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Brief Report

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LHX6 promoter hypermethylation in oncological pediatric patients conceived by IVF

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Abstract

The multifactorial etiology of pediatric cancer is poorly understood. Environmental factors occurring during embryogenesis can disrupt epigenetic signaling, resulting in several diseases after birth, including cancer. Associations between assisted reproductive technologies (ART), such as *in vitro* fertilization (IVF), and birth defects, *imprinting* disorders and other perinatal adverse events have been reported. IVF can result in methylation changes in the offspring, and a link with pediatric cancer has been suggested. In this study, we investigated the peripheral blood methylomes of 11 patients conceived by IVF who developed cancer in childhood. Methylation data of patients and paired sex/aged controls were obtained using the Infinium MethylationEPIC Kit (Illumina). We identified 25 differentially methylated regions (DMRs), 17 of them hypermethylated, and 8 hypomethylated in patients. The most significant DMR was a hypermethylated genomic segment located in the promoter region of *LHX6*, a transcription factor involved in the forebrain development and interneuron migration during embryogenesis. An additional control group was included to verify the *LHX6* methylation status in children with similar cancers who were not conceived by ART. The higher *LHX6* methylation levels in IVF patients compared to both control groups (healthy children and children conceived naturally who developed similar pediatric cancers), suggested that hypermethylation at the *LHX6* promoter could be due to the IVF process and not secondary to the cancer itself. Further studies are required to evaluate this association and the potential role of *LHX6* promoter hypermethylation for tumorigenesis.

Introduction

Assisted reproductive technologies (ARTs) can increase the risk of birth defects and other perinatal adverse events in the offspring.[1,2] Furthermore, there is a positive association between ART, particularly *in vitro* fertilization (IVF), and a higher risk of imprinting disorders, possibly caused by epigenetic modifications in imprinted genes.[3,4]

Considering the Developmental Origins of Health and Disease (DOHaD), some features of ART, like culture media, incubation conditions and embryo manipulation, can impact in the embryo development and epigenome.[5,6] This notion has raised concerns about health problems in IVF individuals during infancy or adulthood, such as low birth weight, diabetes, obesity and cancer.[5,6] A yet controversial link between ART and pediatric cancer has been suggested. In 2005, a meta-analysis based on 11 cohort studies found no relation between increased risk of childhood cancer and ART patients.[7] In 2013, another meta-analysis that evaluated 25 cohort and case-control studies reported that ART and/or fertility treatment increased the risk for specific cancer types, namely leukemia, neuroblastoma, and retinoblastoma.[8]

More recently, two additional large meta-analyses have been published. Following the analysis of 327,884 children conceived after fertility treatment, in which 578 were diagnosed with cancer, Wang *et al.*[9] found an increased risk of developing cancer, especially leukemia and hepatic tumors. The cancer risk is further increased when ART alone is considered, without the use of fertility drugs. Contradicting these findings, a meta-analysis based on 750,138 ART conceived children and 21,400,800 controls did not find an overall increase in risk of pediatric cancer in either ART or IVF.[10]

The investigation of possible epigenetic alterations caused by IVF may offer insights about its potential association with malignancies.[9] Here, we investigated epimutations in the methylomes of a small cohort of eleven patients conceived by IVF who developed pediatric cancer.

Patients and methods

Samples

DNA was extracted from peripheral blood of eleven individuals conceived by IVF who developed pediatric cancer (Table 1). Individuals who underwent bone-marrow transplantation or who were diagnosed with hereditary cancer syndromes were excluded. Patients were referred from the ITACI - Childhood Cancer Treatment Institute (FMUSP), which is a reference pediatric cancer hospital in São Paulo, Brazil. Samples were provided after parents have signed the informed consent.

A control group was composed by peripheral blood samples collected from 12 children without cancer history matched by age and sex with patients (Control group 1 – C1) (Supplemental Table S1a).

A second group was added to this study in order to control the methylation status of children with similar diagnosis of cancer who were not conceived by IVF (Control group 2 – C2) (Supplemental Table S1b). This additional group was based on the recovery of germline Illumina 850K methylation data previously obtained from 16 children who developed pediatric cancer and were naturally conceived: five children with neuroblastoma (NB - peripheral blood methylomes provided by the ITACI center; data not published) and 11 children with acute myeloid leukemia (AML - bone marrow or peripheral blood methylomes public available on GEO[11] accession GSE124413).

Infinium MethylationEPIC (850K) array hybridization

Genomic DNA samples were obtained from standard extraction procedures using the phenol/chloroform method. Evaluation of peripheral blood DNA methylation (DNAm) was performed using the Infinium MethylationEPIC (850K) array, according to the manufacturer's instructions. A total of 500 ng of bisulfite-converted DNA samples (EZ DNA Methylation-Gold Kit; Zymo Research) were hybridized in the Infinium MethylationEPIC BeadChip array (Illumina). The raw image data with signal intensities were captured with the iScan SQ scanner (Illumina) and collected as IDAT files.

DNA methylation analysis

We applied the Chip Analysis Methylation Pipeline (ChAMP) package (version 2.20.1)[12] in the R environment (version 4.0.4)[13] for the methylation analysis. The quality filters removed 3,960 probes with a detection *P*-value above 0.01, 34,605 probes with a bead count <3 in at least 5% of samples, and non-CG sites (2,865). In addition, 94,529 SNP-related probes and 18 probes that aligned to multiple locations were removed. Lastly, 15,818 probes located on the X or Y chromosomes were excluded.[14,15]

The beta-mixture quantile normalization (BMIQ) method[16] resulted in better Infinium I/II normalization compared to PBC[17] or SWAN.[18] Singular value decomposition (SVD)[19] reported the need for array and slide correction, made by ComBat.[20,21] We adjusted the cell-type heterogeneity using the Refbase EWAS method.[22]

Differential DNAm analysis

Methylation differences were identified by comparing patients and controls and using algorithms implemented by ChAMP.[12] Differentially methylated positions (DMPs) analysis, i.e., methylation difference for a single CpG site, was performed using Limma.[23,24] Differentially methylated regions (DMRs) analysis was performed using the Bumphunter algorithm,[25] based on the detection of methylation differences in stretches of the genome in which there are several consecutive CpG sites exhibiting similar methylation alterations. Both analyses were performed considering *P*-value <0.05. In addition, DMRs should have a minimum of seven consecutive probes with changes in DNAm in the same direction (hypo or hypermethylated).

Table 1. Clinical features of 11 individuals conceived by IVF who developed pediatric cancer

ID	Tumor type	Sex	Age at diagnosis (years)
P1	B-Cell Acute Lymphoblastic Leukemia	F	7.0
P2	B-Cell Acute Lymphoblastic Leukemia	F	6.0
P3	B-Cell Acute Lymphoblastic Leukemia	F	1.9
P4	Acute Myeloid Leukemia	M	14.6
P5	Acute Myeloid Leukemia	M	10.2
P6	Neuroblastoma	F	1.8
P7	Neuroblastoma	M	3.6
P8	Neuroblastoma	F	1.4
P9	Ganglioneuroma	M	2.7
P10	Astrocytoma	F	4.6
P11	Melanoma	F	8.5

Results

Eleven patients with pediatric cancer who were conceived by IVF were evaluated (Table 1). Cancer types were hematological malignancies (three patients with B-cell acute lymphoblastic leukemia, and two with acute myeloid leukemia), and nervous system tumors (three patients with neuroblastoma, one with astrocytoma, and one with ganglioneuroma). One patient developed melanoma.

Following exclusion of probes during quality control steps, the methylation analysis proceeded with 714,443 probes for data correction and normalization. The differential methylation analysis compared the group of 11 patients conceived by IVF who developed childhood cancer (IVF/cancer) with 12 unrelated healthy controls (group C1); the goal of this analysis was to detect differences in the blood methylomes of IVF/cancer group possibly related to patient's phenotypes. However, no significant differential methylated position - DMP (adjusted *P*-value <0.05) was found after Benjamini–Hochberg adjustment for multiple testing.[26]

The methylation analysis detected 25 DMRs (Supplemental Table S2), 17 of which were hypermethylated and 8 were hypomethylated in patients. The most relevant DMR was hypermethylated in patients compared to healthy individuals from control C1 ($\Delta\beta = 0.07$). This DMR is located in the promoter region of the *LHX6* gene, mapped to 9q33.2. The *LHX6* DMR encompassed nine CpG sites (Fig. 1), extending on a genomic segment of 803 bp. This genomic segment included only CpGs mapped to transcription start sites 1500 and 200 (TSS1500 and TSS200); four of these CpGs (cg00774728, cg00485681, cg17434149, cg21237939) map to a CpG island (chr9: CpG island 254, hg19), and five CpGs (cg06347782, cg04201727, cg11328695, cg22254104, cg02539128) to the shore (Table 2).

The heatmap using the beta values of the CpGs mapped to *LHX6* of patients and controls C1 (Fig. 2a)[27] revealed that mostly

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Table 2. Promoter region of the *LHX6* gene and its DMR genomic features

ID	Chromosome	Genomic coordinates (hg19)	Strand	Probe type (1)	Gene	Location in the gene (2)	CGI location (2)
cg00774728	9	124990632	F	I	LHX6	TSS1500	Island
cg00485681	9	124990761	R	II	LHX6	TSS1500	Island
cg17434149	9	124990763	R	II	LHX6	TSS1500	Island
cg21237939	9	124991047	R	II	LHX6	TSS200	Island
cg06347782	9	124991182	F	I	LHX6	TSS1500	Shore
cg04201727	9	124991209	F	I	LHX6	TSS1500	Shore
cg11328695	9	124991348	F	I	LHX6	TSS1500	Shore
cg22254104	9	124991432	F	II	LHX6	TSS1500	Shore
cg02539128	9	124991435	F	II	LHX6	TSS1500	Shore

(1) Probe type according to different probe designs (Infinium I and Infinium II) in the Illumina Methylation chip
(2) Annotations for the CGIs genomic location in relation to gene sequence were done according to Illumina's CpG loci database.

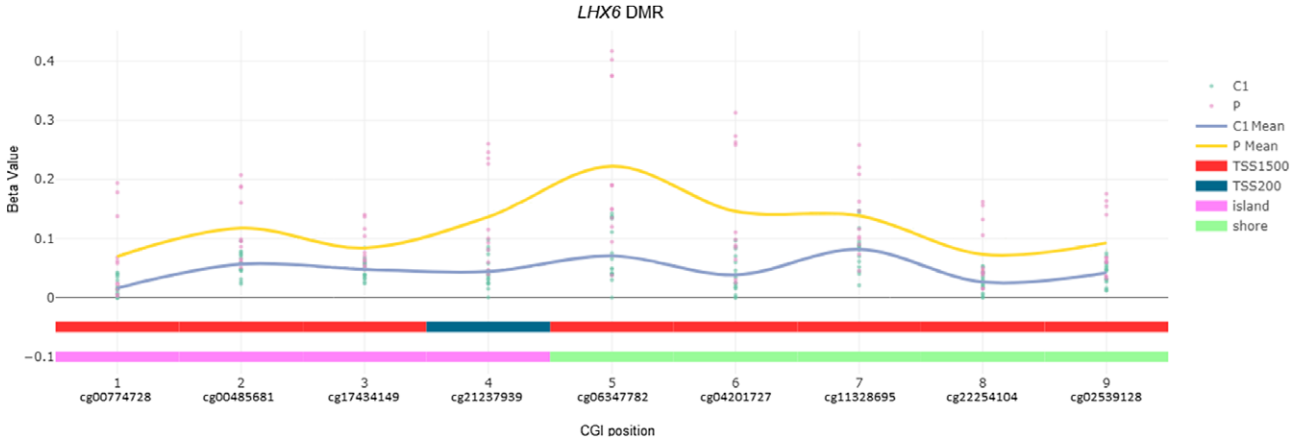

Fig. 1. Hypermethylated DMR at the promoter region of the *LHX6* gene. Plot (image extracted from ChAMP)[12] showing the beta values of methylation of each CpG site mapped to the *LHX6* gene. Green dots: individuals from the healthy control group (C1); Pink dots: individuals from the patient group (P); C1 mean: average beta value from the control group; P mean: average beta value from the patient group; TSS1500: transcription start site 1500; TSS200: transcription start site 200; island: CpG island; shore: CpG shore.

four patients in the IVF group (P1, P4, P5, and P6) contributed to the identification of the DMR, although patients P3, P7 and P8 also contributed to this pattern of increased methylation. Considering this observation, we added a second control group to this study (control C2), based on the recovery of Illumina Infinium MethylationEPIC germline data previously obtained from 16 children who developed pediatric cancer and were naturally conceived: five children with neuroblastoma and 11 children with acute myeloid leukemia. Methylation levels of the nine CpG sites mapped to *LHX6* were retrieved from all cases for comparison between the three groups: the four IVF patients, the control C1 (healthy children) and the cancer control C2 groups (Fig. 2b). Seven out of nine CpGs (cg00485681, cg17434149, cg21237939, cg06347782, cg04201727, cg22254104, and cg02539128) exhibited higher methylation levels in the subgroup of patients when compared to both control groups (Fig. 2c and Supplemental Table S3).

Discussion

Parental subfertility, parental age at conception, children sex, low birth weight, and other environmental factors, such as tobacco and alcohol consumption during pregnancy, can result in a biased risk of childhood cancer associated with ART.[8,9,28] Thus, a link between ART and pediatric cancer remains controversial. Considering the yet disputed association between pediatric cancer and ART,[7–10] in which ART is suggested to cause epigenetic modifications that could increase the risk of cancer, it is crucial to investigate DNA methylation in these patients.[3,9] In previous studies, increased risk rates for specific types of cancer such as leukemia and neuroblastoma were detected among children born following fertility treatment.[8,9] Likewise, our study IVF cohort included eight patients diagnosed with leukemias or neuroblastoma.

Current research regarding epigenetic alterations associated with ART are generally related to imprinting disorders.[3,4] Previous studies that investigated the association between ART and pediatric cancer did not explore the presence of DNAm changes in patients conceived by IVF that developed pediatric cancer.

DMPs were not detected after multiple testing adjustments. A possible explanation for this situation can be the small number of patients. Small group analysis may not identify real changes because they do not reach statistical power when evaluating hundreds of thousands of sites simultaneously.[29] In addition, one could

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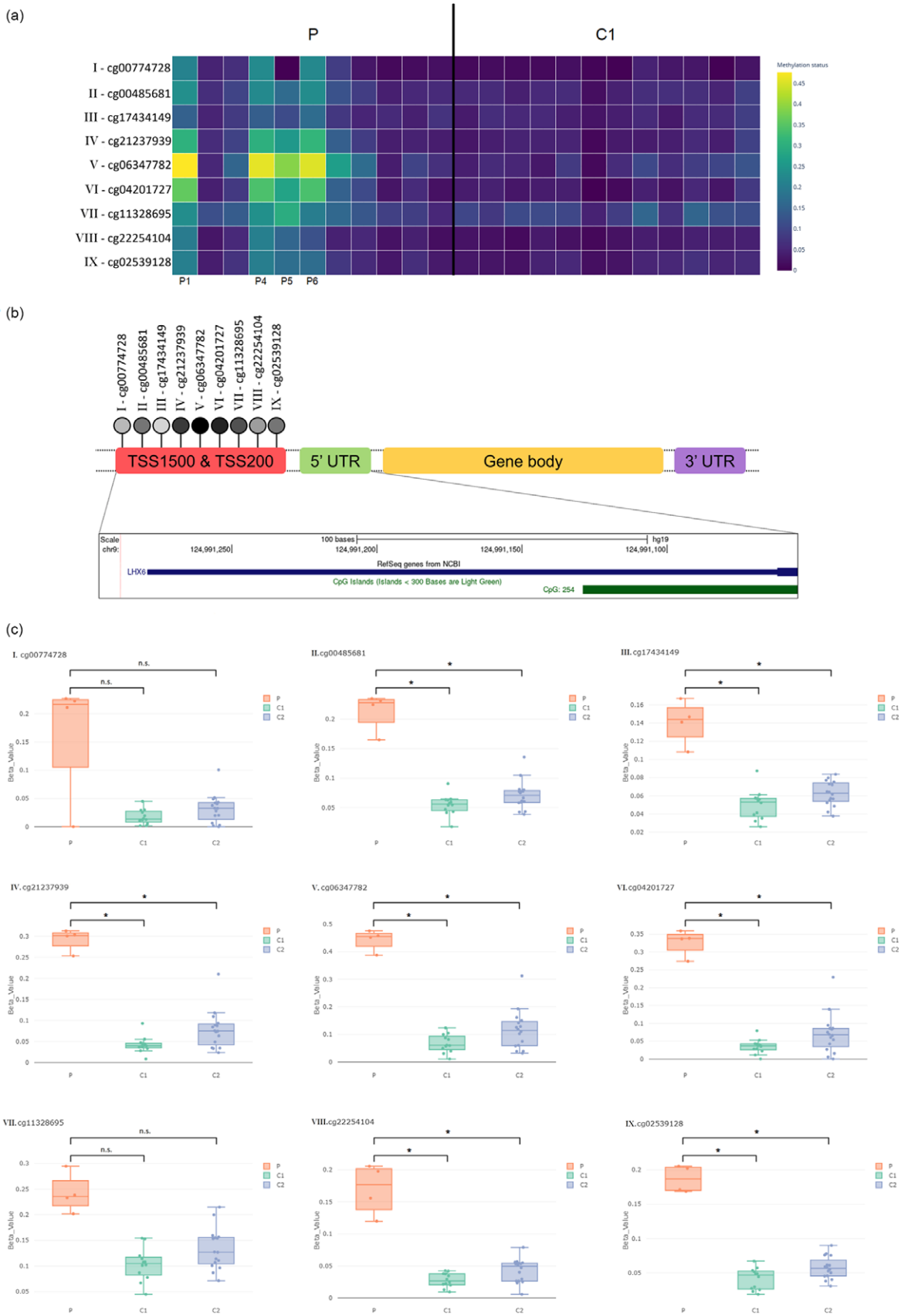

Fig. 2. DNA methylation (DNAm) pattern from the CpGs located in the *LHX6* DMR that was found in all analyses. ***a.*** Heatmap (image extracted from Plotly)[27] showing the DNAm level of nine DMPs located in the original DMR revealed in the analysis (P and C1 groups separated by the black line). ***b.*** Regions of the *LHX6* gene with the CpG sites from the DMR depicted as numbered lollipops. Promoter region image extracted from UCSC genome browser. ***c.*** Boxplots (images extracted from ChAMP)[12] of each CpG site with the respective methylation level for the three groups (orange: 4 patients - P, green: healthy controls - C1, blue: cancer controls - C2). n.s. are non-significant DMPs. *Statistically significant DMPs.

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argue that robust DNAm changes were not detected because the methylation analysis was done in peripheral leukocytes obtained from patients and not on tumor tissues which presents a higher specificity.[19,30] Even though DNAm changes in blood samples are generally small, most epigenetic alterations related to diseases occurring during development can be detected in surrogate tissues.[31] Recently, a large study evaluated the cord blood DNAm from 205 ART cases and 2,439 naturally conceived controls revealing two CpG sites associated with ART, as well as related to cancer, aging and HIV infection by EWAS studies.[32–35] Therefore, the use of peripheral blood samples seems to be a suitable strategy for searching for epigenetic variations, which may serve as good biomarkers for cancer risk.

The most significant DMR found in this study maps to the promoter region of the transcriptional factor *LHX6*, which is involved in embryogenesis and head development.[36,37] Thus an additional control group was included to verify the *LHX6* methylation status in children with similar cancers who were not conceived by ART. These analyses excluded the possibility that this DMR was driven by the occurrence of the cancer and not the IVF itself. The higher *LHX6* methylation levels in IVF patients compared to both control groups, healthy children and children conceived naturally who developed similar pediatric cancers (neuroblastoma and leukemia), suggested that hypermethylation at the promoter of the *LHX6* is likely due to the IVF process and not secondary to the cancer itself. During neurodevelopment, *LHX6* is expressed in the ventral forebrain and in tangentially migrating GABAergic interneurons from the neocortex and hippocampus.[36,38] This gene is a potential tumor suppressor gene in glioma,[39] pancreatic,[40] head and neck,[41] breast,[42] lung,[43] and cervical[44] cancer. Particularly, *LHX6* may affect signaling pathways such as the Wnt/β-catenin in breast,[42] lung[45] and liver cancer,[46] *TP53* in hepatocarcinoma[46] and PI3K/Akt/mTOR in breast cancer.[47] *LHX6* promoter hypermethylation has already been related to transcriptional silencing, and it is described as either hypermethylated or partially methylated in cervical, head and neck, pancreatic, lung and liver cancers.[40,41,43,44,46,48] Likewise, the DMR we found is hypermethylated in the promoter region.

Therefore, the *LHX6* promoter hypermethylation previously associated with cancer and detected in this study, can be suggested as an epimutation that increases the risk of cancer in the patients herein reported. There are possible confounding factors such as low birth weight, parental age, type of infertility leading to IVF, use of fertility drugs, maternal smoking, and patient tumor, which can result in methylation alterations.[9] Another aspect not investigated here was the genetic susceptibility to cancer of this group of patients. A recent study that analyzed the methylation profile of 23,116 individuals[49] reported that 2/3 of the epivariations segregated according to underlying sequence variants, while the other 1/3 occurred post-zygotically. Therefore, the identified epimutations in these patients could also be attributed to genetic variants, driving both the methylation pattern and increased cancer risk.

In conclusion, we searched for possible variations in DNAm that could be linked to increased risk of childhood cancer in children conceived by IVF. A hypermethylated DMR in patients was detected in the promoter region of *LHX6*, a gene previously associated with cancer when its promoter region is hypermethylated. Therefore, this DMR can be an epimutation contributing to increased cancer risk in some children conceived by IVF and thus deserves additional investigation.

Supplementary materials. For supplementary material for this article, please visit https://doi.org/10.1017/S2040174422000526

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Conflict of interest. None

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation (Resolution 466/12) and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the Ethics and Research Committee of ITACI (CAAE 47277115.0.0000.0068), and Institute of Biosciences (University of São Paulo, São Paulo, Brazil) (CAAE: 09163818.4.0000.5464).

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