

## Association of a Functional Inducible Nitric Oxide Synthase Promoter Variant with Susceptibility to Infantile Cerebral Palsy

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### Abstract

**Background:** Recently it has been shown an increase in the interleukin 1 beta and nitrite levels in cerebrospinal fluid as a primary response of the immature brain to oxygen deprivation in newborns that suffered perinatal asphyxia, 30% to 40% of these patients later develop neurological abnormalities including cerebral palsy. Formerly was shown that an increased enzyme activity of NOS2 is responsible for the increase in nitrite levels in cerebrospinal fluid. The NOS2A gene has a polymorphic microsatellite (CCTTT)<sub>n</sub> located at -2.6 Kb from the gene promoter. The expansion of this microsatellite to 13 or 14 repeats increases transcription of the NOS2A gene and triples the nitric oxide level under hypoglycemia and hypoxia conditions. The study aim was shown that the expansion of -2.6 Kb CCTTT microsatellite in the NOS2 gene promoter, constitutes a risk factor for developing cerebral palsy in newborns that suffered perinatal asphyxia

**Methods:** Genomic DNAs purified from peripheral leukocytes of 48 ICP patients and 57 healthy children, the (CCTTT)<sub>n</sub> microsatellite expansion were amplified by PCR, purified from agarosa gel in micro-column method and sequenced using genomelab methods development kit cycle sequencing dye terminator in an automated CEQ8000 sequencer.

**Results:** The presence of a 14-repeat is significantly associated with infant cerebral palsy (Fisher *P* value=0.0122). Multivariate analysis adjusted for age and sex confirmed the association with an increased risk of developing infant cerebral palsy (odds ratio, 1.78; 95% confidence interval, 1.150-2.752; *P*=0.01).

**Conclusions:** Our findings suggest that an expansion to 14 repeats of the CCTTT microsatellite plays a key role in the development of cerebral palsy in children that suffered perinatal asphyxia.

**Keywords:** Perinatal hypoxia; Single nucleotide polymorphism; Asphyxia susceptibility

### Introduction

The hypoxic ischemic encephalopathy (HIE) secondary to perinatal asphyxia, affects a 1.5 to 5% of newborns; unfortunately 10% to 20% of infants with moderate HIE die and 30% to 40% develop neurological abnormalities, whilst 50% of infants with severe HIE, die and the remainder develop neurological conditions [1]. To our knowledge, the reason why some children that suffered perinatal asphyxia develop infant cerebral palsy while other children with similar clinical histories develop memory and learning difficulties but no motor disabilities remains unknown. Infantile cerebral palsy (ICP) is a neurological disease resulting from damage of the immature brain in newborn children, clinically it is a non-progressive brain insult that produces permanent and progressive secondary postural and movement disorders [2] and induces long-term complications such as muscle hypotrophy, deformities in the skull and spine [3]. In Mexico there are approximately 500,000 diagnosed cases of CP, be the leading cause of death in children from 5 to 14 years old [4] and it is the most common cause of physical disability in children throughout the world [5]. The main clinical spectrum of ICP includes quadriplegia, hemiparesis, and diparesis that affect upper and lower limbs to varying degrees. The therapeutic management of ICP is targeted at the lesions caused on the central nervous system, bone deformations and muscle hypotrophy [6]. The initial inflammatory response in the immature brain as a result of a traumatic birth can be mapped from proteins and metabolites that participate in inflammation and oxidative stress [7,8]. The main oxidative metabolite which is produced in the inflammatory response is the Nitric oxide (NO), it is a multifunctional molecule that has been

found to participate in neurotransmission [9], regulation of vascular tone as a vasodilator [10], and apoptosis [11], as well as in certain human pathologies such as Alzheimer's [12] and Parkinson's disease [13]. NO is produced by three NOS (Nitric Oxide Synthase) isoforms; neuronal (NOS1 or nNOS), inducible (NOS2 or NOS2A) and endothelial (NOS3 or eNOS) which produce nitric oxide by oxidation of L-arginine [14]. NOS1 and NOS3 are encoded and expressed by constitutive genes. NOS2 is encoded by an inducible gene that activates in inflammatory and pathological conditions. Other authors have described that the promoter region of the NOS2 gene may influence the transcriptional activity of the gene. Various microsatellites have been associated with the susceptibility to develop specific pathologies. For example there is a pentanucleotide polypyrimidine microsatellite, CCTTT (rs3833912) [15] at position -2.6 Kb in the NOS2A gene promoter which has been associated with malaria [16], pulmonary arterial hypertension [17,18], diabetic retinopathy [19] and nephropathy [20], dementia with Lewy bodies [12], nasal polyposis and atopy [21,22]. Recently, studies

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have described differences in induction of NOS2A depending on the number of CCTTT repeats, importantly, (CCTTT) 14 allele has greater activity than the (CCTTT)9, (CCTTT)12, and (CCTTT)15 alleles; also numerous cytokine responsive elements have been found in the promoter and regulatory region of NOS2A, with a number of them residing in the -4 Kb region upstream of the transcriptional initiation site [23,24]. The -2.6(CCTTT) 13, 14 and 15 alleles of the microsatellite in the NOS2A gene respond to IL-1 $\beta$  drastically increasing NO production under hypoxic and inflammatory conditions [25]. Together, these observations point towards whether the functional -2.6(CCTTT) n pentanucleotide microsatellite in the NOS2A gene is associated with the development of cerebral palsy in patients with clinical history of perinatal asphyxia.

## Methods

### Subjects

A total of 105 children arranged into two groups were studied in a case control study; 47 patients with cerebral palsy and clinical history of perinatal hypoxia-ischemia and low APGAR (<7 at 5 min) matched to 57 healthy children as controls all living in the Mexico City metropolitan area. All patients underwent treatment at the neuropediatric's department of the Centro de Rehabilitación Integral Teleton (CRIT) in Tlalnepanltla, Estado de México. Clinical manifestations of cerebral palsy have been described previously [26,27]. All individuals were between 7-14 years old with a mean age of cerebral palsy patients and control group at 14.3 and 12.6 years, respectively. This study was approved by the institutional review board of the Centro de Rehabilitación Integral "Teleton" and the Hospital Regional "Primero de Octubre" and informed written consent was obtained from all patients' parents.

### Genotyping

Peripheral blood (200  $\mu$ L) obtained by means of puncture with a contact activated lancet (BD Microtainer, NJ, USA) was collected in a tube containing K2 EDTA as an anticoagulant (BD Microtainer, NJ, USA). Once the samples were taken, they were immediately stored at 4°C using plates of cryoconservation gel and hermetic receiving containers for their transfer to the Genomic Medicine Laboratory of the Regional Hospital "Primero de Octubre" ISSSTE. The samples were processed for the genomic DNA extraction by using Illustra microcolumns (Blood Genomic Mini Prep Spin Kit) of General Electric Healthcare (Piscataway, NJ, USA). Polymerase chain reaction (PCR) amplification of the corresponding fragments of the NOS2A promoter region, including the (CCTTT)n microsatellite site, was performed in a 9700 thermal cycler (Applied Biosystems) using the following primers: 5'-ACC CCT GGA AGC CTA CAA CTG CAT- 3' and 5'-GCC ATC GCA CCC TAG CCT GTC TCA-3'. The PCR reaction was performed in a final volume of 25  $\mu$ L including PCR buffer 1X, 100  $\mu$ M dNTPs, 200  $\mu$ M of each primer, 500  $\mu$ M MgSO<sub>4</sub>, 0.2 U Pfx DNA polymerase and 20 ng of genomic DNA. Cycling conditions included an initial denaturing of 95°C for 5 minutes followed by 35 cycles at 95°C for 30 s, 65°C for 30 s and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. In order to determine if there was contamination, negative controls were included in each PCR reaction. Samples were analyzed by electromagnetic electrophoresis in Horizon 20.25 chamber (Whatman) on 2% agarose gels at 100V and 400 mA run for 2 hours, waiting a size bands from 141 bp to 236 bp for alleles with 1 and 20 repeats of the CCTTT microsatellite, expecting a delta of 5 bp for each additional microsatellite. To ensure that heterozygous alleles were correctly identified, bands were recovered and extracted using the QIAquick Gel

Extraction Kit (QIAGEN, Hilden, Germany). Once purified the DNA was used in sequencing PCR using genomelab methods development kit cycle sequencing dye terminator (beckman coulter, galway, ireland) and afterwards cleaned using the agencourt cleanseq kit (Beckman Coulter, Galway, Ireland). Products were then subjected to direct sequencing electrophoresis in an automated CEQ8000 Beckman DNA sequencer (Beckman Coulter, Galway, Ireland). The resulting chromatograms were aligned and viewed with Chromas 2.3 software (Technelysium Pty Ltd, Ewantin, Australia) using the GenBank NOS2A reference genomic sequence accession number AF440785.

### Statistical analyses

To test for differences in frequency distributions of genotypes between groups the  $\chi^2$  test was used. Age and sex adjusted odds ratios (ORs) and 95% confidence intervals (CI) were calculated by logistic regression analysis. In all statistical tests  $P < 0.05$  was considered to be statistically significant. Data analysis was carried out using the PSPP 0.6.0 (PSPP) open source software (GNU licence) and graphical data representation was done with QtiPlot 0.9.6.2 open source software (GNU licence).

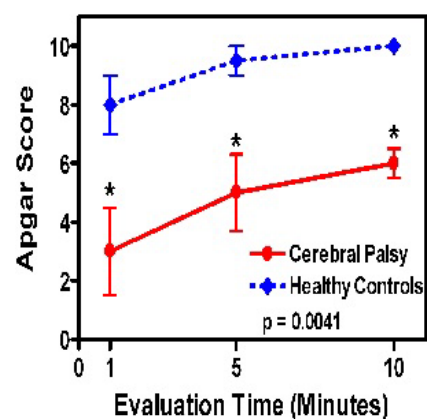
## Results

### Selection of study subjects

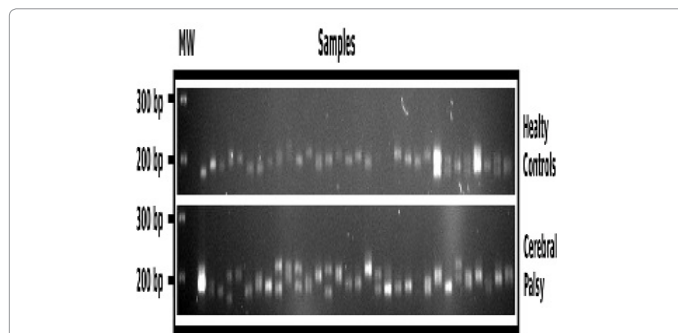
Forty-eight patients for a genomic study of infant cerebral palsy (ICP) were included based on four inclusion criteria: (1) Term babies with more than 36 weeks gestation; (2) Demonstrable history of perinatal asphyxia in neonatal medical records; (3) No metabolic diseases; and (4) No brain malformations. Thus, all individuals included in the patient group had been diagnosed with infant cerebral palsy by clinical, radiological and electroencephalographic criteria. These individuals were matched by age and gender to 57 apparently healthy control individuals in whom neurological diseases had been excluded by clinical neuropediatric evaluation.

### Perinatal asphyxia history

The cerebral palsy group was composed of 67.7% of men and 32.3% of women, while control group was composed by 42.1% of men and 57.9% of women. The Apgar score in patients with infant cerebral palsy was  $3 \pm 1.48$ ,  $5 \pm 1.31$  and  $6 \pm 0.5$  at 1, 5 and 10 minutes after birth respectively, whereas in the control group these were  $8 \pm 1$ ,  $9.5 \pm 0.5$  and 10 at 1, 5 and 10 minutes after birth respectively (Figure 1).



**Figure 1:** Time course of APGAR score. All APGAR scores ratings were obtained from the data in the medical records of the participants in the CRIT Tlalnepanltla, with prior informed parental consent.



**Figure 2:** Polymorphic alleles of CCTTTn microsatellite in the promoter region of NOS2a gene. Representative electrophoretic pattern in agarose gel 2.5% prestained with ethidium bromide, the photograph shows the vast (CCTTT)<sub>n</sub> microsatellite variability in the promoter region -2.6 Kb which regulate the transcription of the inducible nitric oxide synthase type A (iNOSa or NOS2A) by interleukin-1 $\beta$ .

Genotype	(CCTTT) <sub>n</sub>	Groups		p Value	OR (95% CI)
		Healthy Controls	Cerebral Palsy		
Short	n $\leq$ 12	38 (66.7 %)	27 (57.5 %)	0.0001*	0.3328 (0.245-0.451)
Pathological	n = 13	6 (10.5%)	5 (10.6%)	0.1846 NS	0.6053 (0.311-1.178)
	n = 14	7 (12.3%)	10 (21.3%)	0.0122*	1.779 (1.150-2.752)
Long	n $\geq$ 15	6 (10.5%)	5 (10.6%)	0.2652 NS	0.7036 (0.405-1.222)

**Table 1:** General alleles frequency of -2600(CCTTT)<sub>n</sub> microsatellite of NOS2A promoter in patients with cerebral palsy secondary to hypoxia-ischemia perinatal.

### Electrophoretic analysis

Electrophoretic mobility on agarose gels of PRC products of the -2600(CCTTT)<sub>n</sub> microsatellite revealed the presence of different sizes of maternal and paternal alleles with high molecular weight variability in the Mexican population which does not allow direct sequencing analysis from genomic DNA (Figure 2).

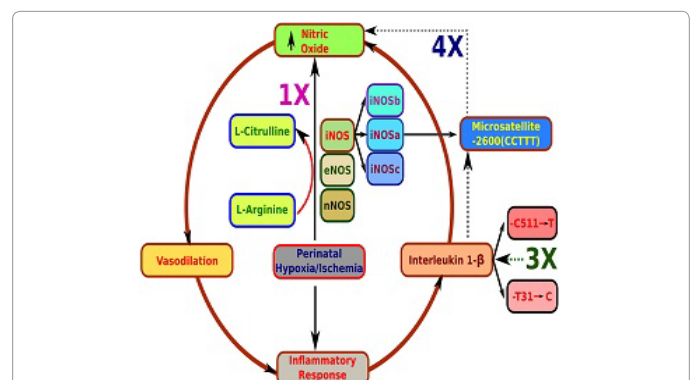
### Allele frequency of the CCTTT microsatellite polymorphisms

A total of 13 different alleles (5 to 17 repeats) were found in healthy individuals and cerebral palsy patients. The frequency distribution of microsatellite alleles followed a normal distribution ranging from 6.4% for 3 repeats, 2.1% for 1 repeat with a peak of 31.9% for 12 repeats (Figure 1). Short-form alleles ( $\leq$ 12 repeats) were found to be present in 57.5% (27 out of 47) of cerebral palsy patients and 66.74% (38 out of 57) of healthy controls; also, the physiological form 13 repeats, was found to be present in  $\approx$ 10% of both cerebral palsy and healthy control groups, with 5 and 6 patients respectively, while the 14-repeat long allele was present in 21.3% of the cerebral palsy patients and 12.3% of healthy controls (10 and 7 individuals, respectively). Long form alleles ( $\geq$ 15 repeats) were found to be present in  $\approx$ 10% (4 out of 48) of cerebral palsy patients and  $\approx$ 10% (6 out of 57) of healthy controls (Table 1).

Statistical analysis revealed that the short form do not have correlation with cerebral palsy development (OR=0.3328, 95% CI=0.245-0.451, P=0.822) while the 14-repeat allele was significantly associated with the development of cerebral palsy in patients with a history of perinatal asphyxia (OR=1.779, 95% CI=1.150-2.752, P=0.0122). Linear regression analysis showed that the risk of developing cerebral palsy significantly increases as the repeat number in an individual increases (OR=1.29, P=0.023).

### Discussion

Nitric oxide acts as an extra- and intercellular messenger participating in vascular homeostasis, neurotransmission, and has been associated with various neuropathologies [28]. However, to our knowledge the role of NO in human infant cerebral palsy has not been studied. Some studies have described the role of NO as protective in various neuropathologies [29] although other authors consider said molecule as pathogenic [30]. Some studies have attributed the appearance of neuropathologies to an increased production of NO by NOS2A causing direct neurotoxicity or vasodilatation and increased cerebral pressure [31]. In this study, we have tried to establish if there is a correlation between NOS2A and the development of infant cerebral palsy in patients with a history of perinatal hypoxia/ischemia by analyzing the association of previously described NOS2A-promoter variants responsible for increased NO production. Furthermore, this is the first study to be carried out in Mexican population and it is also relevant as it provides an insight into the population's mixed race. *In vitro* experiments using a luciferase gene reporter assay have shown that when there are 13 or 14 microsatellite repeats in the promoter region the transcription rate of NOS2A increases 4X when induced by oxygen and glucose deprivation; furthermore, IL-1 $\beta$  also increases the transcription of NOS2A by 4X when there are 13 or 14 microsatellite repeats [19]. We observed an association of the 14 repeat-long allele with the development of cerebral palsy in children with a history of perinatal asphyxia, an increased number of microsatellite repeats is a significant risk factor for severe neurological sequels in Mexican population. We think it is possible that in neonates that suffer perinatal asphyxia with an APGAR score lower than 7 at 10 minutes the 14-repeat allele is responsible for increasing the level of NO by influencing the transcription rate of NOS2A in infant cerebral palsy patients. NO induces the expression of IL-1 $\beta$ , which can up regulate the transcription of NOS2A via stimulation by the (CCTTT)<sub>14</sub>-repeat microsatellite allele (Figure 3). On the other hand, the presence of the shorter 12-repeat microsatellite allele, possibly downregulating the production of proinflammatory cytokines such as IL-1 $\beta$  in response to perinatal asphyxia, might explain the decreased risk of developing infant cerebral palsy in individuals that have short-form CCTTT microsatellite alleles. Since microsatellites are potentially unstable and of unknown functional relevance [32] the presence of some other yet-not-described mutation in the promoter region or the coding gene



**Figure 3:** Polymorphic variations in the NOS2a and IL1b genes implicated in PCI development in patients that suffered perinatal asphyxia. The (CCTTT)<sub>14</sub> microsatellite in the NOS2 gene increase 4 times the NO production. The -511 and -31 polymorphisms in the IL-1b gene increase its transcription and 3 times the NO production, the haplotypes (CCTTT)<sub>14</sub>-511T increase 6 or more times the NO production, conducting to massive neuronal death by oxidative stress, this condition allows the PCI development like a sequel in children that suffered perinatal asphyxia.



itself, which might be increasing the susceptibility for developing infant cerebral palsy in Mexican patients with a history of perinatal asphyxia, cannot be ruled out. Nevertheless, our results suggest that the (CCTTT)<sub>n</sub> microsatellite in the promoter of the NOS2A gene in patients that suffered perinatal asphyxia could be used immediately after birth to include in the treating an unspecific inhibitor of NOS as L-NG-Nitroarginine Methyl Ester (L-NAME) or a selective inhibitor for iNOS such as glucocorticoids (dexamethasone) during HIE status, in order to reduce the overproduction of nitric oxide and subsequent oxidative stress, mainly in patients with an expansion of 14 repeats of CCTTT microsatellite, alternatively the expansion could be used to identify patients that require a strong early stimulation program in order to induce synaptic plasticity in the hypoxic/ischemic brain and prevent the development of infant cerebral palsy

### Author's Contributions

STM carried out the diagnosis and treatment of the ICP patients, MRTB and BALCH obtained the blood samples, extracted DNA and genotyped the -2600(CCTTT)<sub>n</sub> microsatellite, supported the molecular cell biology work. DMF and JAGB conceived the paper, prepared the tables and figures, made the statistical analysis and prepared the final draft of the paper. All authors read and approved the final draft of the manuscript.

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