Exposure to hypertensive disorders of pregnancy increases the risk of autism spectrum disorder in affected offspring.

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 # Joint contribution

Abstract

There is growing awareness that prenatal adversity may increase the risk of autism spectrum disorder (ASD). Here we examined the association between hypertensive disorders of pregnancy (HDP) and ASD risk at 7-years of age using the Millennium Cohort Study (MCS) a representative cohort of 13,192 children born in the UK from 2000-2001. We also sought to examine cytokine expression in the serum of women with the pre-eclampsia, which is the most common HDP, and whether exposure of fetal neurons to this serum could change patterns of neuronal growth. HDP were reported by mothers nine months post-delivery. ASD was parent-reported at age seven, based on a doctor or health care professional's diagnosis. Weighted logistic regression was used for data analysis, adjusting for several potential confounders including maternal alcohol consumption, education, depression, age, and poverty status. Sensitivity analyses were performed excluding pre-term births, small for gestational age (SGA), pre-pregnancy hypertension and depression. There was a significant association between HDP and a two-fold increased risk of ASD (AOR=2.10 [95% CI: 1.20-3.70]). Excluding preterm excluding preterm births, SGA births and offspring of women who had pre-pregnancy hypertension or over the age of 40 did not change the results materially. At the cellular level, exposure of fetal cortical neurons to 3% serum isolated from women with an established HDP, increased neuronal growth and branching *in vitro*. These findings indicate that HDP exposure may increase the risk of ASD in the offspring.

Key Words: Autism spectrum disorder; ASD; autism; pre-eclampsia; pregnancy, hypertensive disorders.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder that is characterised by deficits in socialisation, communication, and repetitive behaviour (Lai et al., 2014). There is also evidence for on-going central and peripheral immune dysregulation (Estes and McAllister, 2015), and dysregulation of the hypothalamic–pituitary–adrenal (HPA) stress response (Spratt et al., 2012). Recent evidence from 11 sites across the USA in the Autism and Developmental Disabilities Monitoring Network at the CDC, has reported that ASD affects 1 in 68 children (Christensen et al., 2016), and ASD is now a World Health Organisation global health priority. Although ASD is highly heritable with a strong genetic basis, increasing evidence suggests that non-genetic factors may play a role in its aetiology (Mandy and Lai, 2016; Sandin et al., 2014). Consequently there is an increasing focus on prenatal adversity as a potential risk factor for ASD (Mandy and Lai, 2016). In addition a recent study has demonstrated focal patches of abnormal laminar cytoarchitecture and cortical disorganization of neurons, but not glia, in the prefrontal and temporal cortical tissue in children with ASD (Stoner et al., 2014). This is suggestive of a potential dysregulation of brain development at prenatal developmental stages, as cortical lamination is on-going during the second trimester of pregnancy (Stiles and Jernigan, 2010). One of the most common forms of prenatal adversity that occur at this stage of pregnancy are hypertensive disorders of pregnancy (HDP).

HDPs are the most common pregnancy complication, affecting 10-15% of pregnancies and they begin most frequently in the second trimester (Olson-Chen and Seligman, 2016). HDP is an umbrella term for several hypertensive conditions of pregnancy, including gestational hypertension and pre-eclampsia (Olson-Chen and Seligman, 2016). Children with ASD are twice as likely to have been exposed *in utero* to pre-eclampsia (Walker et al., 2015), consistent with other studies indicating that pre-eclampsia (Mann et al.,

2010) and maternal hypertension (Polo-Kantola et al., 2014) increase the risk of ASD in affected offspring. In addition, brain structural alterations that share similarities with those seen in ASD have recently been described in non-affected children born of pregnancies complicated by pre-eclampsia (Ratsep et al., 2016). However, the impact of HDP on ASD risk requires further study in additional cohorts, and crucially the biological basis of the increased risk of ASD in affected offspring, which is unknown, requires further investigation.

Here we sought to examine the association between HDPs and ASD in the Millennium Cohort Study, a cohort representative of children born in the UK in the year 2000 (Connelly and Platt, 2014). Recent studies have shown that dysregulation of circulating maternal cytokine signaling can lead to ASD-like behaviour in rodent offspring (Choi et al., 2016) and increased levels of maternal cytokines and chemokines during gestation are associated with ASD with intellectual disability in humans (Jones et al., 2016). Moreover as placental IL-6 signalling is a crucial determinant of ASD-like behaviour (Wu et al., 2017), we also sought to determine if there were elevated cytokines in serum from women with established pre-eclampsia and whether exposure to maternal serum could alter patterns of fetal neuronal growth.

2. Materials and Methods

2.1 Cohort

The Millennium Cohort Study (MCS) (Connelly and Platt, 2014), a nationally representative cohort of children born in the UK in the years 2000-2002, was used for epidemiological analysis. The MCS used a stratified cluster sampling design, stratified first by country within the UK (England, Scotland, Wales and Northern Ireland), then by electoral ward. Sampling methods have previously been described in detail (Plewis, 2007). Surveys of the MCS have been conducted in 5 waves: age 9 months (wave 1), age 3 years (wave 2), age 5 years (wave 3), age 7 years (wave 4) and age 11 years (wave 5). For the current analysis, exposure and co-variate information was collected from wave 1, and outcome information (ASD) was collected from wave 4. All variables were self-reported by the "*main respondent*" or "*partner respondent*." Main respondents for the surveys were the infant's biological mother the majority of the time (99.7%), partner respondents were the infant's biological father the majority of the time (85.7%). The London Multicentre Research Ethics Committee granted ethical approval for these surveys, and as the current investigation is a secondary data analysis no further approval was needed. All survey materials are available online (www.cls.ioe.ac.uk/mcs).

2.2 Variables

Hypertension during pregnancy (HDP) was measured at wave 1 using a two-tiered question. Women were first asked if they had suffered from any illnesses during pregnancy which required medical attention. Those that said "*yes*" were asked to choose from a list, including "*raised blood pressure, eclampsia/pre-eclampsia or toxaemia.*" Women who indicated this were considered cases of HDP. For ASD, parents were asked at wave 4 if a doctor or health professional had ever told them that their child had "*autism, Asperger's Syndrome, or ASD*". *("Has a doctor or health professional ever told you that [cohort child's name] had any of the*

following problems?"). Those that responded "yes" were considered to have ASD and were considered cases for the analysis. The data from this wave of the MCS have previously been used to examine the factors impacting ASD (Curran et al., 2016; Russell et al., 2014), and all survey materials are available online (www.cls.ioe.ac.uk/mcs). Other variables that were considered were smoking during pregnancy, gestational age at birth, small for gestational age (SGA) (calculated using a customised centile calculator (Gardosi and Francis, 2013), poverty, ethnicity, urbanicity, sex, birth order, and maternal age, education, depression, body mass index (BMI) (as defined by the World Health Organisation), pregestational diabetes and pregestational hypertension. Variables were categorised as shown in Table 1.

2.3 Statistical analysis

Logistic regression models were used to examine the association between HDP and reported ASD. Crude analysis was conducted, followed by adjustment for smoking during pregnancy, birth order, and poverty, as well as maternal ethnicity, age, education, depression, BMI, pregestational diabetes and pregestational hypertension. Crude and adjusted analyses were also conducted on several sub-groups: women without pregestational hypertension, women under the age of 40 at birth, babies that were not born pre-term, and babies that were not born SGA. Analysis was then stratified by sex and birth order. To account for the complex sampling design employed in the MCS, analyses were weighted and survey commands were used in SAS. As weights have been previously shown not to be significantly changed between waves (Plewis, 2007), wave 1 weights were used. All analysis was done using SAS version 9.4.

2.4 Multiplex ELISA measurements

Multiplex cytokine analysis was performed on time of disease serum samples from five women with severe term pre-eclampsia from the screening of pregnancy endpoints (SCOPE) study, which had detailed inclusion/exclusion criteria as descried previously (Kenny et al.,

2014). Five control non-pre-eclampsia samples were from nulliparous women with singleton pregnancies that were randomly selected matched for age and BMI. Ethical approval was obtained from the local ethics committee (Cork. ECM5 (10) 05/02/08) and all women provided written informed consent. Serum cytokines and chemokines were measured using a Human multi-Plex Ultra-Sensitive kit (MSD Gaithersburg, MD) in a 96-well format. Each well of each 96-well plate was coated with capture-antibodies to Eotaxin1, MCP-1, IFN-γ, L-6, IL-8 and the assay was run according the manufacturer's recommended protocol. Cytokine concentrations were determined using MSD Discovery Workbench Software (v. 3.0.17). All data were analysed using a Students *t*-test and considered significant when $p < 0.05$.

2.5 Primary neuron culture and analysis

Embryonic day (E) 18 Sprague-Dawley rat embryos were removed by laparotomy and transferred to ice-cold Hank's Balanced Salt Solution (Sigma). Primary cultures of cortical neurons were prepared as previously described (Hegarty et al., 2014). $2x10^5$ cells were electroporated with 0.5μg of an enhanced GFP expression plasmid using the Neon™ Transfection System (Invitrogen) according to the manufacturer's protocol under specific parameters (1100 V; 30 ms; 2 pulses). Dissociated neurons were plated on poly-D-lysine-coated 24-well plates at a density of $1x10^5$ cells per well in DMEM/F12, 33 mM D-glucose, 1% L-glutamine, 1% FCS, 2% B27. Cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. After 24 h, cultures were treated with 3% serum from patients with pre-eclampsia (n=5) or matched controls (n=5) from the SCOPE study (Kenny et al., 2014). Sera-treated, GFP-positive cortical neurons were imaged with an (Olympus IX70) inverted microscope fitted with an Olympus DP70 camera and AnalysisD™ software 24 h later. Neurite growth analysis was performed in a blinded fashion as previously described (Gutierrez and Davies, 2007; O'Keeffe et al., 2016). All data were analysed using a Students t-test and considered significant when $p < 0.05$.

3. Results

3.1 Millennium Cohort Study

There were 18,296 singleton babies that participated in the wave 1 survey. Of these, 13,192 participated in the wave 4 survey, and 13,098 had data on both HDP and ASD. Of the 13,098 children that were included in analysis, 983 (7.50%) were exposed to HDP. There were 199 (1.52%) reported cases of ASD, 30 (3.05%) of which were exposed to HDP (Table 1). In unadjusted analysis, the OR of the association between HDP and ASD was 2.27 (95% CI = 1.43-3.60), this was not materially changed after adjustment (AOR = 2.10; 95% CI = 1.20-3.70) (Table 2). When women over the age of 40 were excluded, the association appeared slightly stronger (AOR = 2.63; 95% CI = 1.27-4.02), but this was not a significant change (chi-square for heterogeneity p = 0.74). No other sub-groups (e.g. women without pregestational hypertension, babies that were not pre-term and babies that were not SGA) were different from the main cohort results (Table 2). Stratification by sex did not have an impact on results (Table 2). When the cohort was stratified by birth order, the association appeared weaker in children that were first born (OR = 2.08; 95% CI = 1.10-3.95), compared to children born second or more (OR = 2.49; 95% CI = 1.27-4.87) but this was not statistically significant (p = 0.70).

3.2 Cytokine and chemokine levels in women with established pre-eclampsia.

We next examined the expression of Eotaxin1, MCP-1, IFN-γ, IL-1β, L-6 and IL-8 (Masi et al., 2015) in the serum of women with established pre-eclampsia which is one of the more common hypertensive disorders, in the SCOPE cohort. Multiplex ELISA analysed revealed no statistically significant differences between Eotaxin1, MCP-1, IFN-γ and IL-8 (IL-1β was not detectable) between cases and controls, however there was a trend towards an increase in IL-6 ($t(8) = 2.18$, $p=0.06$) (Fig. 1). Given the detailed maternal data available in the SCOPE cohort (Kenny et al., 2014), we examined IL-6 levels in individual cases compared to

matched controls, and in four out of 5 cases the level of IL-6 was higher than in their matched control (Table 3).

3.3 Serum from patients with pre-eclampsia promotes excessive neuronal growth.

As aberrant connectivity is a core feature of ASD, we next sought to determine if there were circulating factors present in maternal sera that could affect fetal neuron growth, E18 cortical neurons were transfected with GFP to visualise the neuronal arbors and plated for 24h. Cultures were then treated with 3% serum from patients with term pre-eclampsia (P) (≥ 37 weeks) or matched controls (C) for 24h. Treatment with serum from patients with pre-eclampsia significantly increased the number of neurites, total neurite length (C: 272±17µm v P: 372±19µm; $t(8)=3.6$; $p=0.007$) (Fig. 2a), (C: 1.8±0.1 v P: 2.4±0.2; $t(8)=4.3$; $p=0.003$) (Fig. 2b), and branching (C: 2.5±0.3 v P: 3.9±0.2; $t(8)=4.1$; $p=0.004$) (Fig. 2c), when compared to neurons treated with serum from matched controls (Fig. 2d).

4. Discussion

This study examined the impact of hypertension during pregnancy on ASD, using epidemiological analysis on data from the MCS cohort. In the MCS cohort, a cohort representative of children born in the UK in the year 2000, children exposed to HDP were over twice as likely to be diagnosed with ASD by age 7. This association remained consistent after adjustment for several variables and across various sub-groups of the population.

In agreement with our data, a number of studies have reported an increased risk of ASD in offspring exposed to a HDP. For example, Buchmayer et al. carried out a case-control study which reported that pre-eclampsia exposure lead to a greater than 50% increase in the risk of ASD in the offspring (Buchmayer et al., 2009). A significant association between pre-eclampsia and ASD was also seen by Burstyn et al. in a study cohort of 218,890 live births (Burstyn et al., 2010). These findings support the earlier findings of Huttman et al.

who reported that there was a marginally increased risk for ASD among offspring to mothers with pregnancy-induced hypertension (Hultman et al., 2002). In agreement with these earlier findings, a number of more recent studies have also specifically examined the association between HDP exposure and ASD risk in the offspring. Langridge et al. examined maternal conditions and perinatal factors for children diagnosed with ASD with or without intellectual disability, and reported that pregnancy hypertension, which was defined as pre-eclampsia or essential hypertension, was associated with an increased risk of ASD with intellectual disability (Langridge et al., 2013). Some studies have also specifically examined the association between pre-eclampsia (the most common HDP) and ASD. Mann et al. in a study of 87,677 births reported a significant association between pre-eclampsia and ASD risk whether or not birth weight was controlled for (Mann et al., 2010). In agreement with this, Walker et al. reported that children with ASD were twice as likely to have been exposed *in utero* to pre-eclampsia when compared to controls, even after adjustment for a number of potential confounders including maternal education, parity and pre-pregnancy obesity (Walker et al., 2015).

However it should be noted that in contrast to our present report, other studies that have examined the impact of pre-eclampsia on ASD risk, have reported no association (Glasson et al., 2004; Larsson et al., 2005). In addition to this, data from the CHARGE study (Childhood autism Risks from Genetics and the Environment), which was a population-based, case-control investigation, has shown that although the prevalence of pre-gestational hypertension was more common among ASD case mothers than controls, once covariate adjustment was made this association was not significant (Krakowiak et al., 2012). Moreover similar data were found by Lyall et al, who reported that pregnancy-related high blood pressure had no association with ASD in the offspring (Lyall et al., 2012). These findings are largely in agreement with other studies that have found no association between pregnancy-

induced hypertension and ASD in the offspring (Bilder et al., 2009; Dodds et al., 2011). These reports emphasise the significant variations in relation to whether HDP exposure increases the risk of ASD in affected offspring, and highlight the need for a systematic review and meta-analysis to identify if possible an overall pooled estimate of association (Maher et al., 2017). This also raises the issue of why there is a significant heterogeneity between studies. It is worth noting that previous studies reporting an association between pre-eclampsia, the most common HDP, and ASD have reported that the risk increased with greater pre-eclampsia severity (Walker et al., 2015). This suggests that the severity of the HDP may be a crucial factor in determining the impact on neurodevelopmental outcome. For example, pre-eclampsia may have a stronger impact on development of ASD than hypertension alone, which may help to explain different findings across studies examining sub-types of HDPs. Although in the MCS cohort we were unable to examine the association by sub-type of HDP, however this may be of interest in future research.

In this study we also showed that exposure of fetal cortical neurons to serum from women with pre-eclampsia lead to increased neurite growth. It is important to stress that this does not imply causation with regards to ASD risk, but rather that there is the potential for a circulating factor(s) present in maternal serum as a result of pre-eclampsia that can change patterns of fetal neuronal growth. In future work it will be important to determine whether this happens *in vivo*, whether this leads to ASD-like behavioural changes in affected offspring, and to identify the factor or factors that leads to these changes. It is however interesting to note that it has recently been shown that elevations in maternal IL-17a leads to an abnormal cortical phenotype and behavioural disturbances in affected offspring (Choi et al., 2016). Given that there is an increase in circulating IL-17a in PE (Darmochwal-Kolarz et al., 2012; Toldi et al., 2011), disturbances in the maternal immune system in HDP may be a determinant of neurodevelopmental outcome in affected offspring. It is also interesting to

note that it has recently been shown that elevated IL-6 signalling in the placenta is necessary and sufficient for the development of ASD-like social deficits in exposed offspring (Wu et al., 2017). Given that we found that 4 of 5 pre-eclampsia samples had elevated levels of IL-6 compared to a matched control, it is possible that in some cases of pre-eclampsia, elevated placental IL-6 signalling may relay inflammatory signals to the fetal brain to impact neuronal development.

This study has several strengths. It utilised a large, contemporary, population-based cohort. Due to the sampling method employed in the MCS, the cohort is representative of children born in the UK in the years 2000-2001. However, this study is also subject to some limitations. Firstly, in the MCS, data were reported by parents. As the cohort is anonymised, it was not possible to validate reported HDP or ASD, and these may have been mis-reported. The first survey was conducted 9 months after birth, and exposure variables may have been subject to some recall bias, though HDP has been previously shown to be accurately reported post-delivery (Falkegard et al., 2015). Similarly, in the wave 4 survey, the wording of the question on ASD (e.g. "has a doctor or health professional ever told you…") may have been overly inclusive, as parents may have reported cases that were suspected but not confirmed by doctors. However, previous publications have used the MCS to examine ASD (Curran et al., 2016; Russell et al., 2014) and ADHD (Bohm et al., 2017) in the UK. Also, HDP was reported overall, and could not be examined by sub-group or severity. Previous results indicate that the association between pre-eclampsia and ASD may depend on severity (Walker et al., 2015) and future work should investigate this association further by examining the effect of sub-group on ASD risk.

The findings of this study suggest that children exposed to HDP are more likely to develop ASD that children from a healthy pregnancy. Additionally, exposure to serum from

women with pre-eclampsia may lead to changes in neuronal growth, but further *in vivo* studies are needed to determine what effect this may have on the development of ASD.

Acknowledgments

This study was funded by a research centres grant (Grant#: INFANT-12/RC/2272) (L.K.) from Science Foundation Ireland.

Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Maternal and Neonatal Characteristics of the Millennium Cohort Study by exposure to hypertension during pregnancy

	No Hypertension During Pregnancy	Hypertension During Pregnancy
Maternal age		
14 to 19 years	904 (7.46)	76 (7.73)
20 to 29 years	5451 (44.99)	469 (47.71)
30 to 39 years	5481 (45.24)	416 (42.32)
40+ years	268 (2.21)	76 (7.73)
Missing	11 (0.09)	0 (0.00)
White Ethnicity	10319 (85.18)	881 (89.62)
Urbanicity		
Urban	8021 (66.21)	655 (66.63)
Suburban	747 (6.17)	77 (7.83)
Rural	621 (5.13)	51 (5.19)
Missing	2726 (22.50)	200 (20.35)
Maternal education		
Secondary school	6468 (53.39)	572 (58.19)
Higher degree	3561 (29.39)	295 (30.01)
No degree	1423 (11.75)	82 (8.34)
Missing	663 (5.47)	34 (3.46)
Poverty (income under 60% OECD national median)		
Yes	4035 (33.31)	270 (27.47)
No	8044 (66.40)	713 (72.53)
Missing	36 (0.30)	0 (0.00)

Maternal BMI		
Normal weight	7523 (62.10)	482 (49.03)
Underweight	643 (5.31)	30 (3.05)
Overweight	2272 (18.75)	258 (26.25)
Obese	936 (7.73)	161 (16.38)
Missing	741 (6.12)	52 (5.29)
Smoking during pregnancy		
Never	8049 (66.44)	698 (71.01)
Quit	1408 (11.62)	129 (13.12)
Light	1430 (11.80)	97 (9.87)
Heavy	1208 (9.97)	58 (5.90)
Missing	20 (0.17)	1 (0.10)
Maternal depression		
Not Depressed	9193 (75.88)	683 (69.48)
Depressed, not treated	1855 (15.31)	183 (18.82)
Depressed, treated	1043 (8.61)	114 (11.60)
Missing	24 (0.20)	1 (0.10)
Diabetes unrelated to pregnancy	51 (0.42)	20 (2.03)
Longstanding Hypertension		
Yes	20 (0.17)	47 (4.78)
No	12081 (99.72)	936 (95.22)
Missing	14 (0.12)	0 (0.00)

Table 1 Continued

	No Hypertension During Pregnancy	**Hypertension During Pregnancy**

Sex (Male)	6126 (50.57)	513 (52.19)
First born	4942 (40.79)	531 (54.02)
Small for gestational age (centile <10)	1861 (15.36)	218 (22.18)
Gestational age at birth		
40 weeks	3476 (28.69)	222 (22.58)
24-36 weeks	703 (5.80)	155 (15.77)
37 weeks	631 (5.21)	69 (7.02)
38 weeks	1588 (13.11)	163 (16.58)
39 weeks	2547 (21.02)	201 (20.45)
41+ weeks	3026 (24.98)	165 (16.79)
Missing	144 (1.19)	8 (0.81)

Table 2. The association between hypertension during pregnancy and autism spectrum disorder in the Millennium Cohort Study

	Exposed cases	Adjusted*OR (95% CI)
Hypertension during pregnancy and ASD	30	2.10 (1.20-3.70)
Excluding women with longstanding hypertension	27	2.02 (1.12-3.63)
Excluding women aged 40+	30	2.63 (1.27-4.02)
Excluding pre-term babies	27	2.11 (1.15-3.88)
Excluding small for gestational age babies	22	2.37 (1.28-4.39)
Excluding women with longstanding hypertension	27	2.02 (1.12-3.63)
Excluding women aged 40+	30	2.63 (1.27-4.02)
Excluding pre-term babies	27	2.11 (1.15-3.88)
Excluding small for gestational age babies	22	2.37 (1.28-4.39)
Among girls	7	2.29 (0.88-6.00)
Among boys	23	2.18 (1.29-3.66)
Among first born	17	2.08 (1.10-3.95)
Among birth order 2+	13	2.49 (1.27-4.87)

*Adjusted for: smoking during pregnancy, birth order, and poverty, as well as maternal ethnicity, age, education, depression, BMI, longstanding diabetes and longstanding hypertension

Abbreviations: OR = Odds ratio, CI = Confidence interval

Table 3. IL-6 levels in matched case-control pairs

Control-Case	Control	Case
Matched pair 1	0.26pg/ml	1.69pg/ml
Matched pair 2	0.78pg/ml	0.41pg/ml
Matched pair 3	0.47pg/ml	1.08pg/ml
Matched pair 4	0.32pg/ml	0.55pg/ml
Matched pair 5	0.46pg/ml	1.38pg/ml

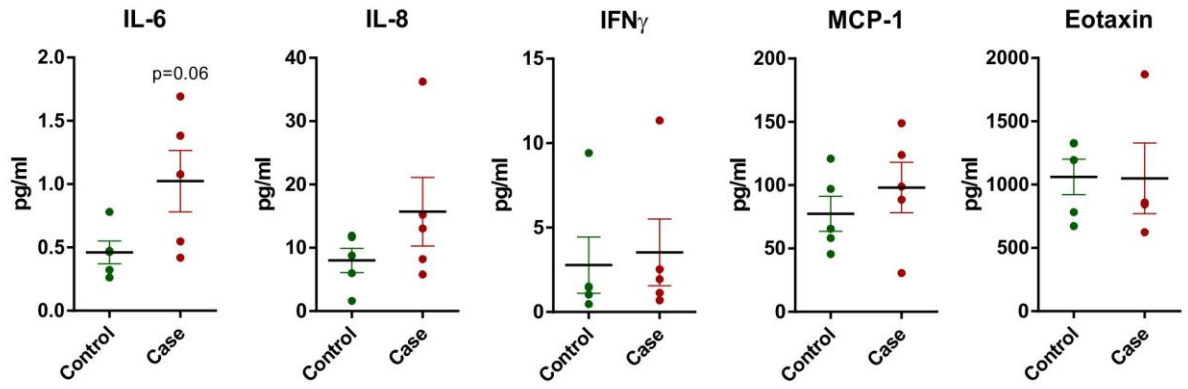

Figure 1: Cytokine and chemokine expression in women with established pre-eclampsia.

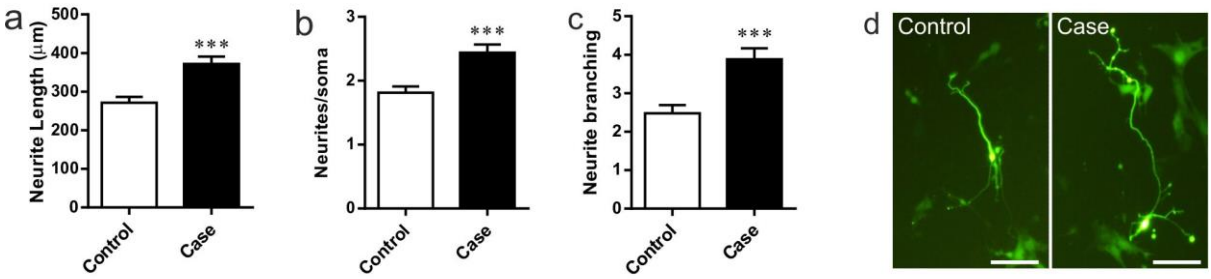

Figure 2. Pre-eclampsia serum promotes neurite growth in fetal cortical neurons.

Bar charts showing (a) total neurite length, (b) number of neurite per soma, (c) neurite branching and (d) representative photomicrographs of GFP-positive E18 cortical neurons cultured for 24 h with 3% serum from patients with term pre-eclampsia or matched controls. Mean ± SEM of data from 75 neurons in each condition from 5 individual serum samples. *** $P < 0.001$, statistical comparison with control; Student's t-test. Scale bar = 50µm.