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Second trimester maternal serum biomarkers and the risk of cerebral palsy

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[illegible]t is already known about this topic? What does this study add?

- [illegible]Abnormal second-trimester levels of biomarkers in maternal serum are associated with later cerebral [illegible]alsy (CP).
- [illegible]Early pregnancy factors have potential importance in causal pathways to CP.
- [illegible]Causal pathways involving placental dysfunction and genetic syndromes may be implicated.

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ABSTRACT

Aims: To investigate whether second trimester screening (T2MSS) biomarkers are associated with cerebral palsy (CP) and identify CP characteristics associated with abnormal biomarker levels.

Method: In this retrospective case-control data linkage study, we linked mothers of 129 singleton CP cases from a population register to their 2TMSS records and selected 10 singleton pregnancy controls per case (n=1290). We compared mean and abnormal levels of alpha-fetoprotein (AFP), beta subunit of human chorionic gonadotrophin (β-hCG), estriol (UE3) and inhibin between cases and controls and within CP subgroups.

Results: Compared to control pregnancies, CP pregnancies had higher mean levels of AFP (1.10 vs 1.01 MoM, p=0.01) and inhibin (1.10 vs 0.98 MoM, p=<0.01). CP pregnancies were 2.5 times more likely to be associated with high levels of AFP (OR 2.52 [95% CI 1.30, 4.65]; p<0.01) and 2.6 times for inhibin (OR 2.63 [95% CI 1.37, 4.77]; p<0.01), and 6.8 times when AFP and inhibin were both elevated (OR 6.75 [95% CI 2.41, 18.94]; p<0.01). In CP cases, high AFP and high inhibin levels were associated with preterm birth and low birthweight.

Interpretation: Abnormal second-trimester biomarker levels suggest abnormal placentation plays a role in the causal pathway of some CP cases.

INTRODUCTION

Cerebral palsy (CP) describes disorders of movement and posture that occur because of non-progressive disturbance to the developing brain.[1] Due to the diversity of timings and types of disturbance, the etiologies of CP are complex. In Australia, 94% of CP cases are deemed to be prenatally or perinatally acquired.[2] Although premature birth is an important factor along one of the most common causal pathways to the development of CP, 60% of CP cases in Australia are born at term and the broad pathways to CP are more heterogeneous.[2,3]

Second trimester maternal serum screening (2TMSS) has been used to provide an estimate of risk for fetal neural tube defects and chromosomal abnormalities based on serum levels of alpha-fetoprotein (AFP), beta subunit of human chorionic gonadotropin (β-hCG), unconjugated estriol (uE3) and inhibin A.[4] AFP is a glycoprotein produced by the developing fetus that reaches its peak in the second trimester, and has been implicated in the regulation of estrogen, neuronal differentiation and immunomodulation.[5,6] Peptide hormone, β-hCG, is involved in many physiological processes during pregnancy, including implantation and formation of the maternal fetal circulation.[7,8] uE3 is produced by the fetal liver and plays a role in maintaining the integrity of the maternal-fetal circulation.[9] Considered a marker of placental function, inhibin A is a glycoprotein hormone produced by the feto-placental unit in early pregnancy.[10]

Individual biomarker levels recorded in the second trimester have also been associated with pregnancy complications that are known to increase the risk of CP.[3] Elevated AFP has been associated with preterm delivery, fetal death and pre-eclampsia, whereas both high and low levels of hCG have been associated with fetal growth restriction and premature birth.[11] Recent literature supports an association between low levels of UE3 and adverse outcomes such as fetal growth restriction and spontaneous abortion.[12] A relationship also exists between high levels of inhibin and both pre-eclampsia and preterm delivery,[13] whereas low inhibin in the first trimester has been associated with non-viable pregnancies.[14,15]

Based on the biological functions of 2TMSS biomarkers and their known associations with adverse pregnancy outcomes, we hypothesised an association between second trimester biomarkers and subsequent development of CP. We aimed to add to the knowledge of causal pathways of CP through investigating these associations.

METHODS

Study design and setting

This retrospective case-control study was conducted at the Murdoch Children's Research Institute in Melbourne, Australia. It involved data linkage between the Victorian Cerebral Palsy Register and the database of the Maternal Serum Screening laboratory at Victorian Clinical Genetics Services. Established in 1987, the population-based Victorian Cerebral Palsy Register holds data on persons with CP born or receiving health services in the Australian state of Victoria from 1970 onwards. Medical records of the two tertiary paediatric institutions in Victoria are the main sources of case identification and data collection. To optimise case ascertainment, an opt-out consent process is in place. Case status is based on the latest consensus definition and criteria for inclusion.[1,16] Victorian Clinical Genetics Services has collected 2TMSS data since 1996. On average, 10 percent of pregnant mothers in Victoria underwent second trimester screening during the study period of 1996 to 2017.

Study groups

The sampling frame for cases was all singleton CP cases registered with the Victorian Cerebral Palsy Register and born in Victoria between 1996 and 2017. This time frame was selected to encompass children born to mothers who had the opportunity to participate in 2TMSS in Victoria. Post-neonatal causes of CP

were excluded, as their CP was deemed unlikely to be related to mid trimester pregnancy factors. Records with missing maternal first and last names were also excluded.

The data linkage process is shown in Figure 1. The maternal serum screening database included 123,692 complete 2TMSS results at the time of linkage. Initial data linkage was performed using maternal name, maternal date of birth or age and estimated date of delivery. Potential matches were checked manually by the Register data custodian (SR) using additional contact details and/or medical insurance number.

Ten control 2TMSS records were extracted for each linked CP case, comprising the five records immediately before and after the case record.

Marker levels

The 2TMSS screening program has been described previously.[17] Serum markers were collected between 14 and 20^{+6} weeks gestation and were measured on a Brahms Kryptor analyser for AFP and β-hCG and on a Beckman Coulter Access2 analyser for uE3 and inhibin. All biomarkers were adjusted for gestation and maternal weight.[18] Each marker value was converted to a multiple of the population median (MoM), representing the extent to which the screening result differed from the median value of the population.[19]

Potential predictor variables

Potential predictor variables were extracted from the VCPR. These included maternal age (<25, 25-34, 35+ years), previous pregnancies and births (0, 1-2, 3+), indicator of socio-economic status (high or low, based on dichotomised Socio-Economic Indexes for Areas deciles for Victoria), birth gestation (<28, 28-31, 32-36, 37+ weeks), birth weight (<1000, 1000-1499, 1500-2499, 2500+ grams), weight for gestation (birthweight under or over the 10^{th} centile for birth gestation and sex), 5-minute Apgar (0-3, 4-7, 8-10), neonatal nursery admission, sex, CP subtype (spastic monoplegia/hemiplegia, spastic diplegia/triplegia, spastic quadriplegia, ataxia, dyskinesia, hypotonia), Gross Motor Function Classification System (GMFCS) level, presence of a birth defect (none, cerebral, non-cerebral), and MRI classification (maldevelopments, predominant white matter injury, predominant grey matter injury, miscellaneous, normal; based on the CP MRI Classification System).[20]

Data analysis

To explore potential selection bias in the CP sample and generalisability to all CP, we used χ-square tests to compare key maternal, perinatal, and child-related variables across linked and unlinked CP groups.

Marker levels were first analysed as continuous variables. As the distributions of marker MoMs were positively skewed, each marker was log-transformed to reduce skewness and approximately conform to normality. Student's *t*-tests were performed on the log-transformed data to compare the mean value for each marker between CP cases and non-CP controls. For presentation of the results, each mean value and its associated 95% confidence limits were back-transformed to obtain the geometric means and confidence intervals. One-way analysis of variance (ANOVA) was performed to investigate the strength of association between the geometric means of each biomarker across specific maternal, perinatal and child-related variables in CP cases.

To evaluate the association between high and low levels of each biomarker and CP, marker MoMs were also analysed as categorical variables. A low biomarker level was defined as a MoM value under the 5^{th} centile cut-off, whereas high MoM levels were defined as a biomarker level over the 95^{th} centile cut-off. The cut-off values in MoMs for the 5^{th} and 95^{th} centiles were based on the non-CP control group for this study (Table II). Logistic regression was used to explore the association of CP with high and low biomarker levels, with odds ratios quantifying the relative odds of low (<5^{th} centile) and high (>95^{th} centile) biomarker levels for CP cases

compared to non-CP controls. χ-square analyses were used to compare biomarker levels across the same CP subgroups as for the continuous data.

Receiver-operating characteristic (ROC) analyses were conducted to assess the utility of the biomarkers, both separately and combined, in predicting the subsequent development of CP.

Analysis was conducted in Stata 15.1 (StataCorp LLC, College Station, TX, USA).

Ethics approval

Ethics approval for this study was received from the Royal Children's Hospital Human Research Ethics Committee in April 2018 (HREC #3805).

RESULTS

Data were available for 129 CP cases and 1290 non-CP controls. The CP cases linked to corresponding second trimester screening records represented 7% of the total sample sent for linkage (Supplementary Figure), consistent with the 10% uptake of 2TMSS across the Victorian population over the study period. In evaluating generalisability to the CP population in Victoria, we compared characteristics of the 129 linked CP singleton cases to the 1630 eligible records from the CP register that were unable to be linked(Table I). Fewer matches with 2TMSS records were noted amongst mothers who were 35+ years of age at the birth of their child with CP and amongst individuals with CP that were born between 28 and 31 weeks' gestation. All other maternal, perinatal and child-related factors were observed to be similar between the linked and unlinked CP groups.

AFP and CP

AFP MoMs in CP cases were positively skewed in comparison to non-CP controls (Supplementary Figure). On average, AFP MoMs were higher in CP case pregnancies (geometric mean 1.10 [95% CI 1.02, 1.20]) than in non-CP control pregnancies (geometric mean 1.01 [95% CI 0.99, 1.03]; p=0.01 for difference; Table III). Amongst CP cases, the highest mean AFP MoMs were associated with a birthweight under 1000 grams (1.49 MoM) and birth before 28 weeks' gestation (1.45 MoM; Supplementary Table I). The odds of having low AFP levels were similar between cases and controls (7.0% vs 4.7%; OR 1.54 [95% CI 0.65, 3.22]; p=0.24; Table IV). In contrast, proportionally more mothers of CP cases had high AFP levels compared to mothers of non-CP controls (11.6% vs 5.0%; OR 2.52 [95% CI 1.30, 4.65]; p<0.01; Table IV). In CP case pregnancies, high proportions (designated as >25%) of raised AFP levels were most frequently seen in association with birth <28 weeks' gestation (35%), birthweight <1000 grams (36%) and non-cerebral birth defects (29%; Supplementary Table II).

β-hCG and CP

β-hCG MoMs showed a similar distribution in cases and controls (Supplementary Figure). Further, no meaningful difference was observed between β-hCG levels in CP cases and non-CP controls when comparing either their geometric means or the proportions in the high or low MoM ranges (Table III; Table IV). In contrast to the lack of difference seen in the whole group data, specific CP subgroups had β-hCG levels that were substantially different to those expected. High mean β-hCG MoMs were seen in association with birthweights <1000g (1.45 MoM) and ataxic CP (1.67 MoM; Supplementary Table I). CP cases with brain maldevelopment on MRI (0.87MoM) and cerebral birth defects (0.88 MoM) were noted to have low mean β-hCG MoMs (Supplementary Table I). Low β-hCG MoMs were seen more often than expected (in >25% cases) in association with 3+ previous births to the mother (27%; Supplementary Table II).

uE3 and CP

uE3 MoMs in CP cases were negatively skewed in comparison to non-CP controls (Supplementary Figure). There was little difference in mean uE3 MoMs between CP cases (geometric mean 0.98 [95% CI 0.90, 1.03]) and non-CP controls (geometric mean 1.00 [95% CI 0.98, 1.02]; p=0.26 for difference; Table III) but the odds of a low uE3 was higher for CP cases than non-CP controls (10.1% vs 4.8%, OR 2.22 [95% CI 1.09, 4.23], p=0.01; Table IV). High uE3 levels were seen more often in CP case pregnancies (9.3% vs 5.0%, OR 1.93 [95% CI 0.92, 3.74], p=0.04; Table IV). The lowest mean uE3 levels in CP cases were seen in association with 3+ previous births to the mother (0.72 MoM) and evidence of brain maldevelopment on the child's MRI (0.72 MoM; Supplementary Table I). The same CP subgroups had the highest proportions of uE3 MoMs in the low range (27% and 29% respectively; Supplementary Table II). The highest mean uE3 MoM levels were observed for CP cases born to primigravid women (1.18 MoM), a birth gestation between 28-31 weeks (1.18 MoM) and hypotonic CP (1.29 MoM; Supplementary Table I). Within CP subgroups, 26% of primigravid mothers and 25% of children with birthweights between 1000 and 1400g were in the high uE3 MoM range (Supplementary Table II).

Inhibin and CP

Inhibin MoMs in CP cases were positively skewed in comparison to non-CP controls (Supplementary Figure). The mean MoM level of inhibin was higher in CP cases (geometric mean 1.10 [95% CI 0.99, 1.22]) compared to non-CP controls (geometric mean 0.98 [95% CI 0.95, 1.00]; p<0.01 for difference; Table II). In CP cases, the highest inhibin levels were associated with birth gestation <28 weeks (1.43 MoM) and with non-cerebral birth defects (1.51 MoM). The lowest mean levels of inhibin were seen in CP cases with hypotonia (0.83 MoM) and those functioning at GMFCS Level of IV (0.92 MoM; Supplementary Table I). Compared to 5.1% of controls, 12.4% of cases had a high inhibin level (OR 2.63 [95% CI 1.37, 4.77]; p<0.01; Table IV). Higher proportions of CP cases (designated >25%) with birth gestation <28weeks (29%) and birthweight <1000g (36%) were associated with a high inhibin level (Supplementary Table II). There was weak evidence to support an association between low inhibin levels and CP cases compared to non-CP control (7.8% vs 4.9%, OR 1.64 [95% CI 0.73, 3.32]; p=0.16; Table IV). Subsequently no high proportions (>25%) were seen in the low MoM range across CP subgroups.

Markers combined

Given the findings for AFP and inhibin in our study, logistic regression was used to further explore the association with CP when both AFP and inhibin were in the high range. A pregnancy was 6.8 times more likely to result in a subsequent diagnosis of CP when both AFP and inhibin MoM levels were high (OR 6.75 [95% CI 2.41, 18.94]; p<0.01 for difference; Table IV).

Predictive utility of biomarkers

All markers showed poor predictive ability on ROC analysis (AFP area under the curve (AUC) = 0.55; β-hCG AUC=0.50; uE3 AUC =0.47; inhibin AUC=0.56). High levels of AFP and inhibin together produced an AUC of 0.52.

DISCUSSION

In this retrospective case-control study of 129 pregnancies resulting in the birth of a child later diagnosed with CP, we showed that compared to 1290 control pregnancies, the occurrence of CP was 6.8 times more likely when AFP and inhibin were both high. Our study also showed associations between biomarkers and adverse pregnancy outcomes that are known to increase the risk of CP.[2,3]

Given the heterogeneous and complex etiology of CP, it is unlikely that one test could predict all cases of CP. Although the literature documents associations between AFP, hCG, uE3 and inhibin and adverse pregnancy outcomes, these markers have shown inconclusive prognostic ability and utility as diagnostic tests for these outcomes.[21-23] This was consistent with our findings that although elevated levels of AFP and

inhibin showed an association with the subsequent occurrence of CP, all biomarkers showed poor predictive ability for CP on ROC curve analysis. Nonetheless, our data provide evidence that some cases of CP originate from processes that are present in the second trimester, and well before labour and delivery.

High AFP and risk of subsequent CP

CP pregnancies were 2.5 times more likely to have high second trimester levels of AFP. In further analyses, more than one third of mothers of CP cases born <28 weeks' gestation, or with a birthweight <1000g, recorded a high AFP level. These findings suggest that the association observed in our study between high AFP levels and CP predominantly involves the prematurity/low birthweight pathway to CP. The association between AFP levels >2.0 MoM and placenta-mediated adverse pregnancy outcomes, including preterm delivery, has been described previously.[24-26] Waller et al. reported that women with an AFP MoM >2.5 were 8.7 times more likely to have a preterm infant.[27] Further to this, Tancrède et al. found that AFP MoMs >2.0 were associated with the combined outcomes of preterm birth, birthweight under the 10th centile for gestation, and placenta-mediated complications.[28] A recent meta-analysis showed an increased risk of delivering an infant with a birthweight <10th centile when AFP was >1.0 MoM, the risk increasing withincreasing AFP levels.[25] Despite these findings, and in contrast to our study, an earlier investigation of antecedent factors in 59 cases of CP born <32 weeks gestation failed to establish an association between AFP levels and CP case status, with only one CP pregnancy having an AFP MoM >2.0.[29]

The mechanisms that link elevated maternal serum AFP with adverse pregnancy outcomes are not known. AFP is thought to originate in the fetal liver, although production in the placenta has also been detected.[30] Elevated AFP in maternal serum might result from either increased AFP production in the fetus or increased fetal-maternal transfer of AFP due to compromised integrity of the placental barrier. Both mechanisms could occur together, such as in the setting of placental inflammation or infection, which could directly increase placental permeability whilst triggering fetal production of AFP as an acute phase reactant.[31] Interestingly, studies in a rat model of placental inflammation found that placental inflammation increased both pregnancy complications and maternal AFP levels without evidence of increased fetal AFP production, favouring increased placental permeability as the primary mechanism.[32] Increased placental permeability is a phenomenon that develops gradually, potentially starting with maternal vascular malperfusion caused by poor trophoblast uterine invasion and impaired transformation of the spiral arteries in the first trimester.[33] Subsequent placental dysfunction induced by placental inflammation and vascular endothelial injury increases the risk of maternal hypertension/pre-eclampsia in the mother and growth restriction in the fetus.[33-35] These adverse pregnancy outcomes increase the likelihood of spontaneous preterm labour or induced preterm delivery and expose the immature neonate to an increased risk of brain injury.

Our study noted 29% of children with CP and non-cerebral birth defects also had a high AFP level. Children with CP have a higher rate of cerebral and non-cerebral congenital abnormalities with the most common non-cerebral defects being cardiac defects.[36-38] Conditions where fetal blood or cerebrospinal fluid is in direct contact with amniotic fluid, such as open neural tube defects and gastroschisis, are associated with high levels of AFP in both amniotic fluid and maternal serum.[6,39] Therefore, results may reflect the cases of CP with multiple congenital abnormalities as part of syndromic diagnosis.

β-hCG and risk of subsequent CP

Although mean β-hCG levels were similar between cases and non-CP controls in our study, further analysis showed an association between CP cases and β-hCG levels at both high and low extremes. CP cases were 1.5 times more likely to have a low β-hCG low level and 1.3 times more likely to have a high β-hCG level. Our β-hCG results are similar to the findings of Eskild et al. who reported an odds ratio (OR) for cerebral palsy of 1.42 (95% CI: 0.94, 2.16) per log-transformed unit of maternal β-hCG in the second trimester, after adjustment for maternal age, week of gestation and parity.

The relationship between β-hCG and adverse pregnancy outcomes remains unclear. Some studies have shown an association between either first or second trimester hCG levels and placenta-mediated pregnancy complications that increase the risk of CP.[3,40] In our study, low levels of β-hCG were associated with multiparity and cerebral maldevelopment whereas high levels were seen in relation to birthweight <1000g and ataxic CP. An association between both high and low hCG levels has been reported in relation to preterm birth and low birthweight infants (<2500g). The conclusion from a subsequent systematic review was that high second trimester hCG MoMs were positively associated with maternal pre-eclampsia and small for gestational age infants.[40] Given the role of hCG in implantation and formation and maintenance of the maternal-fetal circulation, our findings are likely related to CP cases that have placental dysfunction as part of their causal pathway.

High and low uE3 and risk of subsequent CP

CP pregnancies were 2.2 times more likely to have a low uE3 level. Our study found higher than expected proportions of CP case pregnancies had second trimester uE3 MoM levels in the high and low ranges. Low uE3 MoM levels were associated with maternal multiparity and brain maldevelopment in CP cases, whereas high levels were associated with primigravid women and CP cases with a birthweight between 1000 and 1400g. Our result contrast with previous literature, where low uE3 levels were reported to be associated with low birthweight.[41-43] Estriol (uE3) is the primary form of estrogen produced by the placenta and detectable in the maternal serum during pregnancy.[9] Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) are the precursors for estriol, are released by the fetal adrenal glands, and are metabolised by the fetal liver before being converted to uE3 by the placenta.[9,44] This process underlies the role of uE3 in pregnancy, with low levels being associated with congenital abnormalities such as inborn errors of metabolism and adrenal insufficiency.[45] Given the association between low uE3 levels and congenital abnormalities, our study findings may reflect CP cases with congenital abnormalities as part of their causal pathway.

High inhibin and risk of subsequent CP

CP pregnancies were 2.6 times more likely to have recorded high levels of inhibin. We found that high levels (>2.20 MoM) of inhibin were seen in CP cases born <28 weeks' gestation or with birthweights <1000g. High second trimester inhibin levels have previously been associated with placenta-mediated complications known to increase the risk of CP.[3] Singnoi et al. noted an association between high second trimester inhibin levels and adverse outcomes such as pre-eclampsia and fetal growth restriction, with no reported association with preterm birth.[46] In contrast, Yue et al. reported a significant association between high inhibin levels and medically indicated preterm birth, an observation also noted by others,[13,47] who concluded that the relationship between preterm birth and inhibin was secondary to other placenta-mediated complications that may increase the risk of elective or emergency premature delivery.

The relationship between high second trimester inhibin levels and placental dysfunction is thought to be due to abnormal proliferation of early trophoblasts resulting in impaired development of the maternal fetal circulation.[10,48] Abnormal formation of early placental vasculature can then lead to increased permeability of the maternal fetal circulation as well as pathological placental findings that have been reported to increase the risk of CP.[35,49]

Limitations

This study had some limitations. First, as linkage matched records using maternal demographics and expected date of delivery, some matches may have been missed due to missing maternal data on the VCPR. Second, we were unable to exclude control pregnancies that resulted in a stillbirth, neonatal death, alternate chromosomal diagnosis or congenital malformation because the screening service does not collect outcome data for all pregnancies. Third, data on non-CP related characteristics were only available for the CP cases

and not for controls; therefore, we could only make comparisons between CP subgroups. Lastly, given that most pregnant women underwent screening during the first trimester,[50] and only 10% had screening in the second trimester, our study groups may not be representative of the wider population, and findings may not be generalisable to all CP.

CONCLUSION

In this study, high second trimester AFP and inhibin levels were associated cases later diagnosed with CP. These findings suggest that early placental dysfunction may be involved in the causal pathways of some cases of CP.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

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Figure Legend

Figure 1: Data Linkage process.

Table I: Characteristics of CP singleton cases matched to 2nd trimester 2TMSS data and compared with eligible but unmatched CP singletons born in Victoria, 1996-2017

	Linked CP cases	Unlinked CP cases	*p*-value
MATERNAL FACTORS			
Maternal age, n (%)			0.09
<25	19 (15)	243 (17)	
25-34	88 (69)	858 (60)	
35+	20 (16)	333 (23)	
Missing	2	196	
Previous pregnancies, n (%)			0.76
0	34 (33)	408 (34)	
1-2	44 (43)	549 (45)	
3+	25(24)	254 (21)	
Missing	26	419	
Previous births, n (%)			0.40
0	58 (52)	633 (45)	
1-2	43 (38)	622 (45)	
3+	11 (10)	134 (10)	
Missing	17	241	
Socio-economic status, n (%)			0.61
Low	61 (48)	757 (50)	
High	66 (52)	745 (50)	
Missing	2	128	
PERINATAL FACTORS			
Birth gestation, n (%)			0.02
<28 weeks	17 (13)	157 (10)	
28-31 weeks	5 (4)	206 (13)	
32-36 weeks	20 (16)	220 (13)	
37+ weeks	87 (67)	1027 (64)	
Missing	0	20	
Birthweight, n (%)			0.56
<1000g	14 (11)	146 (10)	
1000-1499g	8 (7)	147 (10)	
1500-2499g	21 (17)	270 (18)	
2500+g	81 (65)	909 (62)	
Missing	158	5	
Weight for gestation, n (%)			0.44
<10th centile (SGA)	26 (21)	267 (18)	
>10th centile	98 (79)	1203 (82)	
Missing	5	160	
Apgar @ 5min, n (%)			0.11
0-3	13 (11)	88 (7)	
4-7	21 (19)	309 (24)	
8-10	79 (70)	901 (69)	
Missing	16	332	
Neonatal care, n (%)			0.57
Nursery admission	89 (71)	1035 (69)	
No nursery admission	36 (29)	470 (31)	
Missing	4	125	
CHILD-RELATED FACTORS			
Sex, (n %)			0.14
Male	83 (64)	940 (58)	
Female	46 (36)	690 (42)	
Missing	0	0	
CP subtype, n (%)			0.60
Spastic monoplegia/hemiplegia	42 (33)	562 (35)	
Spastic diplegia/triplegia	36 (28)	454 (28)	
Spastic quadriplegia	28 (22)	315 (20)	
Ataxia	4 (3)	50 (3)	
Dyskinesia: athetoid/dystonic	8 (7)	158 (10)	
Hypotonia	8 (7)	63 (4)	
Missing	28	3	
GMFCS level, n (%)			0.51
I	47 (37)	534 (34)	
II	37 (29)	453 (28)	
III	11 (8)	150 (10)	
IV	10 (8)	200 (13)	
V	23 (18)	240 (15)	
Missing	0	32	
Birth defects			0.74
None	99 (77)	1226 (75)	
Cerebral	19 (15)	224 (14)	
Non cerebral only	11 (8)	173 (11)	
Missing	0	7	
MRI pattern			0.61
Maldevelopment	17 (14)	159 (14)	
Predominant white matter injury	40 (34)	463 (40)	
Predominant grey matter injury	29 (25)	266 (23)	
Miscellaneous	20 (17)	150 (13)	
Normal	11 (9)	126 (11)	
Missing	12	466	

GMFCS, Gross Motor Function Classification System; SGA, small for gestational age at birth; MRI, Magnetic Resonance Imaging

Table II: Cut-offs for 5th and 95th centiles calculated using study control data

	Non-CP Controls	
Marker	5th centile	95th centile
AFP	0.57	1.83
hCG	0.34	3.06
uE3	0.56	1.70
Inhibin	0.47	2.20

Table III: Mean second trimester 2TMSS marker multiples of median (MoMs) for CP singleton cases compared to non-CP singleton controls.

	CP Cases	Non-CP Controls	*p*-value for difference
Marker	**Geometric mean [95% CI]**	**Geometric mean [95% CI]**	
AFP MoM	1.10 [1.02, 1.20]	1.01 [0.99, 1.03]	0.01
β-hCG MoM	1.00 [0.87, 1.14]	1.00 [0.96, 1.04]	0.96
uE3 MoM	0.98 [0.90, 1.03]	1.00 [0.98, 1.02]	0.26
Inhibin MoM	1.10 [0.99, 1.22]	0.98 [0.95, 1.00]	<0.01

CP, cerebral palsy; AFP, Alpha-fetoprotein; Total hCG, beta subunit of human chorionic gonadotropin; uE3, unconjugated estriol; MoM, multiples of median

Table IV: Frequency of high and low levels of 2TMSS markers in MoMs in singleton CP case pregnancies compared to singleton non-CP control pregnancies

	< 5th centile				>95th centile			
Marker	**Cases n (%)**	**Controls n (%)**	**OR [95% CI]**	***p*-value**	**Cases n (%)**	**Controls n (%)**	**OR [95% CI]**	***p*-value**
AFP MoM	9 (7.0)	60 (4.7)	1.54 [0.65, 3.22]	0.24	15 (11.6)	64 (5.0)	2.52 [1.30, 4.65]	<0.01
β-hCG MoM	9 (7.0)	62 (4.8)	1.49 [0.63, 3.10]	0.28	8 (6.2)	65 (5.0)	1.25 [0.50, 2.69]	0.57
uE3 MoM	13 (10.1)	62 (4.8)	2.22 [1.09, 4.23]	0.01	12 (9.3)	65 (5.0)	1.93 [0.92, 3.74]	0.04
Inhibin MoM	10 (7.8)	63 (4.9)	1.64 [0.73, 3.32]	0.16	16 (12.4)	66 (5.1)	2.63 [1.37, 4.77]	<0.01
AFP & Inhibin MoM	7 (1.0)	0	1		6 (4.7)	10 (1.0)	6.75 [2.41, 18.94]	<0.01

CP, cerebral palsy; AFP, Alpha-fetoprotein; β-hCG, *beta subunit of human chorionic gonadotropin; uE3, unconjugated estriol; MoM, multiples of median; OR, odds ratio*