

***NR3C1* gene methylation and cortisol levels in preterm and healthy full-term infants in the first three months of life.**

Genomic DNA (gDNA) extraction

Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells or oral mucosal cells using the DNeasy Blood & Tissue Kit (#69506, Qiagen, Hilden, Germany). Exclusively for the extraction of DNA from cells of the oral mucosa, some initial steps were necessary: centrifugation of the tubes at 1000 rpm for 30 seconds, incubation of the tubes containing the samples at 50 °C for 1 hour, and homogenization in a new 15 mL tube. From these preliminary steps, both biological materials (blood or buccal swabs) followed the same procedures described in the manufacturer's protocol. After the extraction step, the DNA samples were spectrophotometrically quantified at 260, 280, and 230 nm, using the NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, USA). Subsequently, the analysis of the integrity of the genomic DNA was also performed by observing the bands after horizontal electrophoresis in a 1% agarose gel.

Sodium Bisulfite Conversion and PCR

According to the manufacturer's instructions, conversion by sodium bisulfite was performed using the EZ DNA Methylation kit (#D5002, Zymo Research, Irvine, USA), starting from 1000 ng of gDNA that were finally eluted in 24 uL of M-Elution buffer. After DNA conversion by sodium bisulfite, samples were amplified using the PyroMark PCR kit (#978703, Qiagen). Amplifications were performed in a final reaction volume of 50 µL, containing 0.2 µM of each primer and 2 µL of converted DNA, together with the Master Mix provided in the kit. Amplifications were performed at an initial denaturation of 95 °C for 15 minutes, 45 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds and extension at 72 °C for 30 seconds and a final extension at 72 °C for 10 minutes, according to the manufacturer's protocol. The ProFlex™ 3 x 32-well PCR System thermal cycler (ThermoFisher Scientific, Waltham, USA) or the Veriti 96-Well thermal cycler (Applied Biosystems, Waltham, USA) was used. All amplification reaction products were analyzed by electrophoresis in 1% agarose gel to verify the correct amplification size in the L-PIX gel photodocumentation system (Loccus Biotecnologia, São Paulo, Brazil). So that later, only one of the amplified strands was selected, one of the oligonucleotides used in the PCR reaction was labeled with biotin (primer #2, Table A).

Table A. List of primers developed for the amplification of *NR3C1* and for the sequencing of the 40 CpG sites of its 1_F region. In the nomenclature of primers _EX-1F_ means Exon 1 Region 1F, F_1 means Forward primer and R_1 means Reverse primer.

#	Primers	Number of bases	Sequence 5' - 3'
1	NR3C1_EX-1F_F_1	22	AAT TTT TTA GGA AAA AGG GTG G
2	5Bio_NR3C1_EX-1F_R_1	18	/5Biosg/TC CCT TCC CTA AAA CCT C
3	NR3C1_EX-1F_R_1	18	TCC CTT CCC TAA AAC CTC
4	NR3C1_EX-1F_Seq_1	14	GTG AAG TGT GTT AT
5	NR3C1_EX-1F_Seq_2	13	GTT ATT TTT TTT T
6	NR3C1_EX-1F_Seq_3	14	GTT TTG TTT TTT GG
7	NR3C1_EX-1F_Seq_4	10	GTG AGT GGT T
8	NR3C1_EX-1F_Seq_5	13	GAG TTT TTT TAG T
9	NR3C1_EX-1F_Seq_6	13	GTT AGT GTT TTT G

Design of primers used in PCR reaction and sequencing

Primers were designed to amplify the regions of interest that included a total of 40 CpG sites of exon 1_F of the *NR3C1* gene, according to the standard numbering of sites reviewed in Chalfun et al, 2021 (<https://pubmed.ncbi.nlm.nih.gov/34519616/>). The primers were designed with the aid of the PyroMark Assay Design SW2.0 (Qiagen, Germany). The oligonucleotides (primers #1 to #3, Table A) design parameters used in PCR reaction were:

- Minimum oligonucleotide size: 18 nucleotides.
- Maximum oligonucleotide size: 30 nucleotides.
- Optimal amplicon size: 300 nucleotides.
- Maximum amplicon size: 700 nucleotides.
- Annealing of oligonucleotides in variable position (CpG sites) was not allowed.
- Algorithm used to calculate annealing temperature: NM mismatch.
- Oligonucleotide concentration: 0.2 µM.
- Minimum annealing temperature: 50 °C.
- Maximum annealing temperature: 72 °C.
- Maximum difference in annealing temperature between oligonucleotides: 10 °C.
- Maximum difference of GC content: 50%.

Additionally, for the design of the sequencing oligonucleotides (primers #4 to #9, Table A), the following parameters were used:

- Minimum oligonucleotide size: 10 nucleotides.
- Maximum oligonucleotide size: 25 nucleotides.
- Minimum target distance: 0 nucleotides.
- Maximum target distance: 10 nucleotides.
- Annealing of oligonucleotides in variable position (CpG sites) was not allowed.

- Algorithm used to calculate annealing temperature: NM mismatch.
- Minimum annealing temperature: 29 °C.
- Maximum annealing temperature: 59 °C.

For the sequencing of the 40 CpG sites of the study, 6 sequencing primers were used. The *NR3C1*_EX-1F_Seq_1 sequencing primer allowed the analysis of the methylation of a specific region of the *NR3C1* gene that includes the CpG sites (8 to 13). *NR3C1*_EX-1F_Seq_2 was used for the sequencing of CpG sites (27 to 38), *NR3C1*_EX-1F_Seq_4 covered the sequencing of CpG sites (14 to 21). *NR3C1*_EX-1F_Seq_5 made up the CpG sites (37 to 39) and *NR3C1*_EX-1F_Seq_6 the CpG sites (22 to 29). For CpG sites 40 to 47 was used the forward primer *NR3C1*_EX-1F_F_1 (primer 1, Table A), the same primer used in the PCR reactions. The *NR3C1*_EX-1F_Seq_3 primer partially worked in the sequencing tests and was excluded from the study, however without causing damage to the complete coverage of the 40-sites sequence. This was possible because this primer was designed to amplify regions that spanned the CpG16 to CpG32 sites, covered by the sequencing primers *NR3C1*_EX-1F_Seq_2, *NR3C1*_EX-1F_Seq_4 and *NR3C1*_EX-1F_Seq_6, which worked completely. The region where the CpG sites (1 to 7) are located was not analyzed, as it is a region of difficult annealing for the primer, formed by the repetition of a series of thymine nucleotides that lead to polymerase-related “slippage” phenomena. In this region, during the replication action, the DNA polymerase enzyme undergoes a slippage, promoting insertions or deletions of base pairs that could impair the correct sequencing.

Methylation Analysis of CpG Sites by Pyrosequencing

In this step, PCR products were collected using streptavidin-coated beads diluted in binding buffer (10 mM Tris-HCl; 2 mM NaCl; 1 mM EDTA; 0.1% Tween 20 – pH 7.6) with the purpose of binding only the biotinylated strands. After 10 minutes of agitation, the beads were aspirated using the Vacuum Prep Workstation, washed with 70% ethanol, followed by denaturation with NaOH and washing (10 mM Tris-Acetate – pH 7.6). After addition of 40 µL of 0.4 mM sequencing primer solution diluted in annealing buffer (20 mM Tris-Acetate; 2 mM MgAc₂ – pH 7.6), the MIX (beads and oligonucleotides) was denatured by 2 minutes at 80 °C and then cooled for 20 minutes to reach the annealing temperature of the primers. The volumes of dNTPs, enzymes and substrate (all components of the PyroMark Gold Q96 Reagents kit (5 x 96) were defined according to the sequence to be injected. Subsequently, the biotinylated strand was then sequenced with the PyroMark 96 ID Platform system (Qiagen). The percentage of each CpG site was generated automatically in the PyroMark Q96 software (version 2.5.8) using the default quality control settings.