



ORIGINAL ARTICLE

Genetic and clinical contributions to cerebral palsy: A multi-variable analysis

Michael E O'Callaghan,¹ Alastair H MacLennan,¹ Catherine S Gibson,¹ Gai L McMichael,¹ Eric A Haan,^{2,3} Jessica L Broadbent,¹ Peter A Baghurst,^{2,5} Paul N Goldwater,^{2,4} Gustaaf A Dekker¹ and for the Australian Collaborative Cerebral Palsy Research Group

¹Discipline of Obstetrics and Gynaecology, School of Paediatrics and Reproductive Health, Robinson Institute, ²Discipline of Paediatrics, School of Paediatrics and Reproductive Health, The University of Adelaide, ³SA Clinical Genetics Service, ⁴Department of Microbiology and Infectious Diseases, SA Pathology at Women's and Children's Hospital and ⁵Public Health Research Unit, Women's and Children's Hospital, Adelaide, South Australia, Australia

Aim: This study aims to examine single nucleotide polymorphism (SNP) associations with cerebral palsy in a multi-variable analysis adjusting for potential clinical confounders and to assess SNP–SNP and SNP–maternal infection interactions as contributors to cerebral palsy.

Methods: A case control study including 587 children with cerebral palsy and 1154 control children without cerebral palsy. Thirty-nine candidate SNPs were genotyped in both mother and child. Data linkage to perinatal notes and cerebral palsy registers was performed with a supplementary maternal pregnancy questionnaire. History of known maternal infection during pregnancy was extracted from perinatal databases.

Results: Both maternal and fetal carriage of inducible nitric oxide synthase SNP rs1137933 were significantly negatively associated with cerebral palsy in infants born at less than 32 weeks gestation after adjustment for potential clinical confounders and correction for multiple testing (odds ratio 0.55, 95% confidence interval 0.38–0.79; odds ratio 0.57, 95% confidence interval 0.4–0.82, respectively). Analysis did not show any statistically significant SNP–SNP or SNP–maternal infection interactions after correction for multiple testing.

Conclusions: Maternal and child inducible nitric oxide synthase SNPs are associated with reduced risk of cerebral palsy in infants born very preterm. There was no evidence for statistically significant SNP–SNP or SNP–maternal infection interactions as modulators of cerebral palsy risk.

Key words: case control; cerebral palsy; infection; interaction; pregnancy; SNP.

What is already known on this topic

- 1 Clinical risks for cerebral palsy such as gestational age and infection during pregnancy are well established.
- 2 Single nucleotide polymorphism (SNP) associations with cerebral palsy have previously been reported.
- 3 Most previous reports of SNP associations with cerebral palsy do not adjust for clinical confounders and have not examined SNP–SNP or SNP–clinical interactions.

What this paper adds

- 1 SNPs in the iNOS gene are associated with cerebral palsy in infants born before 32 weeks gestation, after adjustment for clinical confounders.
- 2 Tests for interaction between maternal and child SNPs were not significant after correction for multiple testing.
- 3 Tests for interaction between SNPs and maternal infection during pregnancy were not significant after correction for multiple testing.

Cerebral palsy is defined as 'a group of permanent disorders of the development of movement and posture, causing activity limitation that is attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour, by epilepsy, and by secondary musculoskeletal

problems'.¹ Cerebral palsy is a common paediatric condition affecting approximately 1.5 to more than 4 per 1000 children born in the developed world, a prevalence that has changed little over the last 50 years despite large changes in obstetric care.^{2–5} Not only has the prevalence remained unchanged, but also the majority of cases remain without a specific known cause.

A number of clinical risk factors for cerebral palsy have been described in the literature including preterm birth,⁶ low birth-weight centile,⁷ maternal infection during pregnancy,⁸ male gender⁹ and a relative with cerebral palsy,¹⁰ but the currently known clinical risk factors do not explain the majority of cases. Because many of these risk factors may have a genetic component, researchers have investigated candidate single nucleotide polymorphisms (SNPs) as potential factors that increase genetic

Correspondence: Dr Michael E O'Callaghan, Robinson Institute, Discipline of Obstetrics and Gynaecology, Level 3, Norwich Centre, 55 King William Road, North Adelaide, SA 5006, Australia. Fax: +61 8 8313 1333; email: michael.ocallaghan@adelaide.edu.au

Conflict of interest: None declared.

Accepted for publication 30 November 2012.

susceptibility for cerebral palsy. Thrombophilic, inflammatory and apolipoprotein-E SNPs have been the most extensively investigated with variable and weak associations reported (for systematic review see O'Callaghan *et al.*¹¹). They include positive associations in individual studies and in a recent meta-analysis.¹² The largest study investigating candidate SNP associations with cerebral palsy was recently conducted by our group.¹³ Univariable analyses of SNP associations¹⁴ and epidemiological risk factors for cerebral palsy¹⁵ have previously been published from this case control study. The initial analysis showed few significant SNP associations with the exception of prothrombin gene mutation and hemiplegia in infants delivered at term to mothers who reported having an infection during pregnancy. This paper presents the multi-variable analysis.

Cerebral palsy is a complex disorder that is likely to be of multifactorial origin, and therefore SNP associations with cerebral palsy were examined after adjustment for potentially confounding clinical risk factors. Interactions between maternal and fetal genotype that may modulate cerebral palsy risk were also tested; these interactions are potentially critical to understanding cerebral palsy given that many clinical risks described to date involve the maternal–fetal interface. Finally, we sought significant interactions between genotype and reports of maternal infection during pregnancy that may modulate cerebral palsy risk.¹⁵

Materials and Methods

Cohort

This analysis utilises data collected as part of The Australian Cerebral Palsy Research Study cohort. The original study design is described in detail elsewhere.¹³ In summary, mother–child case and control pairs (one child per family) were recruited between July 2008 and March 2010 from around Australia with the following inclusion criteria: children were aged between 5 and 18 years, born in Australia and of Caucasian background. Five hundred and eighty-seven case families and 1154 control families were recruited during the same time period and included in the analysis. Basic demographic details of the cohort are shown in Table 1.

Cases

Children with cerebral palsy were recruited from all states of Australia. Invitations were sent to families by state cerebral palsy registers, cerebral palsy health service providers and special education schools. Advertisement was also made using a web site, flyers and media releases. Comparison of case cohort demographics and clinical details (birthweight, gestational age, maternal age, cerebral palsy sub-type and severity) was made with population wide Australian cerebral palsy data with good correlation (see supplementary tables).

Controls

Children without cerebral palsy were recruited as the control cohort from all states of Australia. Invitations were sent to families by schools and advertisement was also made using a web site, flyers, media releases and recruitment booths in public

Table 1 Participant characteristics

Demographic	Cases	Controls
Number of participants	587	1154
Males (%)	345 (58)	529 (46)
Range of birth years	1990–2009	1990–2009
Average maternal age at birth of participating child (years)	31.3	31.0
Average gestational age (weeks)	35.3	39.3
Number ≤ 32 weeks gestational age (%)†	164 (29.3)	9 (0.8)
Number 32–37 weeks gestational age (%)†	87 (15.5)	65 (5.9)
Number ≥ 37 weeks gestational age (%)†	309 (44.6)	1032 (93.3)
Average birthweight (g)	2538	3456
Hemiplegia	191 (33.4)	N/A
Diplegia	149 (26)	N/A
Quadriplegia	145 (25.3)	N/A

†Gestational age data missing for 27 cases and 48 controls.

places. Comparison of control cohort demographics and clinical details (birthweight, gestational age and maternal age) was made with population wide Australian birth data with good correlation (see supplementary tables).

Clinical data

Clinical data relating to both case and control cohorts were collected by linking to perinatal databases in each state of Australia. Where data were not collected for national statistics (e.g. maternal smoking during pregnancy and maternal recreational drug use during pregnancy in some states), data were collected using a maternal questionnaire.¹³ For the case cohort, linkage was also made to cerebral palsy registers in each state of Australia, and data on cerebral palsy sub-type and severity were returned. Data relating to reported maternal infection during pregnancy (e.g. proven genitourinary tract infection, pyrexia during labour and clinically recorded viral infection) were sourced from state-held perinatal outcome data sets. Although the maternal questionnaire collected further details about type of infection and timing, the data may be subject to maternal recall bias. Any infection when it had been recorded prospectively by health-care professionals during pregnancy and at birth was used in preference to the maternal questionnaire. There were too many infection types to meaningfully conduct infection sub-type analysis. Placental pathology, bacteriology and virology results were not available. Cases were classed as hemiplegia, diplegia, quadriplegia or other based on the diagnosis recorded in cerebral palsy registers. Cases and controls were divided into the following gestational age subgroups: very preterm (≤32 weeks gestational age), late preterm (32–37 weeks gestational age) and term (≥37 weeks gestational age). The number of participants and proportion in each subgroup group is shown in Table 1.

Table 2 SNPs examined

SNPs associated with an altered inflammatory response			SNPs associated with thrombophilia			Other SNPs, most associated with preterm birth		
Number	SNP	RS number	Number	SNP	RS number	Number	SNP	RS number
1	TNF- α 308	1 800 629	24	PAI-2_1	6 098	33	APO-E_1	429 358
2	TNF- α +488	1 800 610	25	PAI-2_2	6 103	34	APO-E_2	7 412
3	TNF- α -238	361 525	26	THBD	1 800 576	35	iNOS (NOS2A)	1 137 933
4	TLR4 299	4 986 790	27	FVL	6 025	36	eNOS-922	1 800 779
5	MBL codon 52	5 030 737	28	MTHFR 677	1 801 133	37	ADD1	4 961
6	MBL codon 54	1 800 450	29	MTHFR 1298	1 801 131	38	ADRB2 Q27E	1 042 714
7	MBL codon 57	1 800 451	30	PGM	1 799 963	39	Y-specific	Amelogenin†
8	MBL-221	7 096 206	31	NPY C4112T	16 135			
9	MBL+4	7 095 891	32	NPY A6411C	16 476			
10	MBL-550	11 003 125						
11	IL-4-589	2 243 250						
12	IL-8	4 073						
13	IL 1B+511	16 944						
14	IL-10-819	1 800 871						
15	IL-6-174	1 800 795						
16	CR2-1	3 813 946						
17	CR2-2	1 048 971						
18	CR2-3	1 715						
19	TGF-B1-29	1 982 073						
20	TGF- β 1-509	1 800 469						
21	CFH Y402H	1 061 170						
22	MMP-3	602 128						
23	MMP-2	243 865						

†Internal control.

Genetic data

Candidate SNPs

DNA samples were collected using buccal swabs. The 39 functional candidate SNPs used were described in the published protocol of this study (Table 2).¹³ The panel included candidate SNPs chosen for their association with (i) thrombophilia; (ii) inflammation; and (iii) other previously reported risk factors for cerebral palsy such as preterm birth.

Genotyping and quality control

SNP genotyping was performed by the Australian Genome Research Facility (AGRF, Brisbane Node, Australia) on a MassARRAY iPLEX Gold System (Sequenom, San Diego, CA, USA). Individuals were checked for Mendelian failures (<1% removed), sibling relationship (0.42% removed), gender inconsistencies between DNA analysis and participant questionnaires (0.62% removed) and samples with greater than 25% of SNP tests failing (0.5% removed as per McMichael *et al.*¹⁶). All SNPs achieved greater than 93% successful genotyping, two were excluded as they were non-polymorphic (rs17516265 – TGF β 1, rs1715 – CR2-2) and one was excluded as it significantly differed from Hardy Weinberg equilibrium (rs1061170 – CFHY402H, $P < 0.05$). Linkage disequilibrium testing showed that two SNPs had a correlation of >0.80% and could not be considered as

independent tests (rs6098 and rs6103, both retained for testing consistent with previous studies).

Statistical analysis

SNP analysis

Each fetal and maternal SNP was individually assessed by logistic regression for association with cerebral palsy. To minimise the number of comparisons made, an additive genetic model for SNP data was used (each SNP coded as the number of minor alleles present). Logistic regression was then repeated for each SNP including adjustment for the following potential confounders: gestational age, multiple birth, maternal infection during pregnancy, maternal age, gender, maternal smoking and recreational drug use during pregnancy. Until the precise sequence of events leading to cerebral palsy is clarified, it remains difficult to select potential confounders. Gestational age was included as a potential confounder (following similar analyses^{17–19}), whereas Apgar score was not included as it may be part of the cerebral palsy phenotype.

Odds ratio (OR) and 95% confidence intervals (95% CIs) are reported for all association tests conducted where $P < 0.05$. To account for the large number of tests conducted (overall cerebral palsy association and each subgroup), SNP associations are corrected using the false discovery rate (FDR) method.²⁰

Table 3 Maternal and child SNP associations with cerebral palsy

Gene affected by SNP	Genotype	Cases		Controls		Unadjusted		Adjusted		
		<i>n</i>	%	<i>n</i>	%	OR (95% CI)	<i>P</i>	OR	<i>P</i>	FDR corrected <i>P</i>
Child MMP-2	C/C	308	53.7	689	60.3	1.2 (1.02–1.42)	0.028	1.28 (1.04–1.56)	0.018	0.6
	C/T	233	40.6	389	34.1					
	T/T	33	5.7	64	5.6					
Child TNF- α	G/G	394	68.2	728	63.5	0.85 (0.71–1.02)	0.09	0.79 (0.62–1.0)	0.047	0.7
	G/A	166	28.7	381	33.2					
	A/A	18	3.1	37	3.2					
Maternal IL 1 β	G/G	276	48.1	505	44.9	0.89 (0.76–1.04)	0.14	0.79 (0.66–0.96)	0.017	0.6
	A/G	244	42.5	493	43.8					
	A/A	54	9.4	127	11.3					
Maternal TGF- β	G/G	275	47.8	626	55.7	1.24 (1.06–1.44)	0.007	1.24 (1.03–1.50)	0.025	0.6
	G/A	246	42.8	405	36					
	A/A	54	9.4	93	8.3					
Maternal TNF- α	G/G	387	66.6	724	63.4	0.86 (0.72–1.03)	0.11	0.80 (0.63–1.0)	0.049	0.7
	G/A	178	30.6	371	32.5					
	A/A	16	2.8	47	4.1					

All tests use an additive genetic model. Adjustments made for gestational age, multiple births, maternal infection during pregnancy as reported in state held perinatal data sets, gender, maternal age, maternal smoking and recreational drug use during pregnancy. Only tests with adjusted $P < 0.05$ after adjustment were shown.

Sub-type analysis

Cerebral palsy is a heterogeneous condition, with this diversity possibly reflecting different causal pathways for sub-types within the condition. Associations with cerebral palsy sub-types hemiplegia, diplegia and quadriplegia, as well as gestational age subgroups very preterm (≤ 32 weeks gestational age), late preterm (32–37 weeks gestational age) or term (≥ 37 weeks gestational age) are therefore described. For all sub-type analyses, the whole control cohort was used for comparison.

Interactions

Interactions between mother and child SNPs were assessed using logistic regression. Although it is theoretically possible to test pair-wise interactions between all SNPs in the child's panel, all SNPs in the mother's panel, all clinical variables and then combinations thereof, this would create a large number of tests, most of which would be of little interest. To limit multiple testing, only *a priori* interaction tests were conducted. Firstly, interactions between each individual maternal SNP and the same SNP in the child (this accounts for inherent genotypic correlation) are examined, and secondly, interactions between each individual SNP and maternal infection during pregnancy as reported in state held perinatal data sets (a possible clinical trigger) are also examined.

Institutional review board

Ethics approval was obtained from the research group's institution (approval number REC 1946/4/10) and from

relevant committees in each state of Australia. All cases and controls gave written consent to participate.

Results

SNP associations with cerebral palsy

After adjustment for potential clinical confounders, two child SNPs (in genes encoding matrix metalloproteinase-2 (MMP-2) and tumor necrosis factor alpha (TNF- α)) and three maternal SNPs (in genes encoding interleukin-1 beta (IL-1 β), transforming growth factor beta (TGF- β 1) and TNF- α) remained significantly ($P < 0.05$) associated with all types of cerebral palsy (see Table 3). These tests were no longer significant after FDR correction for multiple testing.

SNP associations with cerebral palsy sub-types

When considering all SNPs and analysing for cerebral palsy sub-types, SNPs in genes encoding the following remained significantly ($P < 0.05$) associated with hemiplegia – child MMP-2, maternal TNF- α and maternal Factor V Leiden; quadriplegia – child Neuropeptide Y (NPY) and TNF- α ; very preterm birth – child inducible nitric oxide synthase (iNOS), child plasminogen activator inhibitor 2 (PAI-2), maternal iNOS, maternal Factor V Leiden, maternal mannose-binding lectin 4 (MBL+4); late preterm birth – child MMP-2, maternal TGF- β 1 and term birth – child adrenoceptor beta 2 (ADRB2), child TNF- α , child PAI-2, maternal NPY, maternal IL-1 β , maternal TNF- α (see Table 4). After conservative correction for multiple testing only maternal and child iNOS SNPs remained

Table 4 Maternal and child SNP associations with cerebral palsy subtypes

Gene affected by SNP	Genotype	Cases		Controls		Unadjusted		Adjusted		
		<i>n</i>	%	<i>n</i>	%	OR (95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>	FDR corrected <i>P</i>
Hemiplegia										
Child MMP-2	C/C	95	50.5	689	60.3	1.35 (1.06–1.72)	0.016	1.42 (1.07–1.88)	0.015	0.6
	C/T	79	42.0	389	34.1					
	T/T	14	7.4	64	5.6					
Maternal TNF- α	G/G	130	68.8	724	63.4	0.8 (0.6–1.06)	0.12	0.66 (0.47–0.94)	0.016	0.6
	G/A	54	28.6	371	32.5					
	A/A	5	2.6	47	4.1					
Maternal Factor V Leiden	G/G	173	90.6	1099	95.5	2.01 (1.1–3.45)	0.019	1.98 (1.07–3.65)	0.038	0.9
	G/A	18	9.4	50	4.3					
	A/A	0	0.0	2	0.2					
Quadriplegia										
Child NPY	T/T	25	17.9	298	26.2	1.32 (1.02–1.69)	0.031	1.41 (1.02–1.95)	0.036	0.8
	G/T	74	52.9	565	49.7					
	G/G	41	19.3	273	24.0					
Child TNF- α 308	G/G	105	74.5	728	63.5	0.62 (0.43–0.89)	0.007	0.54 (0.33–0.87)	0.008	0.5
	G/A	34	24.1	381	33.2					
	A/A	2	1.4	37	3.2					
Very preterm birth (cases born at gestational age \leq 32 weeks)										
Child iNOS										
Child iNOS	C/C	114	69.1	659	57.7	0.68 (0.50–0.92)	0.009	0.57 (0.4–0.82)	0.001	0.05
	C/T	44	26.7	420	36.8					
	T/T	7	4.2	63	5.5					
Child PAI-2	A/A	100	67.6	632	59.2	0.72 (0.53–0.99)	0.04	0.67 (0.46–0.96)	0.026	0.4
	G/A	43	29.1	373	35.0					
	G/G	5	3.4	62	5.8					
Maternal iNOS										
Maternal iNOS	C/C	114	69.1	645	56.3	0.6 (0.44–0.82)	0.0009	0.55 (0.38–0.79)	0.0007	0.05
	C/T	47	28.5	438	38.2					
	T/T	4	2.4	63	5.5					
Maternal Factor V Leiden	G/G	149	89.8	1099	95.5	2.19 (1.26–3.8)	0.010	2.37 (1.28–4.41)	0.01	0.3
	G/A	17	10.2	50	4.3					
	A/A	0	0	2	0.2					
Maternal MBL+4	G/G	114	70.4	672	59.4	0.66 (0.48–0.9)	0.007	0.7 (0.49–1.0)	0.04	0.5
	G/A	43	26.5	404	35.7					
	A/A	5	3.1	56	4.9					
Late preterm birth (cases born at gestational age 32–37 weeks)										
Child MMP-2	C/C	44	52.4	689	60.3	1.47 (1.05–2.05)	0.03	1.57 (1.1–2.25)	0.016	0.7
	C/T	29	34.5	389	34.1					
	T/T	11	13.1	64	5.6					
Child TGF- β 1	G/G	32	38.1	626	55.7	1.49 (1.08–2.05)	0.02	1.53 (1.08–2.16)	0.02	0.7
	G/A	45	53.6	405	36					
	A/A	7	8.3	93	8.3					
Term (cases born at \geq37 weeks gestational age)										
Child ADRB2	C/C	99	33.3	354	31.0	0.87 (0.72–1.05)	0.14	0.81 (0.66–1)	0.04	0.6
	G/C	155	52.2	576	50.4					
	G/G	43	14.5	213	18.6					
Child TNF- α	G/G	213	69.6	728	63.5	0.78 (0.61–1)	0.04	0.76 (0.59–0.99)	0.04	0.6
	G/A	86	28.1	381	33.2					
	A/A	7	2.3	37	3.2					
Child PAI-2	C/C	199	66.1	666	58.8	0.77 (0.61–0.96)	0.018	0.79 (0.62–1)	0.05	0.6
	C/G	90	29.9	403	35.6					
	G/G	12	4.0	64	5.6					
Maternal NPY	T/T	94	31.0	304	26.7	0.84 (0.7–1)	0.05	0.81 (0.67–0.99)	0.04	0.6
	G/T	151	49.8	564	49.6					
	G/G	58	19.1	269	23.7					
Maternal IL- β 1	G/G	149	49.0	505	44.9	0.87 (0.72–1.05)	0.15	0.79 (0.64–0.97)	0.02	0.6
	A/G	127	41.8	493	43.8					
	A/A	28	9.2	127	11.3					
Maternal TNF- α	G/G	209	68.5	724	63.4	0.78 (0.62–0.99)	0.04	0.76 (0.62–1)	0.05	0.6
	G/A	90	29.5	371	32.5					
	A/A	6	2.0	47	4.1					

All tests use an additive genetic model. Adjustments made for multiple birth, maternal infection during pregnancy as reported in state held perinatal data sets, gender, maternal age, maternal smoking and recreational drug use during pregnancy. Gestational age was adjusted for in tests examining hemiplegia, diplegia and quadriplegia. Only tests with adjusted $P < 0.05$ after adjustment were shown. Bold text represents statistically significant comparisons ($P < 0.05$).

significantly associated with cerebral palsy in very preterm infants (OR 0.55, 95% CI 0.38–0.79, $P = 0.05$; OR 0.57, 0.4–0.82, $P = 0.05$, respectively).

Cerebral palsy associations and SNP-SNP interactions

All SNPs were assessed for interaction when the same SNP was carried by both mother and child. A significant interaction was seen for SNP IL-10-819 ($P = 0.012$); however, this was not significant after correction for multiple testing (data not shown).

Cerebral palsy associations and SNP-clinical infection interaction

All SNPs were examined for interaction with maternal infection during pregnancy as reported in state-held perinatal data sets. There were no significant associations after correction for multiple testing (data not shown).

Discussion

This paper confirms in a multi-variable analysis the association of only a few specific SNPs with cerebral palsy outcome and excludes other nominated candidate genes. With the exception of maternal and child iNOS in children born at less than 32 weeks gestational age, SNPs did not remain significantly associated with cerebral palsy, cerebral palsy sub-types or gestational age subgroups after correction for multiple testing.

iNOS SNPs have previously been associated with cerebral palsy in two small independent cohorts; however, associations were not significant after correction for multiple testing. Gibson *et al.* reported associations in a composite of all gestational age groups (heterozygous/homozygous vs. normal, OR 1.29, 95% CI 1.00–1.67; $P = 0.047$) and in infants born at ≥ 37 weeks' gestational age (heterozygous vs. normal, OR 1.58, 95% CI 1.12–2.23, $P = 0.009$; heterozygous/homozygous vs. normal, OR 1.59, 95% CI 1.14–2.22; $P = 0.006$).²¹ Wu *et al.* report an association of iNOS with cerebral palsy in infants born at ≥ 37 weeks' gestational age (OR 1.9, 95% CI 1.2–3.1).²² The observation of a negative association between iNOS and cerebral palsy in very preterm infants in this analysis is in contrast to previous studies and may reflect the larger cohort used combined with adjustment for clinical confounders and multiple testing. This inverse association is seen whether the SNP is present in the mother or the child. iNOS SNP rs1137933 is a synonymous coding mutation and its effects are therefore likely to be mediated by linkage disequilibrium with other SNPs in the iNOS gene.²³ It has previously been associated with Crohn's disease²³ and multiple sclerosis²⁴ through inflammation mechanisms. In the context of cerebral palsy, either upregulation or downregulation of the immune response may leave the fetal brain susceptible to damage, caused either directly by the inflammatory response or resulting from neurotropic infectious agents. It tempting to speculate that enhanced inflammatory capacity associated with this, iNOS SNP conveys a protective effect for infection-associated cerebral palsy. Further studies, ideally in a prospective cohort with detailed phenotyping of the maternal and fetal inflammatory response and the various biologic pathogens, will be required to support or refute this hypothesis.

It is not possible in a retrospective study of cerebral palsy cases and controls to document the full range of maternal infections that may occur during pregnancy. For this analysis, documentation of maternal infection during pregnancy was ascertained from state held perinatal data sets and may be under-reported. Prospective studies collecting such detail are currently impractical. Thus, interaction of candidate SNPs and maternal infection during pregnancy could have been missed in this analysis.

These results do not provide statistical evidence for SNP-SNP or SNP-infection interactions modulating the risk of cerebral palsy after correction for multiple testing. This lack of evidence for association should not be interpreted as evidence for no association. It is plausible that the application of FDR correction for multiple testing to a small group of candidate SNPs chosen because of prior knowledge is too conservative and significant associations have been missed. Larger sample sizes are required to achieve adequate power for these tests and will likely only be achievable using individual patient data meta-analyses of data acquired from multiple studies.

Other weaknesses of this study include the cohort sample size, although it is the largest reported to date. The control cohort included only a small number of very preterm infants, which while typical of the general population, may mean that adjustment for this variable still leaves residual confounding. As gestational age is associated with cerebral palsy, it would be ideal to match the cohorts for this variable; however, the low incidence of very preterm delivery in the general population makes this impractical to achieve in a prospectively recruited case control study. The results of this study remain to be replicated in an independent cohort. Inclusion of reported maternal infection as a variable in regression models testing inflammatory SNP associations with cerebral palsy may result in over correction. This would be the case if inflammatory SNPs only affected infection susceptibility. They may also modulate inflammation magnitude. The study is also limited by the absence of cord blood samples that could be used to assess gene expression and protein data relevant to the SNPs examined. The strengths of the current report include collection of both child and maternal DNA samples, the conservative use of correction for multiple testing and the parallel collection of well validated clinical data. Potential clinical applications of these findings could include development of predictive tools for cerebral palsy based on genetic profiling and clinical characteristics that might allow identification and monitoring of high risk groups. Additional validation is required before this could occur.

Conclusion

Maternal and child iNOS SNPs are inversely associated with cerebral palsy in infants born very preterm. Interaction between maternal and child SNPs and also SNP interactions with clinical infection were not significantly associated with cerebral palsy.

Acknowledgements

This study was funded by the National Health and Medical Research Council and The Research Foundation of Cerebral Palsy Alliance. We thank all participating families and collaborators in each state of Australia. We also thank perinatal and

cerebral palsy register data custodians for providing data used in this study. No authors have any competing interests to disclose.

[Correction added on 14 June 2013, after first online publication: "nitrous oxide" throughout the article was changed to "nitric oxide".]

References

- Rosenbaum P, Paneth N, Leviton A *et al.* A report: the definition and classification of cerebral palsy April 2006. *Dev. Med. Child Neurol. Suppl.* 2007; **109**: 8–14.
- Stanley F, Blair E, Alberman E. *Cerebral Palsies: Epidemiology and Causal Pathways*. London: Mac Keith Press, 2000.
- Andersen GL, Irgens LM, Haagaas I, Skranes JS, Meberg AE, Vik T. Cerebral palsy in Norway: prevalence, subtypes and severity. *Eur. J. Paediatr. Neurol.* 2008; **12**: 4–13.
- Arneson CL, Durkin MS, Benedict RE *et al.* Prevalence of cerebral palsy: autism and developmental disabilities monitoring network, three sites, United States, 2004. *Disabil. Health J.* 2009; **2**: 45–8.
- Paneth N, Hong T, Korzeniewski S. The descriptive epidemiology of cerebral palsy. *Clin. Perinatol.* 2006; **33**: 251–67.
- Mayer PS, Wingate MB. Obstetric factors in cerebral palsy. *Obstet. Gynecol.* 1978; **51**: 399–406.
- Jacobsson B, Ahlin K, Francis A, Hagberg G, Hagberg H, Gardosi J. Cerebral palsy and restricted growth status at birth: population-based case-control study. *BJOG* 2008; **115**: 1250–5.
- O'Shea TM, Klinepeter KL, Meis PJ, Dillard RG. Intrauterine infection and the risk of cerebral palsy in very low-birthweight infants. *Paediatr. Perinat. Epidemiol.* 1998; **12**: 72–83.
- Tioseco JA, Aly H, Essers J, Patel K, El-Mohandes AA. Male sex and intraventricular hemorrhage. *Pediatr. Crit. Care Med.* 2006; **7**: 40–4.
- Hemminki K, Li X, Sundquist K, Sundquist J. High familial risks for cerebral palsy implicate partial heritable aetiology. *Paediatr. Perinat. Epidemiol.* 2007; **21**: 235–41.
- O'Callaghan ME, MacLennan AH, Haan EA, Dekker G. The genomic basis of cerebral palsy: a HuGE systematic literature review. *Hum. Genet.* 2009; **126**: 149–72.
- Wu D, Zou YF, Xu XY *et al.* The association of genetic polymorphisms with cerebral palsy: a meta-analysis. *Dev. Med. Child Neurol.* 2011; **53**: 217–25.
- O'Callaghan ME, MacLennan AH, Gibson CS *et al.* The Australian cerebral palsy research study – protocol for a national collaborative study investigating genomic and clinical associations with cerebral palsy. *J. Paediatr. Child Health* 2011; **47**: 99–110.
- O'Callaghan ME, MacLennan AH, Gibson CS *et al.* Fetal and maternal candidate single nucleotide polymorphism associations with cerebral palsy: a case-control study. *Pediatrics* 2012; **129**: e414–23.
- O'Callaghan ME, MacLennan AH, Gibson CS *et al.* Epidemiologic associations with cerebral palsy. *Obstet. Gynecol.* 2011; **118**: 576–82.
- McMichael GL, Gibson CS, O'Callaghan ME *et al.* DNA from buccal swabs suitable for high-throughput SNP multiplex analysis. *J. Biomol. Tech.* 2009; **20**: 232–5.
- Hollegaard MV, Skogstrand K, Thorsen P, Norgaard-Pedersen B, Hougaard DM, Grove J. Joint analysis of SNPs and proteins identifies regulatory IL18 gene variations decreasing the chance of spastic cerebral palsy. *Hum. Mutat.* 2012; **34**: 143–8.
- Costantine MM, Clark EA, Lai Y *et al.* Association of polymorphisms in neuroprotection and oxidative stress genes and neurodevelopmental outcomes after preterm birth. *Obstet. Gynecol.* 2012; **120**: 542–50.
- Clark EA, Mele L, Wapner RJ *et al.* Association of fetal inflammation and coagulation pathway gene polymorphisms with neurodevelopmental delay at age 2 years. *Am. J. Obstet. Gynecol.* 2010; **203**: 83 e1–e10.
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Statist. Soc. Ser. B Methodological.* 1995; **57**: 289–300.
- Gibson CS, MacLennan AH, Dekker GA *et al.* Candidate genes and cerebral palsy: a population-based study. *Pediatrics* 2008; **122**: 1079–85.
- Wu YW, Croen LA, Vanderwerf A, Gelfand AA, Torres AR. Candidate genes and risk for CP: a population-based study. *Pediatr. Res.* 2011; **70**: 642–6.
- Martin MC, Martinez A, Mendoza JL *et al.* Influence of the inducible nitric oxide synthase gene (NOS2A) on inflammatory bowel disease susceptibility. *Immunogenetics* 2007; **59**: 833–7.
- Barcellos LF, Begovich AB, Reynolds RL *et al.* Linkage and association with the NOS2A locus on chromosome 17q11 in multiple sclerosis. *Ann. Neurol.* 2004; **55**: 793–800.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Comparisons of cases and controls to population data.