes. To date, there is limited research exploring the relationship between prenatal androgen exposure, later language development (Svetlana Lutchmaya et al., 2001) and autistic traits (Auyeung et al., 2009, 2010, 2012). However, research is yet to explore the association (if any) between prenatal androgen exposure and early sensory development.

6.1.1 Sex Differences in Sensory Development

6.1.1.1 General Sensory Development

As discussed in **Chapter 5**, fetal neurosensory development occurs from 20 weeks' gestation onwards (Graven & Browne, 2008). From as early as 2 years of age, sex differences have been observed in psychomotor development. Peyre et al. (2019) showed females demonstrate better

fine motor and language skills than males[75]. However, this difference was no longer apparent by 5-6 years of age. Observing earlier motor development, Piek et al. (2002) suggested limb movements and coordination from 6-18 weeks of age, were associated with the later emergence of female advantage in fine motor skill and a male advantage in gross motor movement. Both studies (Peyre et al., 2019; Piek et al., 2002) have suggested that these sex differences in motor movements cannot be wholly attributed to social, emotional or behavioural factors, instead potential biological influences (e.g., prenatal androgen and estrogen exposure) should be considered (Peyre et al., 2019; Piek et al., 2002). Fagard et al. (2018) suggests that it is through these displayed early 'primitive motor babbling' behaviours (e.g., rotating of the head, movement of the limbs), the increasing of knowledge of their own body and environment that leads to later sensory development (e.g., tactile development).

Animal models have added to our understanding of how early hormone exposure influences sensory development. In rats, Horvath and Wikler (2001) demonstrated that the influence aromatase (estradiol) dependent mechanisms may play in the differentiation and maturation of sensory pathways. In rats, aromatase and aromatase activity was present in areas associated with sensory processing within the olfactory and visual systems. However, researchers did not explore whether aromatase acted as a hindrance or advantage to these systems. Instead, the authors simply demonstrate the presence of aromatase in sensory pathways. Further research is needed to observe biological influences (e.g., the influence of sex hormones) on the development and maturation of sensory pathways, before we determine the directionality of influence specific sex hormones have on these pathways and development.

6.1.1.2 Visual Development

Research shows on average, males have a larger visual cortex than females (Vanston & Strother, 2017). Previous research in adults has demonstrated visual processing differences between the sexes in a range of areas from visual acuity (compared to women, males perform better on acuity tasks (Abramov et al., 2012)) to colour perception (Rodríguez-Carmona et al., 2008) (e.g., higher rates of colour vision deficiency is found in males[76] (*Colour Vision*

[75] Peyre et al. (2019) used a parental questionnaire derived from the Brunet–Lézine Psychomotor Development Scale to assess fine and gross motor skills at 2 years of age and the Ages and Stages questionnaire at ages 3 and 5-6 years. 2002 families were included in the study, at 2 years of age there were 1772 participants.

[76] According to the NHS, colour vision deficiency (mainly a problem with distinguishing between shades red, yellow and green) affect 1 in 12 men and only 1 in 200 women[26].

Deficiency (Colour Blindness), 2017)). These differences between males and females are potentially explained by the difference in visual cortex size (Vanston & Strother, 2017). A recent study by Shaqiri et al. (2018) explored sex-based differences in visual perception using a number of paradigms[77] finding males to outperform females in 6 of the 15 tasks. In no task did females significantly outperform males. Similarly, research has observed, during infancy males also outperform females on visual tasks. For example, at 2 months' of age males demonstrate a higher level of contrast sensitivity[78] (Dobkins et al., 2009) and at 3 months they display greater accommodative responses[79] (Horwood & Riddell, 2008) (e.g., changes in points of fixation). Conversely, in infants, females have been shown to precede males in the development of depth perception from 4-6 months' of age (Alexander & Wilcox, 2012; Horwood & Riddell, 2008). Stereopsis (depth and 3D perception) has been found to be negatively correlated with serum testosterone levels (Held et al., 1984). Gwiazda et al. (1989) have suggested the potential slower development of stereopsis in males is potentially related to the neurotropic effects of testosterone. However, no further research has been conducted exploring the influence of testosterone on stereopsis development. Therefore, the influence of testosterone on stereopsis development remains unclear. Alexander and Wilcox (2012) conclude that observed sex differences in visual development and performance do not have any long-term consequences for later behavioural outcomes as they appear to be transient (i.e., only apparent at one time point during development).

6.1.1.3 Auditory Development

McFadden et al. (2002; 2011) have suggested prenatal androgen exposure influences cochlea development as well as aspects of the auditory brain. Previous research found females to have

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[77] Shaqiri et al (2018) using a sample of 626 participants (342 females, 284 males) aged between 18-82 years aimed to observe potential sex differences in fifteen common visual perception measures. These measures were used to explore visual acuity (e.g., orientation discrimination task and the Freiburg visual acuity task) contrast detection threshold (e.g., the Ponzo-hallway illusion task) and motion detection (e.g., the simple reaction time task and the Simon task). The full sample size was not included in every task.

[78] The 'contrast sensitivity' observed by Dobkins et al. (2009) was defined as luminance (light/dark) and chromatic (red/green) sensitivity.

[79] Horwood and Riddell (2008) measured the accommodative responses of an infant. These are the reflex action of an eye, in response to focusing on a near then looks at a far object or stimulus (and vice versa).

better otoacoustic emissions (OAE's) and display better auditory evoked potentials (AEP's) when compared to males. This potentially suggests steroid hormones produced by the ovaries (estradiol and progesterone) have a positive influence on auditory development, or testosterone produced from the testes to have a hindering effect. Conversely, Rammsayer and Troche (2012) explored potential differences in pitch and loudness discrimination between males and females. The study demonstrated that males had better performance on pitch and loudness discrimination tasks, compared to women. Whilst not the only influencing factor, this may suggest testosterone has a positive influence on auditory development, contrary to findings by McFadden et al. (2002, 2011).

Cassidy and Ditty (2001) examined sex differences in cochlea sensitivity in a sample of 350 neonates (170 males and 180 females) within the first 48 hours of life. Results showed females had more sensitive hearing than males, demonstrating increased responsiveness to higher frequencies than their male counterparts. Research has suggested this may be due to sex-related anatomical differences in cochlear length (e.g., Sato et al. (1991) reported female cochlear length to be 13% shorter than that of males). Whilst previous research has demonstrated sex differences in hearing and auditory development, currently there is not enough research exploring auditory development and ability between the sexes to determine the influence prenatal androgens and estrogens may have on auditory development.

6.1.2 Sex differences in language development

Differences in language development are apparent from as early as 2-3 years of age (Bornstein et al., 2000; Dionne et al., 2003). For example, studies have shown for girls to have a vocabulary almost three times larger than males at around 16 months' of age (Carpenter, Nagell, & Tomasello, 1998; Fenson et al., 1994). Moreover, being male has been implicated in an increased likelihood for communication, language and speech impairment. This is suggested to be due to the observed sex differences in the prevalence of various conditions affecting language (e.g., specific language impairment is 1.3 times more prevalent in males (Tomblin et al., 1997)).

A handful of studies have explored the link between fetal androgen exposure and later language development (Svetlana Lutchmaya et al., 2001; Quast et al., 2016; Schaadt et al., 2015; Whitehouse et al., 2010, 2010). Whitehouse et al. (2012), found cord serum testosterone levels

to be predictive of pragmatic language ability in girls aged 10. Greater difficulties in pragmatic language were positively correlated with higher prenatal testosterone levels. This potential influence of testosterone on language has also been observed in babbling. Researchers found infant babbling at 5 months' of age to be correlated with both estrogens and androgen levels (Quast et al., 2016). 4-week estradiol levels were predictive of articulatory skills and 5-month testosterone levels were negatively correlated with articulatory skills at 5 months' of age. Similar to other studies by Lutchmaya et al. (2001) and Whitehouse et al. (2010) observing the influence of testosterone on language performance in children, a negative correlation between babbling and testosterone level was found. Lutchmaya et al. (2001) found fetal testosterone (fT) levels (extracted from amniocentesis samples) to be negatively correlated with postnatal language development at 18-24 months' of age. These studies have observed the potential influence of testosterone on language development and findings have consistently shown that prenatal testosterone has a hindering effect on later language ability. Furthermore, both Schaadt et al. (2015) and Quast et al. (2016), found postnatal estradiol to positively influence language performance and ability at 4 years and 5 months' of age, respectively. This suggests, similar to auditory development, language may also be influenced by prenatal androgen exposure.

To date, no research has explored the potential relationship between AGD and early language development; Ajay Thankamony in response to comments in Thankamony et al. (2016) implied a relationship to exist between AGD and later speech abnormalities in boys. However, to date, there is no published data exploring this link.

6.1.3 Sex Differences in Autistic Traits

The rationale for exploring the relationship between prenatal androgen exposure and autism was discussed in the introduction of this thesis (**Chapter 1**, *section 1.3.2.2)*. The greater prevalence in males compared to females is suggestive of an underlining biological basis for autism and autistic traits. Two studies by Auyeung et al. (2012, 2010) have explored the relationship between fT levels and infant autistic traits at 18-20 months using the Q-CHAT (further discussed in detail in **Chapter 5**, *section 5.1.3*). Authors concluded increased fT levels have a positive relationship with infant Q-CHAT scores between 18-20 months' of age. Conversely, a more recent study Kung et al. (2016) was not able to replicate this finding. Combining two studies they (1) explored autistic traits (using the Childhood Autism Spectrum

Test (CAST)) in females with a diagnosis of congenital adrenal hyperplasia (CAH)[80] and (2) using a separate sample tested the relationship between fT levels (collected via amniocentesis) and CAST scores[81]. From study 1 Kung et al. (2016) found no significant differences between females with and without CAH or females with CAH and males. Interestingly, they also found no differences in CAST scores between males and females without CAH in their sample. Similarly, within the second study, researchers found no significant correlations between fT levels and CAST scores at 3-5 years. However, in comparison to the first study, Kung et al. (2016) reported a difference between male and female CAST scores in this second study. The authors acknowledge the lack of difference in CAST scores between males and females in the first study may reduce the theoretical basis for observing the relationship between prenatal androgen exposure and autistic traits. Overall, Kung et al. (2016) conclude that there is not sufficient evidence for a relationship between prenatal androgen exposure and autistic traits.

Overall, the literature suggests there are observable sex differences in the development of language and sensory behavioural, as well as early autistic traits. To date, research has explored the relationship between prenatal androgen exposure (e.g., fT) and early autistic traits using maternal serum (Auyeung et al., 2010, 2012; Kung et al., 2016). Amniocentesis is now being phased out of medical care; therefore, reliable non-invasive and indirect measures of fT are being explored. Research has explored the use of 2D:4D digit ratio[82] as a proxy for prenatal androgen exposure (Manning, 2011; Manning et al., 2001; Markoulakis et al., 2012) (see **Chapter 1**, *section 1.4*). However, the longitudinal accuracy of this measure has come into question. 2D:4D digit ratio has been shown to be influenced by postnatal and pubertal steroid levels, changing throughout development (Dean & Sharpe, 2013). Conversely, postnatal hormone levels have not been shown to influence AGD length (Kita et al., 2016; R. T. Mitchell et al., 2015), and therefore this measure is potentially a more robust marker of prenatal androgen exposure. This study will explore the use AGD as proxy for prenatal testosterone and its relationship with later developmental outcomes.

[80] Within study 1: CAH and autistic traits, authors had a total sample of 153 children aged between 4-11 years: females with CAH (n = 43), males with CAH (n = 38), unaffected female relative (n = 41) and unaffected male relative (n = 31). The included unaffected relatives were brothers & sisters and first cousins.

[81] Study 2: Amniotic testosterone and autistic traits consisted of a total sample of 92 children (48 females and 44 males) aged between 3 and 5 years. Amniocentesis was collected was part of routine care between 15- and 25-weeks' gestation.

[82] 2D:4D digit ratio is the ratio of the second to the fourth digit on an individual's hand.

The following questions are addressed: (1) whether there is a relationship between fetal AGD and early autistic traits (using the Q-CHAT); (2) whether there is a relationship between fetal AGD and early language development (using the MB-CDI); (3) whether there is a relationship between fetal AGD and early sensory development (using the ITSP); (4) if there is a difference between the sexes in the above 3 questions.

6.2 Methods

6.2.1 Participants

See **Chapter 2** (*section 2.2.1)* for details of study population (*section 2.2.3, table* 2 for characteristics of population). For the 18 month-follow up, a subsection of the overall sample was used (177 participants; 92 females and 85 males) (See **Chapter 5,** *section 5.2.3* for further details and *table* 16 for mean questionnaire scores).

6.2.2 Ethics

See **Chapter 2** (*section 2.2.1)* for details on ethics obtained for the study.

6.2.3 Procedure

See **Chapter** 2 for description of how fetal AGD measurement was obtained (*see table 9*, *section 2.2.3* for means and SD of raw AGD length) and **Chapter 5** for description of the 18 month-follow up measures (see *section 5.2.3, table 16* for means and SD for the follow-up questionnaires).

6.3 Statistics

6.3.1 Analysis

Data were examined using a Pearson's correlation for all research questions (1-3). Raw scores for the Q-CHAT, ITSP and MB-CDI were used. (See **Chapter 5**, *section 5.3.1*, for rationale and detail behind chosen statistical analysis).

6.3.2 Results

The study included 177 fetuses (92 females and 84 males). 1 fetus was excluded from the analysis as an outlier.

6.3.2.1 Early autistic traits (Q-CHAT)

No relationship was observed between AGD length and Q-CHAT scores, r(170) = .102, p = .181. Similarly no relationship was observed when split by sex; males, r(82) = .024, p = .828 and females, r(86) = .180, p = .094. Standardised AGD measurements by gestational age and raw Q-CHAT scores are plotted by sex in *Figure 19.*

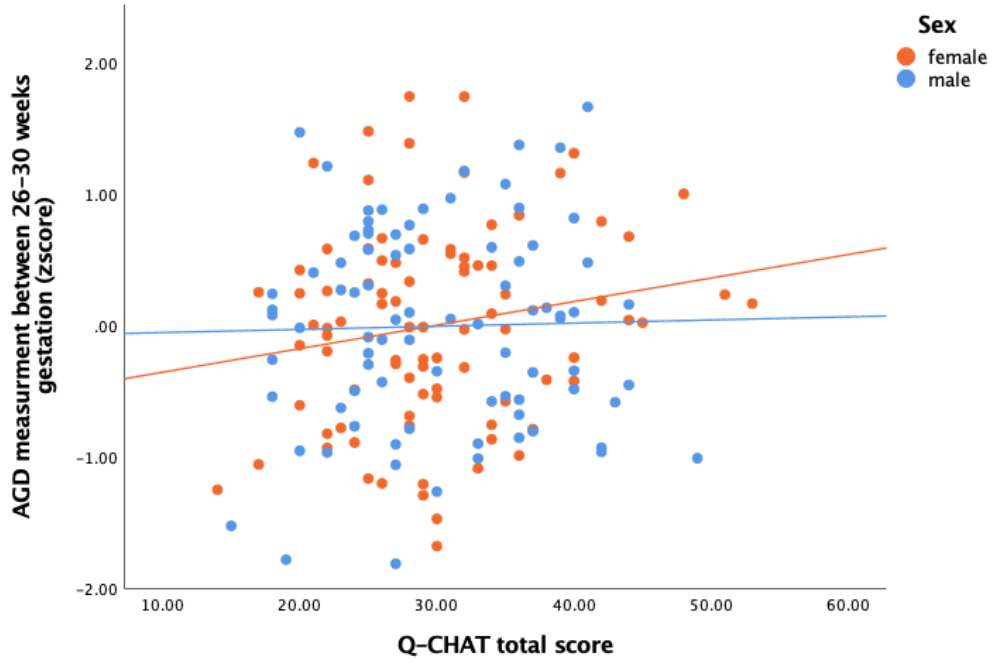

Figure 19: A scatterplot of AGD z-scores and raw Q-CHAT scores split by male (R2 Linear = 5.79, blue) and female (R2 Linear = 0.03, orange) infants between 18-20 months' of age. Mean linear regression lines have been plotted by sex.

6.3.2.2 Early language development (CDI)

No relationship was observed between AGD length and CDI scores, r(169) = .077, p = .316. This was the same when split by sex; males, r(81) = .178, p = .108 and females, r(86) = -.007, p = .945. Standardised AGD measurements by gestational age and raw CDI scores are plotted by sex in *Figure 20.*

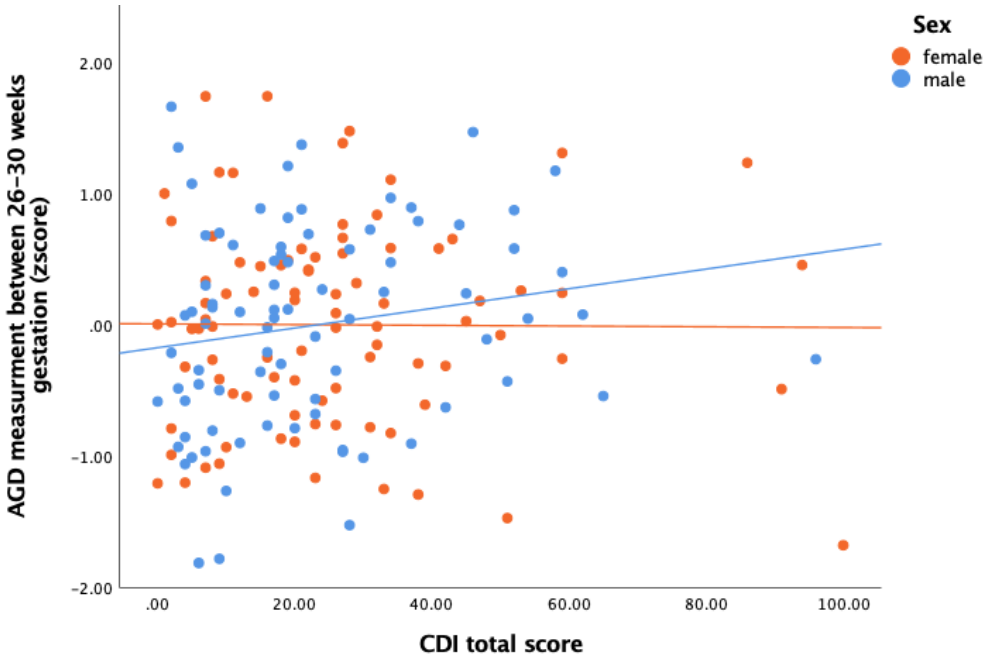

Figure 20: A scatterplot of AGD z-scores and raw CDI scores split by male (R2 Linear = 0.03, blue) and female (R2 Linear = 5.62, orange) infants between 18-20 months' of age. Mean linear regression lines have been plotted by sex.

6.3.2.3 Early Sensory Development (ITSP):

There were no significant differences between AGD length and sensory development in any of the quadrants assessed by the ITSP (auditory, visual, tactile, vestibular and oral processing) (*see table* 21 below for p-values).

	Males	**Females**	**Total**
Auditory Processing	p = .466	p = .393	p = .245
Visual Processing	p = .472	p = .255	p = .197
Tactile Processing	p = .202	p = .707	p = .528
Vestibular Processing	p = .202	p = .906	p = .403
Oral Processing	p = .087	p = .441	p = .863

Table 20: p-values from Pearson's correlation observing the difference between sensory processing and AGD, split by sex.

6.4 Discussion

To date, this is the first study to explore the potential relationship between fetal AGD and later autistic traits, sensory and language outcomes. Results showed no significant relationships between fetal AGD length and autistic traits, language development or any of the sensory processing domains (auditory, visual, tactile, vestibular or oral development).

Differences in sensory development between males and females have been well documented in adults. Research exploring postnatal sensory development and potential sex differences in auditory (Cassidy & Ditty, 2001), visual (Alexander & Wilcox, 2012; Horwood & Riddell, 2008) and motor (Peyre et al., 2019; Piek et al., 2002) development provides a theoretical basis for an underlying biological influence. Research is yet to directly (via maternal serum) explore the influence of prenatal androgens on pre- and postnatal sensory development. In this study there was no relationship between AGD length and sensory processing. One potential explanation for the lack of association could be as a consequence of using the total score from each sensory domain as a continuous variable. Exploring sensory development via analysis of quadrant scores (e.g., a score between 35-43 is categorised as 'typical performance' in auditory processing) was not feasible due to imbalanced sample sizes within each group (see *appendix 7*).

Previous research, however, has implicated that atypical sensory issues occur postnatally (Eeles et al., 2013; Lickliter, 2011), which would also account for these findings. Infants born preterm or with low birth weight (LBW) display stronger sensation seeking, sensory sensitivity and sensation avoiding behaviours as a potential result of their environment (i.e., time spent in the neonatal intensive care unit (Lickliter, 2011)) having an over-stimulating influence on sensory development. This suggests, to an extent, that fetuses whilst *in utero* are protected from outside influences (e.g., via the surrounding of amniotic fluid) which may affect their sensory development. Therefore, further longitudinal research is required with larger and more balanced sample sizes, inclusive of preterm birth, before conclusions can be extrapolated from the observed relationship between AGD length and visual processing.

Previously, research has consistently shown a negative relationship between prenatal androgen (testosterone) levels and language development (Lutchmaya et al., 2001; Quast et al., 2016;

Whitehouse et al., 2010). In addition, Schaadt et al. (2015) and Quast et al. (2016) have demonstrated a positive influence of postnatal estradiol levels on language ability. Using AGD as an indirect measure of prenatal androgen exposure, results were unable to show a relationship between language ability (number of words in vocabulary) and AGD length. However, similarly **Chapter 5** previously the use of the MB-CDI should also be considered a limitation within this study (discussed in *section 5.4*). The lack of relationship may be as a result of the language development measure used.

Lastly, this study was unable to find a relationship between autistic traits and prenatal androgen exposure using AGD as a proxy for fT. Previous research has explored the use of 2D:4D digit ratio as a proxy measurement to observe prenatal androgen exposure and autism. Low 2D:4D ratios have been correlated with autism diagnosis and autistic traits (Al-Zaid et al., 2015; De Bruin et al., 2009; Hönekopp, 2012; Manning et al., 2001). However, more recently Mackus et al. (2017), using a sample of 401 individuals between 18-30 years' of age (mean age: 20.5 years'), was unable to find a significant association between 2D:4D ratios and autism spectrum quotient (AQ) scores. Similarly, Guyatt et al. (2015) in a sample of 6015 of children (mean age: 11.75 years') was also unable to find evidence of a relationship between 2D:4D ratios and autism diagnosis or autistic traits. Both studies (Guyatt et al., 2015; Mackus et al., 2017) suggest this may be due to the samples observed: (1) in an opportunistic university based sample (Mackus et al., 2017) and (2) ALSPAC[83], a prospective population-based cohort (Guyatt et al., 2015). Authors suggested the reduced diversity in autistic traits and autism diagnoses (e.g., Asperger syndrome and high-functioning autism diagnoses) within the sample to bias results. Comparatively this study could also have been influenced by its sample. This study included a prospective population-based cohort, comprising of healthy pregnancies[84]. Whilst the study sample was enriched with mothers with a diagnosis or an existing sibling with a diagnosis of autism. This sub-sample only accounted for 7.8% of the total sample. This

[83] The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective population-based study with an initial recruitment sample of approx. 14,000 pregnancies between April 1991 and December 1992 (*About | Avon Longitudinal Study of Parents and Children | University of Bristol*, n.d.).

[84] As defined in **Chapter 2**, *section 2.2.1,* 'Healthy pregnancies' were pregnant women who had (1) little/no consumption of alcohol during pregnancy, (2) no smoking or recreational drug use during pregnancy, (3) a singleton fetus whose measurements indicated their size to be appropriate for gestational age, (4) the absence of any major fetal anomalies, (5) fetus is not considered to have an intrauterine growth restriction (IUGR) or be large-for-gestational age (LGA) and (6) birth of a clinically healthy baby.

limitation will be further addressed in the overall discussion of the thesis (see **Chapter 7**, *section 7.2*)

This sample was enriched with mothers with conditions associated with increased testosterone levels. Previous studies have shown the maternal conditions influence the developing fetus (Leddy et al., 2008; V. Patel et al., 2004; Rondó et al., 2003) . As discussed in **Chapter 3**, there was no relationship between maternal conditions and fetal AGD, potentially due to limited group sizes of these conditions and inaccuracies in EFW. Similarly, within this chapter no significant correlations were observed between fetal AGD and later behavioural development. The maternal conditions included within this sample were mothers with PCOS, autism and suspected hirsutism. PCOS and hirsutism are categorised as conditions resulting from increased levels of hormones; such as testosterone, luteinising hormone and prolactin (*Polycystic Ovary Syndrome - Diagnosis*, 2017) within the body. However, the severity of the condition may vary dependent upon the level of imbalance within the body, placing this condition on a spectrum. The negative findings from both this chapter and **Chapter 3** maybe a result of mothers within these groups having higher than average (enough to suspect or diagnose PCOS) whilst not having a high enough imbalance to influence the fetuses development. Further research with maternal serum or a method of quantifying severity (see **Chapter 3**, *section 3.4*) is required to assess the influence of maternal endocrine conditions (such as PCOS) on fetal and later infant development.

In summary, within this chapter, AGD as a proxy for testosterone and its relationship with later developmental outcomes was explored. Results suggest the absence of a relationship between AGD length and early autistic traits, language or sensory development. Further longitudinal research (inclusive of low birth weight and preterm birth) is necessary before conclusions can be drawn.

7 Discussion

7.1 Overview

Over the last decade, the importance of the prenatal period on later development (from infant to adult) and outcomes has become apparent. There has been a shift from exploring *whether* the fetal environment contributes to later perceptual, behavioural and cognitive development to *how* this occurs, which has been key in advancing this field of research. However, as stated by Mayer and Joseph (2013) '…fetal growth literature includes some potentially confusing terms and concepts and an enormous body of sometimes conflicting evidence' (p. 145). For example, authors argue there is no universal consensus on how abnormal growth is diagnosed. Currently abnormal growth can be diagnosed using estimated fetal weight, estimated abdominal circumference or both (Mayer & Joseph, 2013). This conclusion may stem from the 'relatively inaccessible nature of the fetus' (Mayer & Joseph, 2013, p. 145), as it presents a challenge when attempting to accurately measure fetal size and development. As discussed throughout this thesis, there are many barriers to determining how fetal development affects later outcomes, such as ethical constraints, population differences, and methodological issues such as small sample sizes. However, advances in imaging techniques, the acknowledgement that there are differences across populations in fetal growth trends, and the increasing understanding about maternal factors that affect fetal development (e.g., maternal smoking (Iñiguez et al., 2013; Jaddoe et al., 2007) and famine (Brown, 2000; Painter et al., 2005)) have been vital in furthering our understanding of what influences fetal development and the potential effects these may have on the unborn child.

The aim of this thesis was to explore whether there is any association between standardised fetal biometric measurements (head circumference (HC), transcerebellar diameter (TCD), ventricular atrium (VA) and anogenital distance (AGD)) and later infant behavioural outcomes.

A further aim was to investigate whether fetal sex, maternal endocrine conditions such as polycystic ovary syndrome (PCOS) and hirsutism, as well as neurodevelopmental conditions such as autism, may influence fetal growth. Neurodevelopmental research has highlighted the importance and potential benefits of early detection for conditions such as autism, and targeted interventions (e.g., towards the specific aetiology sub-group of autism) through improving our understanding of the underlying biological effects, rather than behavioural effects, on development (Zwaigenbaum & Penner, 2018).

Throughout this thesis the limited research into the relationship between later developmental outcomes and fetal growth, both retro- and prospectively using standardised fetal growth measurements (HC, FL and AC) has been made apparent. The research conducted within this thesis provides an insight into the use of both standard (in the UK) and novel fetal measurements and how they can be used to predict later behavioural outcomes. Whilst significant differences may not have been observed within each chapter, this work provides proof of feasibility and reliability of these measurements, in particular AGD. As well as shedding light on future directions research into fetal biometry and behavioural outcomes. As mentioned above, there are many barriers to fetal research, however this thesis has demonstrated the ability for researchers and clinicians alike to be able to reliability explore fetal growth and later outcomes using ultrasound methodology. This is as a result of the advances this in this technology as well as the introduction of newer (i.e. AGD) measurements that can be measured from fetal to adult life. Showing potential in bridging the gap between physical adult and fetal measurements. Lastly this thesis provides additional precursory reasoning and support (from a behavioural perspective) for the introduction of fetal growth measurements, such as TCD into routine clinical care. Fetal TCD measurement is not currently routine across the world[85]. However, research has highlighted its usefulness as a precursor for physical deformities, irregular fetal development and genetic disorders (Atallah et al., 2019) and within this thesis I presented an association between the size of the TCD and infant autistic traits. Suggesting a potential relationship between more novel fetal biometric measures and later behavioural outcomes. Further opportunities are needed to validate these preliminary findings, potential future directions and implications.

[85] As discussed in **Chapter 2** (*section 2.1.5.1*) Atallah et al. (2019), reported TCD in fetuses is a relevant marker to detect associated anomalies such as a high rate of fetal malformations, chromosomal anomalies, severe *intrauterine* growth restriction (IUGR) and genetic disorders. Suggesting the inclusion in routine ultrasound measures.

The first section of this thesis (**Chapters 2-3**) focused on prenatal development, exploring maternal influences (such as PCOS, hirsutism, maternal autism diagnosis) and fetal sex differences using HC, TCD, VA and AGD as the fetal biometric measures. **Chapter 4** explored population-based differences in standardised fetal biometric measurements and discussed whether population specific fetal biometric charts have any clinical relevance and whether this warrants future research. **Chapters 5-6** focused on whether the fetal biometric measurements can predict infant development, specifically language development and atypical sensory experiences as well as autistic traits. Sex differences in these outcome measures were also explored (*see table* 22 for an overview of the findings presented within this thesis).

This chapter will review the main findings of each chapter, followed by a discussion into the implications and potential limitations of the reported studies. Finally, this chapter will conclude by discussing potential directions for future research.

Chapter	Sample	Aim(s)	Key Findings
Two	219 healthy fetuses (104 males and 115 females) Subsamples:	To observe whether there are sex differences in the developing fetal brain structures (HC, TCD and VA).	There were no observable sex differences between these brain structures.
	17 maternal autism or sibling diagnosis 26 maternal PCOS diagnosis 65 Hirsutism but no PCOS diagnosis 108 No maternal condition	To observe whether maternal endocrine (PCOS, hirsutism) or neurodevelopmental (autism) conditions have an influence on the developing brain.	Additionally, there was no effect of maternal conditions on the developing fetal brain (HC, TCD or VA).
Three	219 healthy fetuses (104 males and 115 females) Subsamples: 17 maternal autism or sibling diagnosis	To assess whether previously observed sex differences in AGD can be replicated.	AGD is a feasible and reliable measurement in 2D ultrasound. Results demonstrated strong sex differences, suggesting AGD is influences by prenatal androgen exposure.
	26 maternal PCOS diagnosis 65 Hirsutism but no PCOS diagnosis 108 No maternal condition	To observe if there is an influence of maternal endocrine (PCOS, hirsutism) or neurodevelopmental (autism) conditions on prenatal AGD.	There was no effect of maternal endocrine (PCOS, hirsutism) or neurodevelopmental (autism) conditions on AGD.
Four	UK sample 208 healthy fetuses (101 males and 107 females) Israel sample	To assess whether AGD requires population-based charts if it is to be used to predict fetal and potentially postnatal developmental outcomes (similar to other fetal biometric measures).	There were significant differences in AGD between UK and Israel samples. This significant difference was maintained regardless of sex.

	118 fetuses (59 males and 59 females)		
Five	177 fetuses (92 females and 85 males)	To observe whether a relationship exists between fetal structural brain measurements (HC, TCD and VA) and autistic traits (Q-CHAT).	There was a positive relationship between brain measurements at 20 weeks (TCD and VA) and autistics traits (Q-CHAT) at 18-20 months' of age. This significant correlation was only seen in females. There was no relationship between HC and Q-CHAT score at any gestational age.
		To observe whether there is a relationship between structural brain measurements and language (MB-CDI) development.	There was no relationship between fetal brain measures and language development measures.
		To observe whether there is a relationship between structural brain measurements and early sensory (ITSP) development.	There was no relationship between fetal brain measures and sensory development.
Six	177 fetuses (92 females and 85 males)	To observe whether there is a relationship between fetal AGD and autistic traits.	No correlations were observed between fetal AGD and autistic traits.
		To observe whether there is a relationship between fetal AGD and early language development.	No correlations were observed between fetal AGD and language development.
		Whether there is a relationship between fetal AGD and early sensory development.	No correlations were observed between fetal AGD and sensory development.

Table 21: Summary of key findings from each chapter.

7.2 General Discussion

In **Chapter 2**, fetal sex in relation to fetal brain structure was explored, taking into account maternal conditions such as PCOS, hirsutism and autism. Results indicated that there were no significant sex differences in fetal brain measurements (HC, TCD or VA) or growth velocity, and no difference in these fetal biometric measurements across maternal condition groups.

Researchers and clinicians have suggested that although statistically significant sex differences have been observed in fetal growth measurements, such as VA, these differences are minimal. For example, Kramer et al. (1997) found a mean difference of 0.2mm between males and females in VA width (Kramer et al., 1997) but deemed this to be clinically insignificant (Salomon et al., 2007). Consequently, there has been a focus on identifying specific structural differences in development which may influence later outcomes (e.g., prenatal ventricular atrium size (Mercier et al., 2001; Sadan et al., 2007)). **Chapter 2** found no significant differences between male and female fetuses on any of the brain measures (HC, VA or TCD). This supports previous research which suggests that that there is no clinical reason to adjust for sex differences when examining fetal growth.

To date, no research has explored the influence of endocrine conditions in the mother (e.g., PCOS and hirsutism) on fetal development. Maternal endocrine conditions have been shown to influence fetal development. For example, women with PCOS have been shown to experience a higher rate of *intrauterine* growth restriction (IUGR) (Stirnemann et al., 2017) during pregnancy, compared to women without PCOS. This implies that PCOS in the mother may influence fetal growth. This study found that maternal PCOS and/or hirsutism had no significant impact on any of the prenatal brain measurements (HC, TCD or VA). Based on previous research, the lack of significant findings could suggest these endocrine conditions have an influence on overall growth (i.e., observed through offspring birth weight (Sir-Petermann et al., 2005)) as opposed to influencing the growth patterns of individual measurements or on the developing brain. However, further longitudinal research observing fetal biometry and birth weight between maternal endocrine conditions is needed.

In contrast, previous research has explored the relationship between autism (i.e., infant with a diagnosis of autism) and fetal growth, though results have been contradictory (Abel et al., 2013; Unwin et al., 2016; Whitehouse et al., 2011). Two studies exploring potential prenatal growth

(e.g., HC) in relation to later autism diagnosis have demonstrated atypical prenatal growth trajectories (e.g., in HC and estimated fetal weight (EFW)) in children later diagnosed with autism (Abel et al., 2013; Bonnet-Brilhault et al., 2018). Results from this thesis did not demonstrate that a maternal diagnosis of autism had any influence on gross measurements or growth velocity of brain structures (HC, TCD and VA). These findings add to previous research by Hobbs et al. (2007) and Unwin et al. (2016), who found no significant differences in fetal HC between infants with and without an autism diagnosis.

Currently no longitudinal research exists that examine structural brain development from the pre- to postnatal stages of development in infants with a high likelihood of a later autism diagnosis[86]. This is mainly due to the lack of corresponding methodology that can be safely used throughout pregnancy. As discussed in **Chapter 1** (*section 1.2.2*), whilst magnetic resonance imaging (MRI) is the gold standard for examining brain development, it is not clinically advised to have an MRI during pregnancy before 24-26 weeks' gestation. Additionally, ultrasound is not feasible for longitudinal observations due to the lack of corresponding ultrasound planes for neonates.

Previous research by Hazlett et al. (2017) observing early brain growth in infants later diagnosed with autism, showed hyper-expansion of the cortical surface area between 6-12 months' of age followed by brain overgrowth at 12-24 months. Combined with previous prenatal research observing fetal growth (e.g., HC) (Hobbs et al., 2007; Unwin et al., 2016) and the findings from **Chapter 2**, it could be suggested gross anatomical volumes and growth differences in the brain may only be observed postnatally. Further research is needed however, to better understand the developing brain and the timing of potential pre- and postnatal influences and differentiations (Piven et al., 2017).

Chapters 2 and 3 observed sex-difference in prenatal development in relation to fetal brain growth. No sex differences were found in brain development (HC, TCD or VA), significant sex differences were observed in AGD. Unlike other fetal biometric measures (HC, TCD and

[86] It is important to note, to date, 'The Developing Human Connectome' project has collected and made available over 500 MRI scans of new-borns (MRI's are taken at two time points, once between 20-44 weeks' gestation and another after birth) (*Developing Human Connectome Project (DHCP) | The Developing Human Connectome Project*, n.d.). However, currently no results have been published observing the prenatal to postnatal development of the brain.

VA), AGD has been implicated as a potential predictor of androgen-related outcomes in later life (e.g., male reproductive ability (Eisenberg & Lipshultz, 2015)). **Chapter 3** replicated previous results in Israel of the sexually dimorphic nature of AGD within a UK sample. Similarly, to Gilboa et al (2004), a significant difference between males and females was observed. This adds to previous research implicating AGD as a potential measure for fetal sex identification (Arfi et al., 2016) and predictor of fetal androgen exposure (Barrett et al., 2018; Barrett et al., 2013; Swan et al., 2015; Swan et al., 2005).

Chapter 3 explored the potential influence of maternal conditions (PCOS, hirsutism and autism) on fetal AGD. Existing studies have shown differences in AGD measurement in relation to testosterone levels (Barrett et al., 2018; Hernández-Peñalver et al., 2018; Wu et al., 2017). For example, women with a PCOS diagnosis have a greater AGD length then women without (Wu et al., 2017). In infants, females have a greater AGD if their mother has a diagnosis of PCOS (Barrett et al., 2018). Previous research shows that maternal testosterone levels have an effect on prenatal AGD length. However, results from this thesis showed no significant differences between maternal conditions including PCOS (and also hirsutism and autism) on prenatal AGD. This could be due to small sample sizes in the maternal condition groups (further discussed below in limitations) or because prenatal AGD was measured during a specific 4-week window of development (between 26-30 weeks' gestation). Fetal vulnerability to alternations *in utero* have been shown to influence development. However, as discussed in **Chapter 1** (*section 1.1),* this period of vulnerability has been shown to be present from the first trimester of pregnancy til birth (Reissland & Kisilevsky, 2016). Currently, not enough is known to identify 'critical' timepoints for specific adversities (e.g., increased exposure to prenatal testosterone). Therefore, the chosen 4-week window of development (between 26-30 weeks' gestation) might not be reflective of the potential influence on fetal growth. Future research should explore prenatal AGD measurement longitudinally from the first trimester (based on previous demonstration of its feasibility from 11 weeks' gestation (Arfi et al., 2016)) of development to observe potential influence of maternal endocrine conditions on the developing fetus across gestation.

Chapter 4 highlighted the importance of population-based growth charts. As discussed within *section 4.1*, fetuses are being misidentified (e.g., large-for-gestational age (LGA)) and unnecessary interventions (e.g., caesarean delivery) are being taken due to outdated growth

charts (Ben-Haroush et al., 2004; Buck Louis et al., 2015; M. S. Kramer et al., 2002) [87]. Current research by the World Health Organization (WHO) (Kiserud et al., 2017), INTERGROWTH 21st project (Buck Louis et al., 2015) and the National Institute of Child Health and Human Development (NICHD) (Hirst et al., 2016) have demonstrated a need for updated growth charts that are in line with today's population. This chapter explored whether there were differences between Israeli and UK AGD measurements, and if so, whether countries would benefit from population-based charts. Results indicated that there were significant differences between UK and Israeli populations in AGD length using an existing AGD growth chart by Gilboa et al. (2004). As discussed in **Chapter 4,** (*section 4.4)* these differences could be attributed to varied biological and environmental factors between populations (e.g., genetic predispositions in Israel to congenital adrenal hyperplasia (Paperna et al., 2005)(CAH)[88]). Overall, results demonstrated that there is a need for population-based growth charts to be developed, if AGD is to be used to inform research or used clinically as an early predictor for later outcomes (e.g., precursor for diagnosis of PCOS (Hernández-Peñalver et al., 2018)). Similarly, to those created by Gilboa et al. (2014), future research should consider measuring AGD at different gestational ages with larger sample sizes to create more detailed growth charts of AGD development *in utero*. In addition, other populations should be explored using this measure to see whether there is a significant difference in mean AGD across gestation in other populations.

The last two chapters (**5 and 6**) examined whether the fetal biometrics described within prenatal chapters (**2 and 3**) of this thesis, are related to later infant behavioural outcomes. **Chapter 5** explored the relationship between prenatal brain measurements (HC, TCD and VA) and later sensory (Infant-Toddler Sensory Profile (ITSP)) and language (MacArthur Bates Communication Development Inventory (CDI) short form) development. In addition, the relationship between HC, TCD and VA measured prenatally and infant autistics traits (Quantitative Checklist for Autism in Toddlers (Q-CHAT)) was examined. Results demonstrated a positive correlation between autistics traits and TCD and VA size at 20 weeks'

[87] In the UK we use fetal reference charts created by Chitty et al. (1985) As discussed in the introduction these charts were created using relatively small, white, middle class sample. Since the creation of these charts the UK has become more ethnically diverse (*Population of England and Wales - GOV.UK Ethnicity Facts and Figures*, n.d.) and worldwide changes in maternal anthropometry and sociodemographic factors (e.g., diet and education) has resulted in mothers, on average, giving birth to larger babies (Kramer et al., 2002).

[88] CAH is defined as having elevated levels of prenatal androgens. This would potentially explain why mean AGD distances observed in the Israeli population was significantly larger than UK mean AGD across gestations in both sexes.

gestation. To our knowledge, this is the first time this relationship has been observed. The data presented within this chapter supports previous research associating later autism diagnosis with brain overgrowth (Abel et al., 2013; Bonnet-Brilhault et al., 2018). Whilst both studies found a difference using HC as a proxy for brain development, this finding was not replicated within this study. Instead, an association between early autistic traits and overgrowth in subregions (TCD and VA) of the brain was observed.

Interestingly this association between TCD and VA width and autistic traits was only observed at 20 weeks' gestation. Furthermore, this association was only observed in females. The reason for this remains unclear, however it could potentially be explained by fetal sex and timing of exposures (to potential adversities) during pregnancy. Recently research has demonstrated the moderating effects of both fetal sex and potential timing of exposure on the neurological development of an individual (Glynn & Sandman, 2011). Throughout this thesis (**Chapter 1**, *section 1.1.1* and **Chapter 5**, *section 5.4*), sex differences in response to adversity have been discussed. Females demonstrate the ability to make multiple adaptations whilst male fetuses 'take a minimalist approach' (Glynn & Sandman, 2011) when faced with the same *intrauterine* adversity (Clifton, 2010). This places the male fetus at risk if further adversity is encountered. For example, during periods of famine there is a marked increase in the number of females born, compared males (Song, 2012). Based on this theory, it is suggested that females 'conserve resources' and adjust to adverse maternal conditions (e.g., famine) in different ways, such as through gene and protein changes (Rosenfeld, 2015; Sandman et al., 2013). The observed increase in TCD size and VA width could be an adaptation in response to experiencing *intrauterine* adversity.

Moreover, Glynn and Sandman (2010) (Glynn & Sandman, 2011) consider the potential influence of 'timing' on fetal neurological development. Previous research has established that the timing of certain exposures (e.g., maternal cortisol at 15 weeks' gestation has been associated with poorer cognitive functioning at 1 year of age (Davis & Sandman, 2010)) have different effects on the developing fetus, depending on the stage of organ development. The same signal (e.g., endocrine changes) may produce different outcomes at different gestational ages. The ability of the female fetus to adapt to adversity throughout pregnancy suggests they may become less sensitive to small alternations in their environment across gestation. The adaptability of females could explain why this increase in TCD size and VA width is not maintained as gestation advances, and it is only apparent at 20 weeks' gestation. Additionally,

animal models have suggested that the placental barrier (which modulates fetal exposure to circulating maternal hormones) becomes less active between approximately 19-26 weeks' gestation, coinciding with the second trimester scan at 18-22 weeks' gestation. It is during this time point the fetus may become more vulnerable (and respond to) fluctuations in hormone levels (Seckl, 2006). However further research observing brain development as well as maternal and fetal hormones levels is warranted to assess the above reasoning and speculations.

Chapter 6 considers the relationship between another fetal biometric (AGD) and later sensory and language development, as well as autistic traits. The aim of this chapter was to explore whether AGD could be a proxy for fetal androgen exposure whether it affects later infant behavioural development. To date, there is no research exploring the relationship between AGD and later behavioural development. Results showed no relationship between fetal AGD and autistic traits, language or sensory development. However, as discussed in **Chapter 6**, (*section 6.4)*, the lack of findings within this chapter could be attributed to; the use of MB-CDI to assess language development, the lack of diversity within the sample (only 7.8% were grouped as 'high risk' when observing autistic traits and method of analysis when examining sensory development. Therefore, further research is required, taking into account these limitations, before implications and conclusions can be drawn about whether there is any significant association between AGD length later behavioural development.

The relationship between general fetal biometric measures (i.e. AC, HC and FL) has been well documented. These standardised measurements are used to help assess and monitor fetal health, for example, intrauterine growth restrictions and EFW (Froehlich et al., 2016; F. Hadlock et al., 1983). Within this thesis the potential correlation between early brain development (HC, VA and TCD) and AGD was not explored. Whilst it is acknowledged there may be an association between these measurements (as all are measurements thought to grow proportionally throughout gestation) this was chosen not to be explored for the following reasons. Firstly, the above mentioned fetal biometric measures (e.g. HC) were initially observed separately, creating standardised growth charts for each measurement without assessing correlations (Chitty et al., 1994a, 1994b; Chitty & Altman, 2002). As discussed in **Chapter 4**, when creating standardised growth charts there are a number of variables that need to be taken into consideration such as ethnicity, sex and SES. Additionally these charts need to be generated using a sample with no maternal conditions, pregnancy complications or substance use (for example maternal smoking has been found to reduce HC size and decrease

EFW (Jaddoe et al., 2007; Pereira et al., 2017)) to name a few. Secondly, the creation of growth charts, then enabled researchers to identify associations between measurements that could be clinically useful in early prediction. For example, the relationship between fetal HC and FL has been shown to be useful in detecting dwarfism (Hadlock et al., 1984) and AC and HC in predicting risk of shoulder dystocia during birth (Endres et al., 2015). this in turn helps potential early intervention and monitoring of the pregnancy and fetus.

To date the research on fetal AGD is limited. Whilst a reference chart has been created (Gilboa et al., 2014) this thesis has shown it is not applicable for the UK population, as well as being limited in its exploration in the influence of maternal health and environmental effects (e.g. SES and substance use). Within the scope of this thesis it would not have been possible to take into account all the potential variables and additional information needed to accurately assess whether there was an observable correlation between the fetal biometric measurements used. Further investigation is required taking into account potential influences before correlations between these growth measures can be assessed and in turn to potentially help inform early intervention and monitoring.

Within each chapter, specific limitations have been discussed (e.g., the use of MB-CDI in a UK population in **Chapters 5** & **6**). However, there are some limitations to this study that are consistent throughout the thesis, including the fetal sample size, the size of the maternal conditions' groups and the narrow gestational age range of 26-30 weeks. All of these were constrained by time limits in data collection, recruitment and financial restrictions. Furthermore, research has suggested that maternal anxiety has an influence on fetal and infant maturation (Ellman et al., 2008). At the time of scanning, no fetus showed IUGR, was LGA or had a lower than expected EFW, therefore maternal anxiety was not controlled for in this study. Additionally, socio-economic status and level of education were not collected. As the first and second trimester scans were obtained retrospectively, it was not possible to revisit the fetal biometric measurements (e.g., HC) where the sonographer had been unable to obtain a measurement at the time of the scan. However, this resulted in a maximum loss of 13% data at each time point.

It is also important to acknowledge that this cohort presented with no physical anomalies (i.e. ventriculomegaly or cerebellar overgrowth) during the fetal stages of development. As discussed in **Chapter 6** (*section 6.4*), this may influence the findings of this study as only

typically developing fetuses were selected. Inclusion of higher risk (e.g., fetuses with ventriculomegaly or macrocephaly[89]) fetuses might allow for more diversity within the data. Research observing prenatal growth has suggested atypical growth to be a potential marker to an altered *intrauterine* environment. Therefore, inclusion of high-risk pregnancies, such as those with ventriculomegaly is likely to create more diversity within the sample and in turn the developmental outcomes observed in the unborn child.

The heterogeneity of autism (Betancur, 2011) and PCOS (Hernández-Peñalver et al., 2018) may also have impacted the findings of this thesis. Both conditions are associated with a number of different manifestations (e.g., for a PCOS diagnosis only 2 of 3 criteria need to be fulfilled (*Polycystic Ovary Syndrome - Diagnosis*, 2017)) this potentially influences the *intrauterine* environments differently (e.g., differences in circulating levels of androgen and estrogen within the mother and maternal-fetal unit). However, not enough is known about the potential biological differences (e.g., circulating sex steroid levels) between the different phenotypes of these conditions (e.g., if one women has irregular periods and polycystic ovaries, whilst the other has irregular periods and high levels of testosterone in serum samples) to assess the possible influence on the *intrauterine* environment and as a result the developing fetus.

Lastly, hirsutism is defined by excess hair growth. The definition used within the study was 'during your adult years', have you found coarse, dark, hair growing in any of the following areas: upper lip, chin, breasts, back, belly, chest between breasts, upper thighs and/or upper arms?'. Mothers were included in this group if they answer yes to any of these. However, the wording of this question within the pregnancy history questionnaire (PHQ) (see *appendix 2*) should be acknowledged as a limitation. The use of the phrase 'during your adult years'' could be inclusive of the time during pregnancy. The hormonal changes experienced during pregnancy can cause additional hair growth in the areas implicated above. Therefore, the women within the hirsutism group may be reporting on the hair growth experienced as a result of pregnancy which is not reflective of true hirsutism.

[89] Macrocephaly is defined as a head circumference above the 95th centile.

7.2.1 Future Directions

Overall, this thesis has demonstrated that it is feasible to use fetal biometry to observe later behavioural outcomes. Future studies should address the limitations of this preliminary study by observing brain and AGD development in a larger cross section (inclusive of fetal complications, such as ventriculomegaly and SGA), with a larger number of pregnancies within each maternal condition. Inclusion of fetal complications is suggested because all fetuses included within this study were deemed by existing growth charts to be typically developing 'healthy fetuses'. This may explain the lack of significant findings in relation to maternal conditions and later infant behavioural outcomes, therefore requiring further investigation.

The current study was designed taking into account potential burden on these families (i.e., length of visit and time taken to complete questionnaires). Future studies may wish to include a greater number of developmental measures, such as the ages and stages questionnaire (to assess general development), and a neonatal assessment (e.g., Neonatal Behavioural Assessment Scale (NBAS) to examine neurobehavioral functioning postnatally). Additionally, studies may wish to extend the longitudinal aspect, following up not only infant behaviour but physical and new-born development of the measures explored in this thesis (e.g., HC and AGD).

The reported studies within this thesis also highlighted a number of additional measures that should be observed if this research is to be replicated on a larger scale. Questions into maternal influences, such as mental health, socio-economic status and education level should be included in any future research. As discussed in **Chapter 1** (*section 1.6.1*), maternal influences during pregnancy are thought to influence prenatal programming. These influences having been shown to result in long term effects of cognitive, social and physical development of the offspring (Doherty et al., 2015; Knopik et al., 2016; Ronald et al., 2011; Weinstock, 2005). Therefore, future research may wish to include basic questions into maternal demographics (e.g. household income and highest educational qualification) and maternal mental health questionnaires (i.e. the inclusion of a measure of stress, anxiety or depression, such as the Hospital Anxiety and Depression Scale (HADS) of the Perceived Stress Scale (PSS) to name a few commonly used). The inclusion of such variables would allow the exploration or control of potential influences of maternal social determinants on fetal growth and later behavioural outcomes.

The studies within this thesis were enriched with a sample of maternal endocrine conditionals, namely PCOS and suspected hirsutism. Future research should develop a targeted recruitment strategy for these groups (e.g. recruiting from fertility clinics) as opposed to the opportunistic/convenience sampling used within this thesis. Moreover, this type of research would benefit from the addition of bio-sampling (i.e. maternal serum and amniocentesis) (further discussed in *sections 3.4 and 6.4*). This will help future research quantify levels of hormones, such as testosterone in relation to observable fetal growth. The current project (CUSP) has accessed maternal serum samples at 12- and 20-weeks' gestation and these are in the process of being re-analysed to look at maternal hormone levels during pregnancy. However, this data has not been included within this thesis, as analysis is still ongoing. Within **Chapter 1**, there is discussion into the use of amniocentesis by previous research to observe hormone levels in the developing fetus. Whilst I acknowledge the usefulness of observing fetal hormone levels in relation to growth and later outcomes, the process comes with risks to mother and baby (*Amniocentesis - Risks*, 2018) and is being phased out of medical care. Therefore, in its current form the addition of fetal bio-sampling is risky and not a recommended for research purposes. However, advances in medical techniques may allow for future studies to observe fetal hormone levels without the use of such invasive procedures.

To address physical and further behavioural development, the current project (CUSP) will further observe physical and behavioural development, following infants in this cohort until 36 months' of age. These data have not been included within this thesis due to time constraints. Infants will be invited in for an in person follow up between 18-20 months' of age where infant HC and AGD is measured postnatally to observe the growth trajectories from pre- to postnatal life. Currently there is no research examining the longitudinal development of AGD between these ages and research observing pre- to postnatal HC growth has been inconsistent (Blanken et al., 2018; Bonnet-Brilhault et al., 2018).The study will also observe the potential influence of increased *in utero* maternal androgen exposure (maternal diagnosis of PCOS or autism and potential hirsutism) may have on these measurements during infancy. In particular we will aim to replicate the study by Barrett et al. (2018) observing the relationship between offspring AGD and maternal diagnosis of PCOS or potential hirsutism. This study observed females born to mothers with a PCOS diagnosis had a longer AGD than females born to mothers with potential hirsutism or no endocrine condition.

Those who scored high (+39) on the Q-CHAT during the initial follow up phase (as well as those in the high likelihood of autism group) will be followed up again at 30 months' with another Q-CHAT and a developmental, dimensional and diagnostic interview (3Di). This aims to assess whether the autistic traits observed at 18-20 months' of age remain consistent, are related to their score on the 3Di (which assesses the severity of features associated with an autism diagnosis) between 30-36 months', and if there is a relationship between prenatal measures and development when the infant is older.

7.2.2 Clinical Implications

Within **Chapters 3, 4** and **5**, the clinical implications of the significant findings have been discussed. In **Chapter 3**, the sexually dimorphic nature of AGD demonstrated the potential to assist in early diagnosis for a number of outcomes (e.g., male genital malformation, PCOS and other sexually dimorphic developmental conditions such as autism). The feasibility and reliability of the measure suggests an ease at which it can be applied to routine ultrasound and fetal care. However, the potential usefulness of AGD in early diagnosis and identification in testosterone related disorders remains to be thoroughly explored. **Chapter 4** demonstrated not only the need for growth charts (as standard for other fetal biometric measures) but a need for population-based growth charts. The significant differences observed between populations is an important consideration, with future research into means and ranges between populations and the sex differences within these populations required, in order to accurately assess clinical usefulness in early identification of testosterone related disorder (e.g. PCOS).

Chapter 5 demonstrated that prenatal brain development is associated with early neurodevelopmental outcomes in infants. Specifically, that an increase in TCD, as early as week 20, is associated with autistic traits in both males and females at 18-20 months'. This finding further supports the potential inclusion of prenatal TCD as an early marker of later neurodevelopmental outcomes. Clinically, the observed positive relationship between prenatal VA and TCD size and Q-CHAT score could be introduced into models of early postnatal screening for autism, with the aim of improving access to early interventions to those that would benefit the most. In addition, this finding better informs our understanding of how autism and related traits are associated with prenatal brain development. Further investigation is required to support this finding, using different populations, larger samples and observing

development (both physical and behavioural) longitudinally to observe brain growth in these regions and the later diagnosis of autism.

7.2.3 Final Conclusions

In conclusion, this thesis discussed the possible use of novel biometrics (AGD) for research and clinical application, in addition to standardised fetal biometrics (HC, TCD and VA). It also discussed the potential for using fetal biometrics (e.g., HC and TCD) to help assess later infant outcomes, in particular autistic traits. Whilst, there was no influence of maternal conditions on fetal biometric measures (potentially as a consequence of sample and sub-group sizes), the data provided in this thesis provides the first insight into the influence of maternal conditions on prenatal development. In addition, this thesis provides the first preliminary evidence of prenatal brain development in TCD size and VA width at 20 weeks' gestation and its relationship with infant autistic traits, as well as reaffirming the sexually dimorphic nature and ability to reliability measure AGD *in utero*. Conclusions from this thesis require further investigation if these results are to be generalisable to the world population and to be applied within a clinical setting for early diagnosis and/or monitoring of endocrine related disorders.

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Appendix

Appendix 1: Example of Participant Information Sheet

Professor Simon Baron-Cohen
Director

Ezra Aydin
Research Coordinator,
Autism Research Centre, University of Cambridge
[email]; [telephone]

Participant Information Sheet for Pregnant Mothers – v6, 09/01/2019

Participant Information Sheet
Study Title: The Cambridge Ultrasound Siblings and Parents (CUSP) Research Project

We would like to invite you to take part in a research study about your baby. Before you decide we would like to explain why the research is being done and what it would involve. Please take the time to read the following information carefully. Please feel free to talk to others about the study if you wish.

What is the purpose of the study?
The aim of the study is to investigate how growth during pregnancy may affect the later development of your baby's behaviour. This study will use ultrasound and measures of physical development and later measures of behaviour and cognitive development.

Why have I been invited?
You are attending the hospital for antenatal care or have registered your interest to take part in research. We only ask mothers who have had their 20-week anomaly scan showing a healthy baby.

Do I have to take part?
It is entirely up to you to decide. If after reading this information sheet, you want to take part, we ask you to contact Ezra Aydin (whose details can be found at the bottom of this information sheet) before you are 28 weeks pregnant. Participation in the study will not affect your care in any way and you are free to withdraw from the study at any time.

What happens if I'm interested in taking part?
If you are interested in taking part in the study, please contact Ezra Aydin and she will arrange a suitable time to call and discuss the study with you and answer any questions you may have at this point. She will also be asking you a several 'yes/no' questions about your smoking, alcohol consumption and recreational drug use to assess your eligibility for the study. Once these questions have been answered and we are happy that you are eligible for the study/you

are still happy to take part, we shall arrange for you to come to the Rosie Maternity Hospital, Cambridge for your ultrasound scan. We shall email you a consent form before you arrive for you to have a look over, however you are not required to print it out or sign it at this stage.

When you come in for your ultrasound, Ezra Aydin will meet you and provide a printed copy of the consent form and answer any questions you may have. It is at this point that you will be asked to sign the consent form, to confirm that you have agreed to take part in the study. You will also be asked to fill in a pregnancy history questionnaire before your scan.

What do I have to do if we choose to take part?
If you agree to take part, we would like to ask for your consent to obtain:

1. information about your baby's growth from your routine prenatal ultrasound scans.
2. the birth records of your baby.
3. your previous 12- and 20-week ultrasound scans.
4. your routine blood samples from your pregnancy, for analysis for research purposes.
5. an additional blood sample from the time of the research scan, for analysis for research purposes.
6. a copy of any formal diagnosis of an autism spectrum condition.
7. your baby's blood spot routinely collected at birth, for research.

Antenatal visit
During your 20-week scan we will ask you to have a single research ultrasound scan, where we will collect images and measurements of your baby. The scan will take place between 26-28 weeks of your pregnancy and will last about 45 minutes. You will be able to see the baby on the screen as you did when you had your 12 and 20 week scans, and you will be lying on your back or on your side if you prefer. We will also ask for your permission to collect information about your delivery for our research.

Questionnaires at 18 months' of age
We will contact you by post (or email if you prefer) when your baby reaches 18 months' of age to complete some questionnaires about your child's personality, behaviour and growth. A freepost envelope will be included with this questionnaire if you return it by post. You will also be given the option to complete the questionnaires online.

Assessment visit at 24-36 months' of age
Lastly, you and your child will be invited for an optional follow-up assessment when your baby is 24-36 months old. This will be the same as the 18-month follow up with an online questionnaire (which is mobile friendly) and will take approximately 50 minutes to complete. Depending on the scores of one of the questionnaires we will ask you if you are willing to take part in a telephone interview which will last approximately 40 minutes to 1 hour. If you would like to receive a brief report with a summary of this assessment please tell a member of the research team and we would be happy to produce this for you. Please note that our research assessments are not

diagnostic, and the brief report will simply describe what tests we conducted and your child's behavior. Because this is purely a research study we cannot interpret the results in terms of clinical significance, since the nature of

research is that we do not know if specific scores indicate clinical risk. If you have concerns about your child's development we would advise you to visit your GP.

What are the possible benefits of taking part?
Research is designed to gain new knowledge about the early development of autism. As such, by participating you are helping to advance scientific understanding. You will be given a picture from your ultrasound scan that you may enjoy as a special souvenir of your baby during pregnancy. The results of our research will appear on our website in the form of journal articles at the end of the study.

What happens if we find an anomaly?
The scan is not intended to look for problems with your baby. If any problems are observed during the non-medical scans, you would be referred to the scan clinic and a doctor would make appropriate arrangements for follow-up, as per normal hospital guidelines.

What if I am unhappy about how I have been treated?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions, on 01223 746157. You are also free to contact the Addenbrooke's Hospital Patient Advice and Liaison Service (PALS), by phone (01223 216756) or email (pals@addenbrookes.nhs.uk). In the extremely unlikely event that something does go wrong and you are harmed during the research study and this is shown to be due to someone's negligence then you may have grounds for a legal action for compensation (University of Cambridge/Cambridge University Hospitals NHS Foundation Trust) but you may have to pay your legal costs. Our research is covered by the Cambridge University insurance policy.

What happens at the end of the study?
Your participation in this study will be complete once we have seen you and your child for a final assessment between 24-36 months. We aim to publish data from the study, the data will be presented in terms of group averages and it will not be possible to identify individuals that have participated in the research. We may show pictures of the scan in publications, however we will not include any information that would allow identification of you or your baby. Your privacy and confidentiality is assured and we follow the Data Protection legislation.

Expenses and Payments
If you decide to participate, we will reimburse all travel expenses and you will receive an additional £20/visit for the follow-ups. A cheque will be sent to you after each visit. Additionally we shall provide a picture from your scan.

Will my taking part in the study be kept confidential?
Yes. All data will be kept anonymous and no names will appear in any published or presented material.

What will happen if I don't want to carry on with the study?
If you withdraw from the study, we will destroy all identifiable data, but we will ask you if we can use the unidentifiable data collected up to your withdrawal from the study, unless you do not want us to do so.

Who is organising and funding the research?
This study has been organised by the University of Cambridge and funded by the Autism Research Trust.

Will my GP be informed of my participation?

We will inform your GP of your participation if you give us consent to do so.

I have also consented to take part in the CHILD study; will you require me to do the same assessments twice?
If you have already consented to take part in the CHILD study, we would like to share the data between the two studies. This would reduce the amount of follow-up sessions and assessments you would be asked to complete.

To take part in both studies you *must* be separately consented. If you'd like to learn more about our partner study (CHILD) please ask Ezra Aydin.

Further information and contact details:
If you are happy to take part, please complete the enclosed consent form and return it in the FREEPOST envelope provided or hand it to the researcher on your first visit. If you gave us your contact details then we may contact you to follow up with you to see if you would be interested in taking part. If you would like to learn more about the study, please feel free to contact Ezra Aydin on the email or telephone number given below. We hope that you will be interested in, and will choose to participate in, this project. We appreciate your involvement in any way.

If you do not wish to participate, then you do not have to do anything.
You can contact Ezra Aydin with any questions, by email [email] or telephone [telephone]

*Form redacted for GDPR reasons.

Appendix 2: CUSP Pregnancy History Questionnaire – V1

This questionnaire will ask you questions about your pregnancy, any previous pregnancies, your medical history and your current health.

Name:			
Date of birth:			
Date today:		Due date:	
How many weeks pregnant are you today?			
The father's name of the baby you are carrying:			
Father's date of birth:			

1. What was your height at age 18?	Ft	in
		cm

2. What was your weight at age 18?	Kg	lbs
	St	lbs

3. What is your current height?	Ft	in
		cm

4. What is your current weight?	Kg	lbs
	Kg	lbs

5. At what age did you reach your adult height?	years

6. What is the father of your baby's current height?	Ft	in
		cm

7. Compared to your peers, how much did you weigh as a child?		
Very underweight	Slightly Underweight	Average
Slightly overweight	Very overweight	

8. Compared to your peers, when did your breasts first begin to develop?		
Much earlier	A little earlier	About the same
A little later	Much later	

9. During your adult years, how long was (is) your average menstrual cycle?	
25 days or less	25-34 days
34-60 days	60 days or greater

10. During your adult years, how consistent was (is) the length of your menstrual cycle?	
Highly consistent	Fairly consistent
Fairly variable	Highly variable

11. During your adult years, have you found coarse, dark, hair growing in any of the following areas? Please tick all that apply:		
Upper Lip	Chin	Back
Breasts	Chest between breasts	Belly
Upper thighs	Upper arms	

12. Have you **ever** been diagnosed with any of the following conditions? Please tick all that apply.		
Anovulation (failure to ovulate)	Polycystic ovary syndrome (PCOS)	Breast cancer/ tumours/growths
Cardiac arrhythmia/atrial fibrillation/other cardiac conditions	Congenital adrenal hyperplasia (CAH)	Ovarian cancer/ tumours/growths
Type I Diabetes	Type II Diabetes	Epilepsy
High Blood Pressure	High Cholesterol	Hyperthyroidism
Hypogonadism	Hypothyroidism	Precocious puberty
Uterine cancer/tumours/growths	Delayed puberty	Pre-menstrual syndrome (PMS)
Autoimmune Disorder (please specify):		

13. Do you have autism or Asperger Syndrome?	Yes	No
14. Does the father of your baby have autism or Asperger Syndrome?	Yes	No
15. Do you have any relatives with an autism spectrum condition?	Yes	No
	If Yes, please specify who:	

16. Have you ever had, or do you currently have, any of the following conditions? Please tick all that apply.		
Excessive bodily or facial hair (hirsutism)	Excessive menstrual bleeding	Sudden, unexplained weight loss
Extreme thirst	Severe acne	Unusually painful periods
Hair loss or thinning	Frequent need to urinate	

17. What is your sexual preference?			
Male	Female	Both	Neither

18. Do you identify as male, female or non-binary?		
Male	Female	Non-binary

19. Do you identify as transsexual or transgender?	Yes
	No

20. Have you used any form of hormonal contraceptive? Please tick all that apply.		
hormonal intrauterine device (Mirena)	intra-vaginal ring (NuvaRing)	contraceptive injections (Depo Provera)
contraceptive implant (Implanon, Nexplanon)	contraceptive pill (please specify the brand(s):	

21. Have you ever been diagnosed with primary or secondary infertility?	Primary	Secondary	Neither

22. Have you ever had in-vitro fertilization (IVF) treatment?	Yes	No
	If 'yes' how many times?	

23. If you have experienced infertility issues, did any of the following contribute to your difficulties in conceiving? Please tick all that apply.		
Hypothyroidism	Anovulation	Age
Autoimmune Disorder	Obesity	Hyperthyroidism
PCOS	Other (please specify):	

24. How many times have you been pregnant? Please include all pregnancies, even if the pregnancy did not go to term.	time(s)

25. What was the outcome of your previous pregnancies (if any)? Please put a number under each of the headings on the right, and include all pregnancies, even if the pregnancy did not go to term	live birth	elective termination	still birth/ miscarriage

26. If your current pregnancy was planned, how long did it take you to conceive (i.e. time spent trying to conceive a child)?	years(s)	months

27. If you used hormonal contraception prior to conceiving this pregnancy, for how long did you use hormonal contraception?	years(s)	months
28. How much time had elapsed between any previous pregnancies and this pregnancy? If this was your first or only pregnancy, write "0".	years(s)	months

29. Did you have any of the following pre-existing conditions **prior** to this pregnancy? Please check all that apply.		
Type I Diabetes	Type II Diabetes	Hypertension

30. Did you develop any of the following conditions **during** this pregnancy? Please tick all that apply.		
Gestational Diabetes	Preeclampsia	Hypertension
Eclampsia	Other condition (Please specify):	

31. Were you prescribed any other type of hormonal medication during this pregnancy? If so, could you please give the treatment prescribed, and the condition for which the treatment was prescribed?	Yes	No
	Treatment:	
	Condition:	

32. Were you ever diagnosed with any of the following complications during this pregnancy? Please tick all that apply.	
Infection of amniotic sac and/or membranes	Polyhydramnios (too much amniotic fluid)
Placental abruption (premature detachment of the placenta)	Placenta previa (overattachment of the placenta)
Other complication (Please specify):	

33. Did you ever experience any spotting or vaginal blood loss during the **first** trimester of this pregnancy?	Yes	No
34. Were any anomalies spotted on your routine ultrasound?	Yes	No
35. Have you experienced any vaginal bleeding during the **second** or **third** trimester of this pregnancy?	Yes	No

36. Is your baby small for gestational age?	Yes	No
37. Is your baby large for gestational age?	Yes	No

38. Was this child ever identified as being beneath the 10th percentile or above the 90th percentile for size for gestational age via an ultrasound?	
Beneath 10th percentile	Above 90th percentile
Neither	Don't know

39. Have you had a triple screen test? (This is a blood test to help your doctor see if your baby is potentially at higher risk of certain birth defects, i.e. Downs Syndrome)		Yes	No
39b.	If Yes, what was the result of this test?		

40. Were you ill with a bacterial or viral infection, such as the 'flu, during or immediately prior to this pregnancy?	Yes	No	
	If yes, did you receive antibiotics?	Yes	No

41. Did you ever fail a urine glucose test (i.e. high levels of glucose in your urine) or an oral glucose tolerance test* during this pregnancy?	Yes	No	Don't Know
*This test is sometimes called the "Lucozade/Gatorade Challenge" and involves measuring your blood glucose levels before and after drinking a glucose solution.			

42. Have you been taking any prescription medications to manage your blood sugar or insulin levels during this pregnancy? If possible, can you give the name of the drug(s)?	Yes	No
	Drug Name:	

43. Did you receive any of the following treatments to prevent preterm labor during this pregnancy? Please tick all that apply	
Atosiban (sometimes given under the brand name Tractocile)	No
Progesterone (delivered as an injection as 17P, 17-alpha-hydroxy-progesterone, sometimes under the brand name Makena; also delivered vaginally as a suppository or gel, sometimes under the brand names Crinone, Endometrin or Prochieve; also delivered orally, sometimes under the brand name Prometrium)	If you are unsure whether you received progesterone treatment, or if you have any additional information you would like to provide, please use the space below:
Other (please specify):	

	Yes	No

44. Were you prescribed any other type of hormonal medication during this pregnancy? If so, could you please give the treatment prescribed, and the condition for which the treatment was prescribed?	Treatment: Condition:

45. Are there any other details about this pregnancy that you would like the research team to know? If so, please use the space provided below:

THANK YOU FOR HELPING AUTISM RESEARCH

If you have any queries, please contact Ezra Aydin, Research Coordinator, on email [Email].

Appendix 3: Example of initial email sent to participants

Hi [insert participant name],

Thank you for getting in touch and showing interest in my study. Please find below an information sheet that tells you all about the study. If you have any questions please ask!

We will be looking at early development in pregnancy in families with no history of autism as well as those with, so there may be aspects of the information sheet that is for the latter families. If there are any queries you have or would like any more details please do let me know, I would be more than happy to answer them.

If you are still interested, the next step is to take you through a few screening questions (below) if you wouldn't mind. I'm happy for you to answer the questions via email or over the phone, whichever suits you.

1. Have you smoked during your pregnancy?
2. Have you used any drugs recreationally during your pregnancy?
3. Have you consumed any alcohol during your pregnancy?
4. Are you having twins?
5. Do you know the gender of your baby?
6. How far into your pregnancy are you? (weeks + days)
7. Have you ever been diagnosed with Polycystic ovary syndrome (PCOS)?
8. Have you or your child been diagnosed with ASC?

The reason I ask whether you know the gender of your little one is because we will be exploring an non-invasive measure of testosterone. For this we measure the distance between the baby's anus and genitals. If you do not want to know the gender of your baby, this is not a problem and you can still take part (if you'd like) my sonographer just turns away the screen and does the measure discretely whilst I act as a distraction/entertainment. We've done this successfully with all the parents whom haven't wanted to find out.

If you have any other questions please don't hesitate to contact me.

I look forward to hearing from you.
Best wishes,
Ezra

Appendix 4: Example of reminder email sent to participants

Hi [insert participant name],

Just a gentle reminder that you are booked in for an additional ultrasound scan at the Rosie Maternity Hospital at [insert time] on the **[insert date],** we will be scanning upstairs in *fetal clinic 22*.

When you arrive if you could let reception know, you'll be directed to sit in the second waiting room where I'll come and meet you (I will be the one wearing a purple CPFT/NHS lanyard). Could please bring your pregnancy pack with you.

Before the scan mothers are asked to fill out a questionnaire, AQ and a screening form (attached previously to your confirmation email). I've attached them again here, could you please fill them out at home and bring them with you, or return them to me via email before the scan.

If you have any questions please do not hesitate to contact me, I will have my mobile with me on Monday.

I look forward to meeting you both.
Best wishes,
Ezra

Appendix 5: Example of Participant Consent Form

UNIVERSITY OF CAMBRIDGE
Cambridge University Hospitals NHS
NHS Foundation Trust

Professor Simon Baron-Cohen
Director

Ezra Aydin
Research Coordinator,
Autism Research Centre, University of Cambridge

Participant Consent form – v2, 15/02/2016
Title: The Cambridge Ultrasound Siblings and Parents (CUSP) research project.

Please initial box

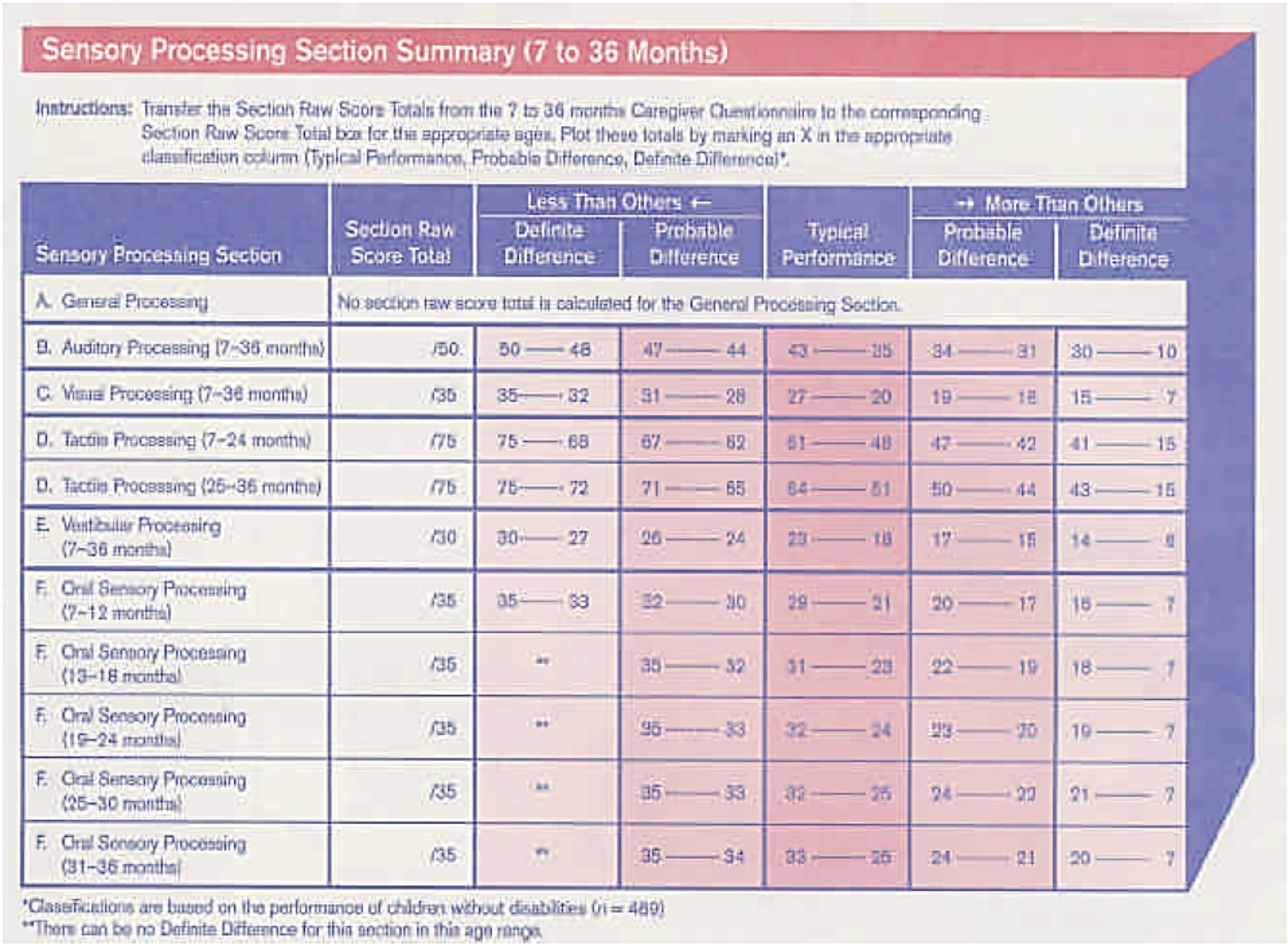
1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐
2. I understand that my and my child's participation are voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by the research team from the University of Cambridge, and by the regulatory authorities or the NHS Trust where it is relevant to my taking part in research. I give permission to these individuals to have access to my child's records. ☐
4. I agree to my antenatal blood samples and my baby's blood spot test both collected as part of routine clinical care, to be re-analysed. ☐
5. I agree for my previous 12 and 20-week routine ultrasound scans to be accessed and used in the study. ☐
6. I agree taking part in the above study and to have the research ultrasound scan ☐
7. I would like the research team to inform the GP about my child's participation in the study. ☐
8. I give permission for the study team to access my medical records relating to my pregnancy, the birth of my baby, and my autism diagnosis. ☐
9. I would like a brief report on my child's progress after the 24-36month follow-up, if I participate in this aspect of the study. ☐ Y ☐ N
10. If I have already consented to take part in the CHILD study, I agree to the data being collected as part of this study to be shared with the CHILD study team. ☐
11. I agree to provide the research team a copy of my ASC diagnosis (if applicable) ☐
12. I am happy to be contacted to hear about future studies. ☐ Y ☐ N

Name of Parent *(Please print)* _____ Date _____ Signature _____

Department of Psychiatry
Douglas House
18b Trumpington Road
Cambridge CB2 8AH

*Form redacted for GDPR reasons.

Appendix 6: Infant/Toddler Sensory Profile Scoring Summary.



Sensory Processing Section Summary (7 to 36 Months)

Instructions: Transfer the Section Raw Score Totals from the 7 to 36 months Caregiver Questionnaire to the corresponding Section Raw Score Total box for the appropriate ages. Plot these totals by marking an X in the appropriate classification column (Typical Performance, Probable Difference, Definite Difference)*.

Sensory Processing Section	Section Raw Score Total	Less Than Others ← Definite Difference	Probable Difference	Typical Performance	→ More Than Others Probable Difference	Definite Difference
A. General Processing	No section raw score total is calculated for the General Processing Section.					
B. Auditory Processing (7–36 months)	/50	50 — 46	47 — 44	43 — 35	34 — 31	30 — 10
C. Visual Processing (7–36 months)	/35	35 — 32	31 — 28	27 — 20	19 — 18	15 — 7
D. Tactile Processing (7–24 months)	/75	75 — 68	67 — 62	61 — 48	47 — 42	41 — 15
D. Tactile Processing (25–36 months)	/75	75 — 72	71 — 65	64 — 51	50 — 44	43 — 15
E. Vestibular Processing (7–36 months)	/30	30 — 27	26 — 24	23 — 18	17 — 15	14 — 8
F. Oral Sensory Processing (7–12 months)	/35	35 — 33	32 — 30	29 — 21	20 — 17	16 — 7
F. Oral Sensory Processing (13–18 months)	/35	**	35 — 32	31 — 23	22 — 19	18 — 7
F. Oral Sensory Processing (19–24 months)	/35	**	35 — 33	32 — 24	23 — 20	19 — 7
F. Oral Sensory Processing (25–30 months)	/35	**	35 — 33	32 — 25	24 — 22	21 — 7
F. Oral Sensory Processing (31–36 months)	/35	**	35 — 34	33 — 25	24 — 21	20 — 7

*Classifications are based on the performance of children without disabilities (n = 469)
**There can be no Definite Difference for this section in this age range.

Appendix 7: Table of sample sizes in each ITSP sensory processing section

	Oral Processing			Vestibular Processing			Tactile Processing			Visual Processing			Auditory Processing		
n	Female	Male	Total	Female	Male	Total	Female	Male	Total	Female	Male	Total	Female	Male	Total
Less than definite difference	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
Less than probable difference	2	3	5	-	1	1	4	7	11	4	3	7	7	6	13
Typical Performance	14	15	29	37	36	73	38	46	84	52	52	104	51	47	98
More than probable difference	6	1	7	25	23	48	21	6	27	16	9	25	6	12	18
More than definite difference	4	-	4	20	23	33	18	14	32	10	10	20	18	9	27
Too low to code	-	-	-	1	-	1	2	2	4	2	-	2	1	-	1
Total	26	19	45	83	83	156	83	75	158	84	74	158	84	74	158

Appendix 8: Example of an ultrasound output given to parents.

Department of Obstetrics & Gynaecology

Delivering high quality care for our next generation

Addenbrookes NHS
NHS Trust

Rosie Ultrasound
Rosie Hospital
Tel: 01223-217621
Fax: 01223-217622

Box 228
Addenbrooke's NHS Trust
Hills Road
Cambridge CB2 2QQ
Tel: 01223 245151
www.addenbrookes.org.uk

Examination date:
Hospital number:
Gestational age:

Patient Data

Name	
Other names	
Date of birth	
Address	
Town	
Count(r)y	
Postcode	
Ethnic group	white (European, Middle Eastern, North African, Hispanic)
GP	

Examination

Time	
Department	Obstetric Ultrasound Dept, Rosie
Indication	Routine, Research

Ultrasound

Operator	
US system	Voluson E8
	transabdominal
Gestational age	
EDD by scan	07/12/2017

Biometry / Anatomy

HC	272.9 mm
TCD	31.4 mm
Ventricular atrium	4.8 mm
AC	244.0 mm
FL	53.0 mm
HC/AC	1.12
Estimated fetal weight	Hadlock (HC, AC, FL)
	1,266 g
	2 lbs 12 oz
Centile	59.0
Fetal heart activity	visualised
Fetal movements	normal
Presentation	breech
Placenta site	low posterior, extending to, but not covering, the os
Amniotic fluid	normal

Amniotic Fluid Index

AF Index	13.6 cm

Doppler ultrasound

Umbilical artery

PI 1.04

EDF positive

Thank you for referring patient

CUSP - Research study

Ultrasound demonstrates a normal growth velocity and a normal amniotic fluid level.
Normal fetal movements reported and seen on scan.
Normal umbilical artery and MCA Doppler studies.
Posterior and low placental, extending to but not covering the os.
Breech presentation.

A scan is arranged for 36 weeks to assess the placental position.

Best wishes,

Daren Chaplin, Sonographer