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妊娠期のPFAAs・OH-PCB曝露による次世代への甲状腺機能攪乱作用と生後の神経発達へ与える影響の解明

平成26年度　総括研究報告書

研究代表者

北海道大学環境健康科学研究教育センター　伊藤　佐智子

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研究代表者　伊藤　佐智子　北海道大学環境健康科学研究教育センター 特任助教

研究要旨

有機フッ素化合物（PFAAs）やPCBは人体へ長期間蓄積し、胎児移行率が高い。これまで血中PFAAs濃度と注意欠陥・他動性障害（ADHD）等児の発達障害との関連や（Hoffman et al. 2010）、PCB代謝物の水酸化PCB（OH-PCB）曝露と5-6歳の注意力低下との関連が報告された（Roze et al. 2009）。そのメカニズムとして脳神経の発生・発育時期の甲状腺機能異常は脳神経発達の障害を招く（Haddow et al. 1999）ことから、化学物質の胎児期曝露が胎児成長に必須である甲状腺ホルモン値を攪乱して神経行動障害を引き起こす可能性が考えられた。特にPFNA、PFUnDAはわが国で生産量が多いが、胎児期の長炭素鎖PFAAs曝露が甲状腺ホルモン値撹乱および脳神経系発達への影響についてこれまで全く検討されておらず、OH-PCBの先行研究も少ないため、わが国における早急な検討と予防対策が急務である。そこで、申請者らは出生前向きコホート「環境と子どもの健康に関する北海道スタディ」大小2つのコホートで、母児260組の代謝関連SNPs解析および母児1000組（予定）の母体血中および臍帯血中新生児甲状腺ホルモン値（TSH、FT3、FT4）と抗甲状腺抗体（TgAb、TPOAb）の測定を行い、長炭素鎖PFAAsおよびOH-PCBの妊娠期曝露による母児甲状腺機能攪乱作用への影響を解明と幼児期や学童期の発達障害との関連を明らかにし、化学物質胎児期曝露による次世代への健康リスク評価を行う。

平成26年度は小規模コホート内で妊娠期母体血中OH-PCB濃度と代謝関連SNPsデータおよび母児甲状腺ホルモン値データが揃う母児260組で個々の代謝関連SNPsを解析し、SNPsを考慮した母体OH-PCB濃度と児甲状腺ホルモン値への影響検討を行った。PCBからOH-PCBへの代謝に関連するとされるAhR、CYP1A1のSNPsごとに層別化し重回帰分析にて検討したところ、AhRのAG/GG型では妊娠期母体血中ΣOH-PCB、4-OH-CB187濃度が高くなると児のFT4値が有意に高くなる（p=0.016、0.031)が、AA型では有意な関連がみられなかった(p=0.856、0.400)。また、CYP1A1、GSTM、GSTTの各SNPsでも同様の検討を行ったが、SNPs間での差はみられなかった。今後は他のSNPsでの検討および複数のSNPsの組み合わせによる層別化での検討の必要があると考える。大規模コホートでは妊娠中母体血液・臍帯血の両検体を有し、8歳のADHD調査を終えた母児500組において母児甲状腺ホルモン値および抗甲状腺抗体測定を行った。平成27年度はSNPs解析、甲状腺ホルモン、抗甲状腺抗体値の測定を引き続き行い、大規模コホート内でPFAAsの妊娠期曝露濃度が甲状腺機能へ与える影響検討を行う。平成28年度は両コホートで甲状腺機能が生後の神経行動発達へ与える影響について解析する。

A．研究目的

有機フッ素化合物 PFAAs や PCB は人体へ長期間蓄積し、かつ胎児移行率が高い。これまで血中 PFAAs 濃度と ADHD 等児の発達障害との関連や(Hoffman, Webster et al. 2010)、PCB 代謝物の OH-PCB 曝露と 5-6 歳の注意力低下との関連が報告された（Roze et al. 2009)。そのメカニズムとして脳神経の発生・発育時期の甲状腺機能異常は脳神経発達の障害を招く（Haddow et al. 1999）ことから、化学物質の胎児期曝露が胎児成長に必須である甲状腺ホルモン値を攪乱して神経行動障害を引き起こす可能性が考えられている。

PFOS、PFOA に代表される PFAAs は、絶縁性・撥水撥油性をはじめとする優れた特性を有することから、衣類・建材・界面活性剤など幅広い分野で使用されている。人は主に飲料水や赤肉、魚介類を通して曝露され、胎児への影響が懸念されているが、十分な研究が行なわれていない。わが国でも 2010 年に PFOS、PFOA が化学物質の審査及び製造等の規制に関する法律の第一種特定化学物質（一部用途以外の製造・輸入禁止）に指定された。しかし、最近、「環境と子どもの健康に関する北海道スタディ（以下、北海道スタディ）」に参加している約 2000 名の妊婦の PFAAs11 種類の濃度を調べたところ、PFOS/PFOA 濃度は年々低下しているが、代わりに長炭素鎖（C=10 以上）の PFNA、PFDA は血中濃度が増加していることがわかった（Okada et al. 2013)。

炭素鎖の長い（C=10 以上）の PFNA、PFDA は日本において諸外国と比較して国内生産量が多いため、次世代の胎児への影響検討が必要だが、胎児期の長炭素鎖 PFAAs 曝露が甲状腺ホルモン値変動へ影響を与える可能性および脳神経系発達への影響についてはこれまでほとんど検討されていない。Hoffman et al.（2010）は、PFAAs4 種と ADHD との関連を検討した米国の横断研究では、血中 PFAAs 濃度が高いほど ADHD 発症のリスクを上げると報告されているが、これまで PFNA、PFDA の胎児期曝露と生後の発達障害との検討を行った報告はなく、神経発達に重要な役割を示す甲状腺ホルモンへの影響も調べられていない。

PCB は毒性が発見されたのち国内で 1972 年に製造が中止され、2004 年にストックホルム条約でその使用と廃棄が禁止された。しかし PCB を含む製品は現在も使用され、安定性と長期にわたる蓄積性のため、環境中や生体から検出され続けている（Schecter 2001）。これまで PCB 胎児期曝露が生後の神経発達を妨げるという報告があり（Grandjean et al. 2001, Jacobson and Jacobson 1996）、成人よりも環境物質に脆弱とされる胎児への影響検討が注目されてきた。

PCB の一部は生体内で Cytochrome450 による酸化を受けた後、大部分が OH-PCB へ代謝され速やかに体外へ排出されるとされてきたが、近年 OH-PCB は PCB 同様生体内や環境中に蓄積することが報告されている（Letcher et al. 2000）。そのため OH-PCB のヒトへの健康影響が懸念されているが、生体内における PCB の代謝経路は明らかになっていない。またこれまで PCB の健康影響とされてきたものが本来は OH-PCB の影響である可能性があり、早急に解明が必要である。OH-PCB は PCB よりも甲状腺ホルモンによく似た構造を有し、Transthyretin(TTR)と強い結合力を有することから（Brouwer et al. 1998)、PCB よりも体内の甲状腺機能維持へ強く影響を与えるとされており、これまで血清中の OH-PCB 濃度と甲状腺ホルモン FT4 との間に負の関連がみられたが、PCB 濃度と FT4 とは関連がみられなかった（Sandau

et al. 2002）という報告やPCB、OH-PCB濃度ともにT3との関連がみられたという報告（Dallaire et al. 2009）があるが、一致した結果は得られていない。甲状腺ホルモンは胎児発育において重要な役割を示し、胎児は自らの甲状腺が分泌を開始するまでの妊娠初期は母親の甲状腺ホルモンに依存している（de Escobar et al. 2004：Calvo et al. 2002）。OH-PCBは胎盤通過性を有し、母体血中より臍帯血中のOH-PCB/PCB濃度比が高い(Kawashiro et al. 2008)ことから、感受性が高い胎児への影響を直ちに明らかにする必要があるが、OH-PCB胎児期曝露による児甲状腺機能への影響についての疫学報告はわずか3報である（Hisada et al. 2012, Dallaire et al. 2009, Otake et al. 2007）。これまでにRoze et al. (2009)によるOH-PCB曝露と5-6歳の注意力低下との関連の報告や、男児において臍帯中のOH-PCB濃度と2歳、5歳時の体重との関連がみとめられたとの報告があるが（Yonemoto et al. 2011, Yonemoto et al. 2012），これらの結果はOH-PCB曝露による甲状腺ホルモン値変動を介している可能性がある。しかし、胎児期OH-PCB曝露による生後の行動情緒や体格成長へ影響を与える可能性については研究が不足しており十分な結果が得られていないため、わが国における早急な検討と予防対策が急務である。

そこで本研究では、一般生活環境レベルでの長炭素鎖PFAAs OH-PCBの妊娠期曝露が及ぼす母児甲状腺ホルモン値への影響を検討することを目的とした。

B．研究方法

2003年から前向き出生コホート研究「環境と子どもの健康に関する北海道研究」を実施中であり、そのうち2003年～2005年に札幌市内同一産科医院にて参加登録を行った妊婦514名の小規模コホート参加者のうち、妊娠中母体血中OH-PCB濃度と妊娠中の母親および出生時の児甲状腺ホルモン値データが揃う260組の母児と、2003年～2012年に北海道内の産科37施設で参加登録を行った妊婦20,929名の大規模コホート参加者のうち、2013年（平成25年）までに児が8歳を迎え、妊娠中の母体血液と出産時の臍帯血の両検体とPFAAs濃度のデータが揃い、8歳時のConners3P調査票返送がある母児1000組を対象とした。

1. 代謝関連SNPsの違いによる胎児期OH-PCB曝露と児の甲状腺ホルモン値との関連検討：

妊婦とその配偶者の既往歴、教育歴、世帯収入、喫煙状況などの対象者の属性は妊娠中期から後期に実施した自記式調査票，児の性別、出生時体重、出産経歴などの出生時所見は医療診療録から得た。小規模コホートでは母体血中OH-PCB濃度は福岡県保健環境研究所で測定し、LC/MS/MSで母体血中OH-PCB濃度を測定し、OH-CB107、OH-CB146+153、OH-CB172、OH-CB187の各異性体について分析した。また児甲状腺ホルモン値（TSH, FT4）は、札幌市が実施しているマススクリーニングの結果を用いた。SNPs解析については、母体血よりAhR, CYP1A1, GSTM1, GSTT1の解析を行った。

母体血中∑OH-PCB濃度と児甲状腺ホルモン値の関連については多変量解析を行った。多変量解析の独立変数は、母体血中∑OH-PCB、OH-CB146+153、OH-CB187濃度とした。OH-CB107、OH-CB172濃度は全対象者の50%未満の検出率であったため、多変量解析は行わなかった。なお、∑OH-PCB濃度、児TSH、FT4値は常用対数変換して解析に用いた。従属変数は、児TSH、FT4値とし、母親の出産時年齢、

非妊娠時 BMI、出産回数，魚摂取量、世帯年収、OH-PCB 測定用採血時期に加え、在胎週数、出生時体重、生下時採血日数で調整して各 SNPs の多型ごとに層別化し、重回帰分析を行った。統計解析には SAS 社 JMP11 を用い、$p<0.05$ を統計学的有意とした。

2. 大規模コホート内母児血中甲状腺ホルモン値（TSH、FT3、FT4）および抗甲状腺抗体の測定（TgAb、TPOAb）：

大規模コホートでは妊娠期母体血より PFAAs 濃度を測定した 500 組の母児において、冷凍保存している妊婦の採血(妊娠 13 週未満)と 出産時の妊婦の臍帯血から妊娠期の母児の甲状腺ホルモン値および抗甲状腺抗体の測定を行った。測定は株式会社エスアールエルに委託して行った。H27 年度も引き続き 500 組の母児において測定を行う。

（倫理面への配慮）

疫学調査は北海道大学環境健康科学研究教育センター，北海道大学大学院医学研究科医の倫理委員会および遺伝子解析審査小委員会および共同研究施設の倫理規定に従って実施し，インフォームドコンセントは「ヒトゲノム・遺伝子解析研究に関する倫理指針」，「疫学研究に関する倫理指針」およびヘルシンキ宣言に基づいて行った。研究への参加は自由意志により，自発的に中止しても不利益を被らないよう配慮し，対象者のプライバシーの保持には細心の注意を払った。すべての実験・研究は，北海道大学大学院医学研究科で規定されている「ヒト組織及び動物を用いた実験指針」に従い，本研究は倫理面の十分な配慮のうえ行った。

C．研究結果

H26 年度は「北海道スタディ」小規模コホートにおいて妊娠期母体血中 OH-PCB 濃度および母児甲状腺ホルモン値（TSH、FT4）データの揃っている母児 260 組の母体血から、当初の研究計画で予定していた代謝関連 SNPs のうち、AhR、CYP1A1、GSTM、GSTT の解析を行い、妊娠期母体血中 OH-PCB 濃度が児甲状腺ホルモン値へ及ぼす影響をこれらの異物代謝関連 SNPs を考慮しながら検討した。SNPs で層別化した集団間での OH-PCB 異性体 4-OH-CB 146 + 3-OH-CB 153、4-OH-CB 187 および ΣOH-PCB 濃度差を検討したところ、有意差はみられなかった。母体血中∑OH-PCB 濃度と濃度の平均は 35.1pg/g-wet であった。この値は諸外国および国内と比較して低い数値であった。

OH-PCB の母体血中濃度が出生時の児 TSH、FT4 値へ与える影響について、PCB から OH-PCB への代謝に関連するとされる AhR、CYP1A1 の SNPs ごとに層別化し重回帰分析にて検討したところ、AhR の AG/GG 型では妊娠期母体血中 ΣOH-PCB、4-OH-CB187 濃度と児の FT4 値に有意な正の関連がみられた($p=0.016$、0.031)が、AA 型では有意な関連がみられなかった($p=0.856$、0.400)。また、CYP1A1、GSTM、GSTT の各 SNPs でも同様の検討を行ったが、SNPs 間での差はみられなかった。

D．考察

本研究では、一般生活環境レベルでの妊娠中 OH-PCB 曝露による児甲状腺ホルモン値への影響について異物代謝関連 SNPs を考慮しながら検討を行い、AhR の SNPs によって児の FT4 値への影響が異なることを見出した。

本研究の母体血中 ΣOH-PCB 濃度 35.1pg/g-wet は先行研究における妊婦と比較して低い濃度であった（Kawashiro et al.

2008; Park JS et al. 2007）。また、「北海道スタディ」の妊婦 256 名の体内 OH-PCB 濃度を異性体ごとに測定したところ、血中ΣOH-PCB/ΣPCB 濃度比は先行研究の妊婦血中比(0.19 : Soechitram et al. 2004、0.18 : Kawashiro et al. 2008)と比較して低い値(0.08)を示した。これは申請者らのコホート内妊婦では他のコホート対象者と比較して、環境中からの曝露量が低いことに加え、体内の PCB から OH-PCB への代謝能力が低い、または OH-PCB 体外排泄能力が高いため、体内 OH-PCB 濃度が低くなっている可能性も考えられた。妊娠期は体内の異物動態が変化する時期であるため PCB の代謝環境の変化や個々の代謝能力の違いによることが考えられるが、これまでヒトにおける PCB、OH-PCB の代謝経路は明らかになっておらず、個々人の生体内代謝環境による血中濃度差についての疫学研究はない。H26 年度に行った SNPs 解析では主に異物代謝第 1 相、第 2 相とされる SNPs（AhR、CYP1A1、GSTM、GSTT）を解析し、検討を行ったが、今後は AhRR, CYP1A2, CYP1B1, GSTP1, NQO1 の追加解析に加え、複数の SNPs 組み合わせによる層別化を行い、検討の必要がある。

本研究では、母が AhR の AG/GG 型の児では胎児期ΣOH-PCB、4-OH-CB187 曝露濃度と出生時 FT4 値に有意な正の関連がみられ、この結果は SNPs による層別化はしていなかった過去の報告と一致していた（Otake et al. 2007）。甲状腺ホルモンについては、母親の妊娠初期における甲状腺機能低下症と児の IQ 低下や神経心理学発達悪化との関連を示す報告があることから（Haddow et al. 1999）、胎児初期における母親の甲状腺ホルモン値が非常に重要であることが知られている。ラットでの動物実験では母ラットの OH-PCB 曝露後に胎児血清中 T4 値の低下と TSH 値の増加がみられた（Meerts et al. 2002）。T4 値の低下という点では PCB 曝露と同様の結果がみられ（Morse et al. 1996）、これは本来生体内で代謝された OH-PCB による影響であった可能性もある。しかし、ヒトについては報告が少なく、代謝前の物質である PCB との結果の比較を行いながらさらに検討が必要である。

また、本研究ではH26 年度、H27 年度で「北海道スタディ」大規模コホート内の母児 1000 組において、冷凍保存している妊婦の採血(妊娠 13 週未満)と 出産時の妊婦の臍帯血から妊娠期の母児の甲状腺ホルモン値および抗甲状腺抗体の測定を行う。特に、甲状腺ホルモン値は FT4 に加え FT3 を測定することで、FT4、FT3 値がほぼ同様の変動をする臨床的な甲状腺疾患と区別する形で環境化学物質曝露による甲状腺機能攪乱の影響を比較して明らかにできると考えられる。

E．結論

母体血中 OH-PCB 濃度と児甲状腺ホルモン値との関連を母親の SNPs で層別化し解析を行ったところ、AhR の AG/GG 型では妊娠期母体血中 ΣOH-PCB 、4-OH-CB187 濃度と児の FT4 値に有意な正の関連がみられたが、AA 型では有意な関連がみられず、遺伝子多型の違いによって OH-PCB 曝露が甲状腺ホルモン値へ及ぼす影響が異なることが示唆された。また、CYP1A1、GSTM、GSTT の各 SNPs でも同様の検討を行ったが、SNPs 間での差はみられなかった。今後、甲状腺機能に加えて、OH-PCB 曝露濃度が及ぼす神経行動発達への影響を検討・評価していく必要があると考える。

F．健康危険情報
特になし

G．研究発表
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H. 知的財産権の出願・登録状況

該当なし

II 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mitsui T., Araki A., Imai A, Sato S., Miyashita C., Ito S., Sasaki S., Kitta T., Moriya K. Cho K., Morioka K., Kishi R., Nonomura K.	Effects of prenatal Leydig cell function on the ratio of the second to fourth digit lengths in school-aged children	PLOS ONE	13	e437	2014
Araki A., Mitsui T., Miyashita C., Nakajima T., Naito H., Ito S., Sasaki S., Cho K., Ikeno T., Nonomura K., Kishi R.	Association between maternal exposure to di(2-ethylhexyl) phthalate and reproductive hormone levels in fetal blood: The Hokkaido Study on Environment and Children's Health	PLOS ONE	9 (10)	e109039	2014

PLOS | ONE

RESEARCH ARTICLE

Effects of Prenatal Leydig Cell Function on the Ratio of the Second to Fourth Digit Lengths in School-Aged Children

Takahiko Mitsui[1]*, Atsuko Araki[2], Ayako Imai[1], Sakiko Sato[1], Chihiro Miyashita[2], Sachiko Ito[2], Seiko Sasaki[3], Takeya Kitta[1], Kimihiko Moriya[1], Kazutoshi Cho[4], Keita Morioka[4], Reiko Kishi[2], Katsuya Nonomura[1]

1 Department of Urology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan, 2 Hokkaido University Center for Environmental and Health Sciences, Sapporo, Hokkaido, Japan, 3 Department of Public Health, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan, 4 Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan

* mitsui68@med.hokudai.ac.jp

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Abstract

Prenatal sex hormones can induce abnormalities in the reproductive system and adversely impact on genital development. We investigated whether sex hormones in cord blood influenced the ratio of the second to fourth digit lengths (2D/4D) in school-aged children. Of the 514 children who participated in a prospective cohort study on birth in Sapporo between 2002 and 2005, the following sex hormone levels were measured in 294 stored cord blood samples (135 boys and 159 girls); testosterone (T), estradiol (E), progesterone, LH, FSH, inhibin B, and insulin-like factor 3 (INSL3). A total of 350 children, who were of school age and could be contacted for this survey, were then requested via mail to send black-and-white photocopies of the palms of both the left and right hands. 2D/4D was calculated in 190 children (88 boys and 102 girls) using photocopies and derived from participants with the characteristics of older mothers, a higher annual household income, higher educational level, and fewer smokers among family members. 2D/4D was significantly lower in males than in females ($p<0.01$). In the 294 stored cord blood samples, T, T/E, LH, FSH, Inhibin B, and INSL3 levels were significantly higher in samples collected from males than those from females. A multivariate regression model revealed that 2D/4D negatively correlated with INSL3 in males and was significantly higher in males with <0.32 ng/mL of INSL3 ($p<0.01$). No correlations were observed between other hormones and 2D/4D. In conclusion, 2D/4D in school-aged children, which was significantly lower in males than in females, was affected by prenatal Leydig cell function.

analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The ratio of the 2nd finger to 4th finger lengths (2D/4D) in humans has been reported to be smaller in males than in females [1]. This sexual difference has been attributed to the prenatal hormonal environment, such as exposure to higher levels of androgens and some other gonad-specific hormones [2] through androgen receptors, which are located in fetal cartilaginous tissue [3]. This hypothesis for the underlying mechanism for this difference is supported by the following findings; lower 2D/4D in girls with congenital adrenal hyperplasia [4], higher 2D/4D in individuals with complete androgen insensitivity syndrome [5], and the existence of a relationship between 2D/4D and polymorphisms in androgen receptors [6].

Prenatal exposure to sex hormones is known to affect human development, including that of the fetal digits, and one of the most important periods for the fetus is from the first to second trimester of pregnancy. Although most organ systems are developing during this period, the endocrine control systems have already been formed. The sexual difference in 2D/4D has already been established during early prenatal development under the influence of sex hormones [7, 8], and 2D/4D is considered to be stable after the early prenatal stages. Therefore, 2D/4D has been used as an easily measurable and stable anthropometric index of prenatal androgen exposure. However, the mechanism responsible for the sexual difference in 2D/4D has not yet been elucidated in detail.

There is currently no established approach for measuring prenatal hormone exposure when investigating the relationship between 2D/4D and the hormonal environment earlier in pregnancy in order to elucidate the mechanism underlying the sexual difference in 2D/4D; measuring prenatal hormone levels is difficult and not feasible for ethical reasons during a normal pregnancy. On the other hand, umbilical cord blood is obtained immediately after delivery, and its hormone levels are broadly considered to reflect the hormonal environment of the fetus at late gestation [9, 10]. Previous studies have been performed using cord blood to investigate the relationship between fetal hormonal exposure and human development [11–13].

In the present study, as a part of the Sapporo Cohort, Hokkaido Study on Environment and Child Health [14, 15], we investigated whether sex hormone levels in cord blood influenced 2D/4D in school-aged children.

Participants and Methods

Participants

This prospective birth cohort study was based on the Sapporo Cohort, Hokkaido Study on Environment and Child Health [14, 15]. Study details regarding the population, data collection, sampling of biological specimens, and contents of the questionnaire have been described previously [14, 15]. Briefly, native Japanese women living in Sapporo City or its surrounding areas were enrolled into the study at 23–35 weeks of gestation at Sapporo Toho Hospital between July 2002 and October 2005. Of the 1796 women approached, 25% were excluded as they decided to enroll in the Japanese cord blood bank or deliver the baby at another hospital; therefore, 514 pregnant women were enrolled in this cohort study (participation rate of 28.6%).

This study was approved by the Institutional Ethical Board for Epidemiological Studies at Hokkaido University Graduate School of Medicine and Hokkaido University Center for Environmental and Health Sciences. All participants provided written informed consent. Informed consent on behalf of the children enrolled was provided by their parents.

Measurement of 2D/4D

Ten out of 514 participants were excluded from the study due to miscarriage, stillbirth, relocation, or voluntary withdrawal from the study before delivery. As 7 sets of twins were born, a total of 511 children (246 males and 265 females) were finally included in the Sapporo Cohort study. Of these, 350 children (68.1%), who are currently school-aged and could be contacted for this survey, were requested via a mail to send black-and-white photocopies of the palms of both the left and right hands. Measurements of digits were made from photocopies of the ventral surface of the right and left hands. The participants were instructed to straighten their fingers and lightly place their hands palm down on the photocopy machine. Measurements were made to the nearest 0.5 mm from the mid-point of the finger crease proximal to the palm to the tip of the finger using steel Vernier calipers. The ratio was calculated by dividing the length of the second digit by that of the fourth digit[1]. All measurements were taken twice by two observers blinded to participants' information in order to confirm the measurements obtained.

Sex hormone measurements in cord blood samples

At the time of delivery, a blood sample of 10–30mL was collected from the umbilical cord and stored at -80°C for later analysis.

The following hormone levels in 294 stored cord blood samples (135 boys and 159 girls) were measured. Testosterone (T), estradiol (E), and progesterone (P) levels were measured using LC-MS/MS [16, 17]. An immunoradiometric assay was used to measure luteinizing hormone (LH) (Spac-S LH Kit, TFB, Inc., Tokyo Japan) and follicle-stimulating hormone (FSH) levels (Spac-S FSH Kit, TFB, Inc., Tokyo Japan). Inhibin B levels were measured using an enzyme-linked immunosorbent assay (Inhibin B Gen II ELISA, Beckman Coulter, Inc., CA, USA). An enzyme immunoassay (Insulin-like 3 (INSL3) / RLF (Human)—EIA Kit, Phoenix Pharmaceuticals, Inc. CA, USA) was used to measure INSL3 levels. INSL3 was measured in males because it reflects Leydig cell function. It was also measured in 20 randomly selected samples from females. All sex hormone measurements were performed by Aska Pharma Medical Co., Ltd. (Kanagawa, Japan).

Statistical analyses

Data on the characteristics of participants, 2D/4D, and sex hormone levels were presented as a group mean ± standard deviation and were analyzed between groups using a one-way ANOVA. Sex hormones were converted to a log10 scale as these data did not fall into a normal distribution. A half of the detection limit was used when levels were below the detection limit for individual hormones. The relationship between 2D/4D and sex hormone levels in cord blood samples was calculated using a multiple linear regression analysis. The inclusion of covariates was based on biological considerations and adjustments were made for maternal age (continuous), birth weight (continuous), maternal smoking during pregnancy (yes or no), and maternal alcohol consumption during pregnancy (yes or no). All statistical analyses were performed using JMP pro 10 (SAS institute Inc., NC, USA), except for the intra-class correlation coefficient for right and left 2D/4D measurements, which was calculated using SPSS statistics version 19 (IBM, IL, USA). Significance levels were set to 0.05 for all comparisons.

Results

1) Patient characteristics

A total of 190 children from the 189 participants, including 88 males and 102 females, sent back photocopies of their palms. The characteristics of the participants and their children who

sent back photocopies for 2D/4D were compared to their children without 2D/4D. 2D/4D was derived from the following participants; older mothers, a higher annual household income, higher educational level, and fewer smokers among family members. No significant differences were observed in gender, birth weight, or gestational age (Table 1).

2) 2D/4D

In all right hand, left hand, and mean values, 2D/4D was significantly higher in females than in males (Fig. 1). 2D/4D fell into a normal distribution in all right hand, left hand, and mean values.

The intra-class correlation coefficient (1, 2) for right and left 2D/4D measurements was 0.720 (95% confidence interval: 0.627–0.789). The mean 2D/4D value in both hands was used to determine its relationship with sex hormones as a representative value of each participant.

3) Sex hormones in cord blood samples

T, E, P, and INSL3 were detected in all samples. INSL3 was only measured in 20 randomly selected samples from females. The detection percentages of LH in males and females were 25.7% and 0.7%, respectively, while those of FSH in males and females were 46.8% and 0%, respectively. Inhibin B was detected in 99.2% of males and 26% of females (Table 2). The mean intra-assay and inter-assay coefficients of variations in terms of sex hormone measurements were as follows; T: 1.4%–5.3%, E: 3.2%–11.3%, P: 2.7%–6.3%, LH: 4.8%–6.5%, FSH: 2.3%–3.7%, Inhibin B: < 3.8%, and INSL3: 1%–5% in the mean intra-assay coefficients of variations, and T: 3.4%–5.1%, E:

Table 1. Patient characteristics.

		2D/4D (+)		2D/4D (-)		
		n	Mean ± SD	n	Mean ± SD	
Maternal characteristics						
Age at delivery (years old)		189	31.4 ± 4.2	315	30.7 ± 5.2	**
Pre-pregnancy BMI (m^2/kg)		189	21.0 ± 3.1	315	21.6 ± 3.4	
Parity	Primiparous	92 (48.7)		148 (47.0)		
	Multiparous	97 (51.3)		167 (53.0)		
Annual house hold income (million yen per year)	<5	108 (57.1)		237 (75.2)		**
	≥5	81 (42.9)		78 (24.8)		
Educational level (years)	≤12	58 (30.7)		166 (52.7)		**
	≥13	131 (69.3)		149 (47.3)		
Smoking during pregnancy	Nonsmoker	174 (92.1)		232 (73.7)		**
	Smoker	12 (7.9)		83 (26.3)		
Alcohol consumption during pregnancy	Nondrinker	120 (63.5)		235 (74.6)		
	Drinker	69 (36.5)		80 (25.4)		
Infant characteristics						
Gender	Males	88 (46.3)		158 (49.2)		
	Females	102 (53.7)		163 (50.8)		
Birth weight (g)		190	3037.6 ± 379.7	321	3003.9 ± 444.5	
Gestational age (weeks)		190	38.9 ± 1.5	321	38.6 ± 1.6	

The values in brackets represent percentages.
**: $p<0.01$.

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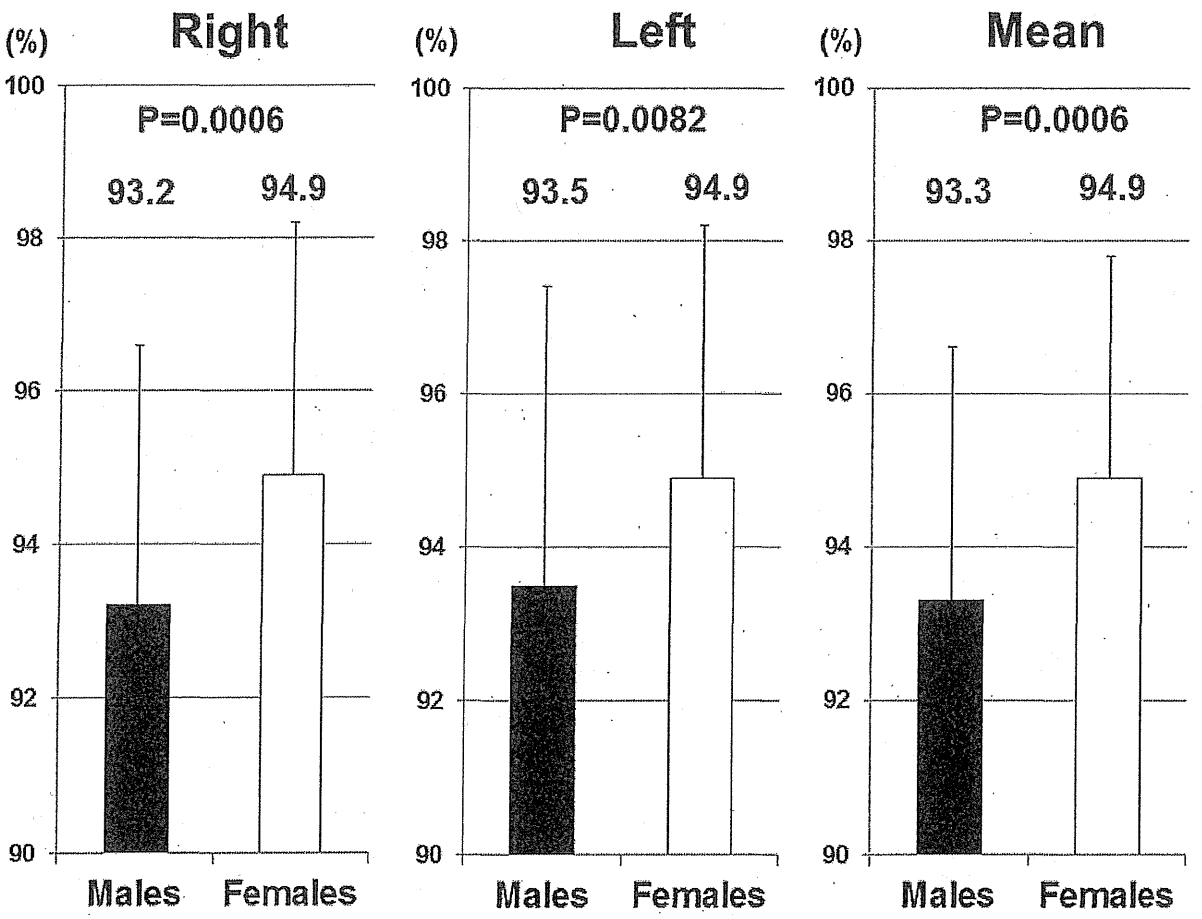

Fig 1. 2D/4D in right hands, left hands, and mean values. 2D/4D in right hands, left hands, and mean values were significantly higher in females than in males.

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Table 2. Sex hormone levels in cord blood in males and females.

		Males				Females				
	DL	n	50th	25th-75th	>DL (%)	n	50th	25th-75th	>DL (%)	p-value
Testosterone (pg/mL)		135	98.9	76.5–126	100	156	69.9	51.9–96.3	100	<0.001
Estradiol (ng/mL)		135	4.86	3.33–7.42	100	159	4.67	3.15–6.48	100	0.227
Progesterone (ng/mL)		135	226	184–286	100	159	210	167–276	100	0.184
T/E		135	18.5	13.9–25.7	100	156	15.9	11.8–21.8	100	0.002
LH (mIU/mL)	0.5	132	<DL	<DL-0.82	25.7	155	<DL	<DL-<DL	0.7	<0.001
FSH (mIU/mL)	0.5	132	<DL	<DL-0.66	46.8	154	<DL	<DL-<DL	0.0	<0.001
Inhibin B (pg/mL)	11	134	44.0	33.9–58.3	99.2	159	<DL	<DL-11.8	26.0	<0.001
INSL3 (ng/mL)	0.01	132	0.29	0.25–0.34	100	20	0.18	0.17–0.23	100	<0.001

DL: detection limit.

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4.8%–9.5%, P: 4.7%–6.0%, LH: 7.2%–26.0%, FSH: 5.4%–6.7%, Inhibin B: < 5.6%, and INSL3: 6%–15.0% in the mean inter-assay coefficients of variations.

The median concentrations of T, LH, FSH, Inhibin B, and INSL3, which indicate androgen activity, were significantly higher in males than in females (Table 2).

4) Relationship between 2D/4D and sex hormones

No significant differences were observed in the hormone levels of children who sent back photocopies for 2D/4D and those who did not (Table 3).

A multivariate regression model showed that 2D/4D negatively correlated with INSL3 only in males. Regarding the other sex hormones in both males and females, no correlations were observed with 2D/4D (Table 4). The application of 0.32 ng/mL of INSL3 from the receiver operating characteristic curve as a cut-off value revealed that 2D/4D was significantly higher in males with <0.32 ng/mL of INSL3 ($p<0.01$) (Fig. 2). This result indicated that 2D/4D could be affected by prenatal Leydig cell function.

Table 3. Sex hormones in cord blood and 2D/4D.

	Males					Females				
	2D/4D (+)		2D/4D (-)			2D/4D (+)		2D/4D (-)		
	n	50th Min Max	n	50th Min Max	p-value	n	50th Min Max	n	50th Min Max	p-value
Testosterone (pg/mL)	45	90.9 12.2 483	90	101 5.45 620	0.240	69	64.9 12.3 457	87	71.3 6.25 168	0.255
Estradiol (ng/mL)	45	4.05 1.91 26.6	90	5.38 0.01 33.5	0.200	72	4.86 1.66 31.2	87	4.42 1.44 17.4	0.143
Progesterone (ng/mL)	45	183 13.7 455	90	234 0.43 471	0.378	72	201 6.25 467	87	216 8.86 514	0.457
T/E	45	21.7 2.05 52.1	90	17.5 2.73 21839	0.477	69	15.7 1.9 47.6	87	15.7 0.68 40.3	0.424
LH (mIU/mL)	45	<DL <DL 2.39	87	<DL <DL 3.37	0.986	70	<DL <DL 0.61	85	<DL <DL <DL	0.263
FSH (mIU/mL)	45	<DL <DL 1.43	87	<DL <DL 1.89	0.765	72	<DL <DL <DL	82	<DL <DL <DL	N/A
Inhibin B (pg/mL)	44	43.3 <DL 90.6	90	<DL <DL 104	0.957	72	<DL <DL 76.6	87	<DL <DL 65.7	0.947
INSL3 (ng/mL)	44	0.28 0.1 0.48	88	0.29 0.07 0.75	0.454		N/A N/A		N/A N/A	

N/A: not applicable.

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Table 4. Relationship between 2D/4D and sex hormones in cord blood.

Hormone levels	Total			Males			Females		
	n	B (95%CI)	R^2	n	B (95%CI)	R^2	n	B (95%CI)	R^2
T (pg/mL)	114	-0.021 (-2.449, 1.956)	0.113	45	-0.209 (-8.080, 1.754)	0.060	69	0.151 (-0.835, 3.909)	0.214
E (ng/mL)	117	-0.070 (-2.893, 1.257)	0.111	45	-0.051 (-4.956, 3.625)	0.022	72	-0.104 (-3.346, 1.219)	0.180
P (ng/mL)	117	0.036 (-1.323, 1.977)	0.107	45	-0.020 (-4.461, 3.971)	0.020	72	0.078 (-1.114, 3.647)	0.175
T/E	114	0.010 (-2.259, 2.514)	0.113	45	-0.138 (-6.331, 2.650)	0.036	69	0.200 (-0.440, 5.190)	0.228
LH (mIU/mL)	115	0.017 (-2.167, 2.610)	0.104	45	0.207 (-1.335, 5.346)	0.055	70	0.126 (-6.313, 21.64)	0.180
FSH (mIU/mL)	117	-0.038 (-3.696, 2.448)	0.105	45	0.180 (-2.162, 7.177)	0.048		N/A	N/A
INSL3 (ng/mL)		N/A N/A	N/A	44	-0.377* (-30.17, -2.318)	0.145		N/A N/A	N/A
Inhibin B (pg/mL)	116	-0.139 (-2.238, 0.331)	0.124	44	-0.068 (-5.877, 3.891)	0.024	72	-0.082 (-1.387, 2.732)	0.172

*: $p<0.05$,
N/A: not applicable.

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Discussion

In the present study, the ratio of the digit length of the 2nd finger to that of the 4th finger, which has been used as an easily measurable and stable anthropometric index of prenatal exposure to androgens, was calculated in school-aged children, and sex hormone levels in cord blood samples were then measured. The levels of sex hormones indicating androgen activity in cord blood were significantly higher in males than in females. 2D/4D was significantly higher in females than in males, and negatively correlated with INSL3 only in males.

The biosynthesis of testosterone hypothetically occurs at a gestational age of 9 weeks, whereas 2D/4D dimorphism appears as early as at 14 weeks of gestation [7, 8], which indicated that early levels of sex hormones can influence 2D/4D. A previous study reported that 2D/4D reflected a genetic background subjected to a given level of exposure to prenatal androgens [1]. A gestational peak in testosterone production due to the development of Leydig cells occurred between 14 and 18 weeks. Thus, compelling evidence currently shows that 2D/4D is affected by prenatal exposure to androgens in humans.

In the present study, we used the mean 2D/4D value in both hands as a representative value of each participant, as previously reported, because the influence of the stronger side of the hands in 2D/4D on correlations with any factors has not yet been established and the intraclass correlation coefficient (1, 2) for right and left 2D/4D measurements was 0.720 (95% confidence interval: 0.627–0.789). 2D/4D in the left hand negatively correlated with INSL3 (β = -0.414, p = 0.0125), whereas 2D/4D in the right hand was not correlated with INSL3 (β = -0.268, p = 0.1093). We attributed these differences in 2D/4D between the right and left hands to various factors including measurement errors, the relatively small sample size, and the

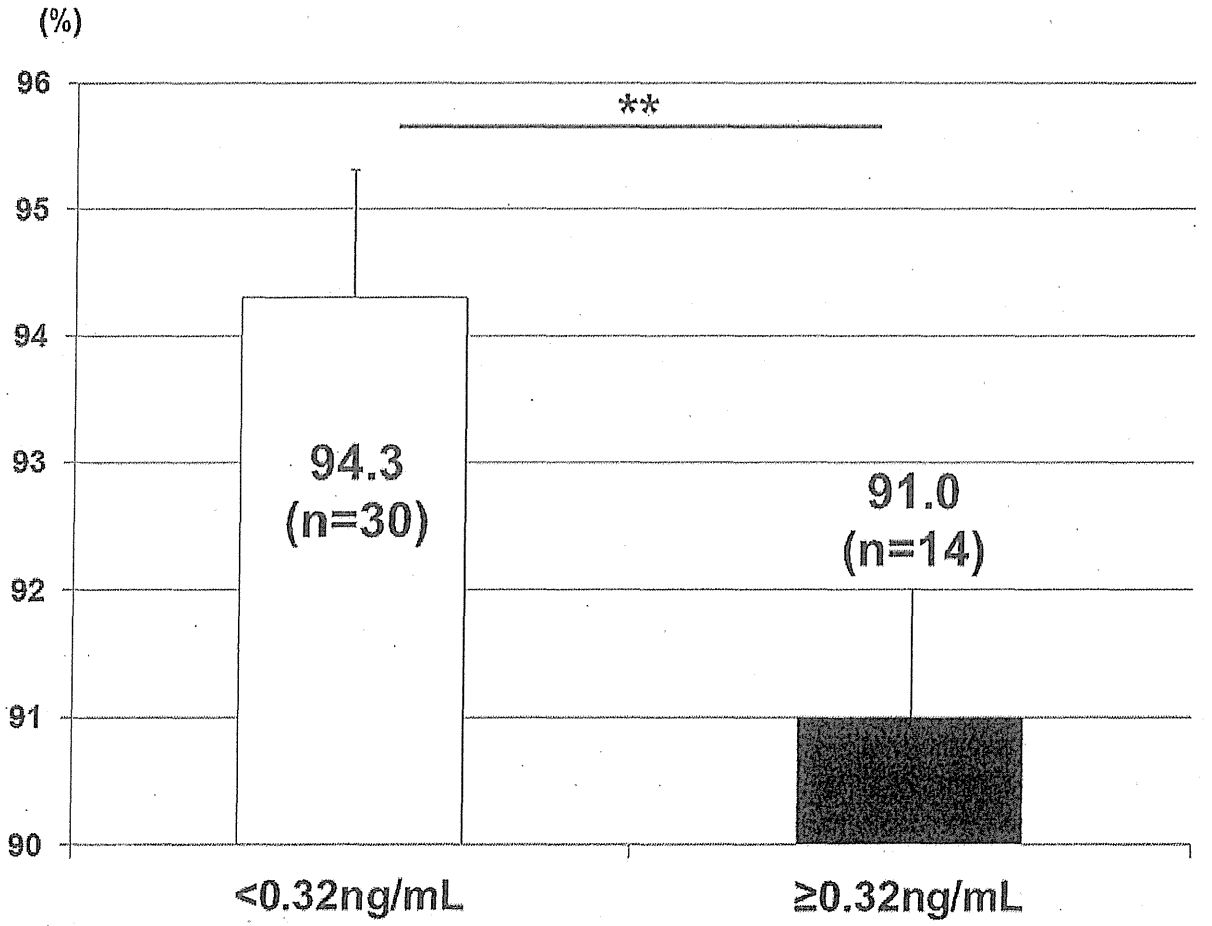

Fig 2. 2D/4D and INSL3. 2D/4D was significantly higher in males with <0.32 ng/mL of INSL3 in cord blood ($p<0.01$). **: $p<0.01$.

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limitations associated with physical measurements. Thus, we considered it reasonable to use the mean value of 2D/4D as a representative value of each participant.

In the present study, no correlation was observed between the level of testosterone in cord blood and 2D/4D. This result was compatible with previous findings, which demonstrated that the concentration of testosterone in cord blood could not predict 2D/4D[18]. Furthermore, a previous study suggested that amniotic fluid, but not cord blood, was the best candidate for investigating the effects of early fetal exposure to androgens [19]. These findings taken together with our results indicated that testosterone in cord blood did not influence 2D:4D or reflect fetal exposure during the critical period of digit development at approximately 14 weeks of gestation. The measurement of sex hormones in cord blood may be affected by obstetric and maternal factors, such as prematurity, labor onset, placental weight, intrauterine infection, and preeclampsia, which have not yet been established in detail [9].

INSL3 levels in cord blood samples correlated with 2D/4D in males. INSL3 is constitutively produced by Leydig cells in the fetal testis, not by other organs, after sex determination [20], and is a gender-specific fetal hormone. The fetal testis is established at approximately 7 weeks of pregnancy and the *INSL3* gene in fetal Leydig cells is detectable by 8–10 weeks of pregnancy

in humans [21]. This period of transition from the first to the second trimester is important for development, and is very vulnerable to a range of endocrine-disrupting insults to male reproductive development. Thus, the detection of INSL3 in fetal blood during mid-gestation reliably indicates a male fetal gender [21]. INSL3 in cord blood reflects prenatal Leydig cell function, which serves in the production of testosterone, and may also reflect androgen exposure during the important developmental window of earlier pregnancy for the digits as well as male reproductive development. In the present study, a correlation was observed between INSL3, but not testosterone, in cord blood and 2D/4D, and a previous study also demonstrated that 2D/4D was significantly related to adult testosterone levels and the presence of testosterone deficiency syndrome [22].

No correlation was noted between other hormones with androgen activity, such as LH, FSH, and Inhibin B, and 2D/4D. This may have been due to more than 50% of the stored cord blood samples being below the detection limit for LH and FSH. Therefore, more sensitive kits are needed to measure LH and FSH. Since Inhibin B reflects Sertoli cell function, its levels may not directly indicate androgen exposure *in utero* for digit development. Furthermore, a previous study using mice showed that receptors for androgen and estrogen were particularly located in the 4^{th} digit and the growth of this digit was stimulated by androgen, but arrested by estrogen[23]. Although it has already been reported that 2D/4D cannot be determined by prenatal testosterone alone and the balance between prenatal testosterone and prenatal estrogen is another important factor in fetal digit development [24], our results showed that T/E in cord blood did not correlate with 2D/4D. Thus, the present study revealed that only prenatal Leydig cell function, indicating early exposure during gestation to androgens, could be implicated in 2D/4D.

As one of factors that affects sex hormones during gestation, endocrine-disrupting chemicals, e.g. phthalates, dioxins, polychlorinated biphenyls (PCBs), and perfluorinated alkyl acids (PFAAs), have been shown to induce a broad spectrum of toxic effects on the reproductive system and genital development in the prenatal period in humans. Our cohort study already demonstrated that maternal exposure to phthalates reduced the levels of T/E, P, inhibin B, and INSL3 in cord blood, suggesting that exposure to DEHP *in utero* may have adverse effects on both Sertoli and Leydig cell development in males [25]. Previous studies also revealed that other endocrine-disrupting chemicals affected the hormonal environment during the prenatal period in humans. Cao et al. demonstrated that maternal exposure to dioxins decreased T and E in cord blood [26]. Furthermore, Hsu et al. showed that maternal exposure to PCBs decreased T/E in boys at puberty [27]. Regarding PFAAs, Vested et al. reported that maternal exposure to perfluorooctane sulfonate (PFOS) during gestation decreased the concentration and counts of sperm and increased LH and FSH levels in males after puberty, suggesting that maternal exposure to PFOS may affect semen quality and reproductive hormone levels in adult human males. Thus, maternal exposure to endocrine-disrupting chemicals influences sex hormones during gestation, as demonstrated by anti-androgen activity in males. These findings indicate that maternal exposure to endocrine-disrupting chemicals affects sex hormone levels during gestation and induces physical changes to the digits of children. An animal study has already showed that prenatal exposure to low doses of endocrine-disrupting chemicals induced feminized digit ratios in male rats[28]. Further studies are warranted to confirm this in humans.

Polymorphisms in androgen receptors (AR) may also affect sensitivity to androgen exposure in 2D/4D. AR are produced by the AR gene, which is located on the X-chromosome and repeats the nucleotide sequence CAG on exon 1. Furthermore, the number of CAG repeats varies in length among individuals and code for the length of a polyglutamine stretch on the N-terminal domain of AR. Although previous studies revealed that there was no evidence for a