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A.O. Nikulina

SIGNIFICANCE OF SINGLE-NUCLEOTIDE VARIANTS OF ANOREXIGENIC HORMONE GENES IN CHILDHOOD OBESITY

Dnipro State Medical University
Volodymyra Vernadskoho str., 9, Dnipro, 49044, Ukraine
Дніпровський державний медичний університет
вул. Володимира Вернадського, 9, Дніпро, 49044, Україна
e-mail: anna.nikulina.201381@gmail.com

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Ключові слова: *однонуклеотидні варіанти генів, гени анорексигенних гормонів, метаболічно здорове ожиріння, метаболічно нездорове ожиріння*

Abstract. Significance of single-nucleotide variants of anorexigenic hormone genes in childhood obesity. Nikulina A.O. *Obesity-induced dysregulation of hypothalamic neurons is not completely eliminated by restoring body weight, therefore the most urgent task of modern precision medicine is to predict the trajectory of development of metabolic disorders associated with obesity in children. The aim of the study was to determine the level of association of single-nucleotide variants of genes that determine eating behavior – Neuronal growth regulator 1, Fat mass and obesity associated gene, Glucagon-like peptide-1 receptor, ghrelin, leptin receptor, cholecystokinin, in the development of metabolically unhealthy obesity. 252 obese children aged 6-18 years were examined. The main group (n=152) consisted of children with metabolically unhealthy obesity (MUO) according to Identification and prevention of Dietary- and Lifestyle-induced Health Effects in Children and Infants 2014 criteria. The control group (n=100) consisted of children with metabolically healthy obesity (MHO). All children underwent a general clinical, immunobiochemical examination at the Synevo laboratory (Ukraine). Whole-genome sequencing (CeGat, Germany) was performed in 31 children of the primary and 21 children of the control group. Static analysis: variance analysis ANOVA, method of estimating data*

dispersion, ROC-analysis, method of testing statistical hypotheses. The level of single nucleotide variants association of anorexigenic hormone genes with MUO that exceeded the threshold accepted by 75% of the available data was, respectively, in ascending order: leptin receptor (LEPR) rs1137101 (40.38%), Glucagon-like peptide-1 receptor (GLP1R) rs1126476 (40.38%), GLP1R rs2235868 (42.31%), GLP1R rs1042044 (42.31%), LEPR rs3790435 (48.08%), cholecystokinin (CCK) rs754635 (50%), LEPR rs2186248 (55.76%), GLP1R rs6918287 (55.76%). Genotypes of the GLP1R gene, such as CC rs10305421 determine insulin resistance (F=5.6); GA/AA rs3765468 – meta-inflammation (F=5.8); AA rs6918287 – basal hyperglycemia (F=6.3) and triglyceridemia (F=51.3), p<0.05. Single-nucleotide variants of the gene GLP1R rs6918287, LEPR rs2186248, CCK rs754635 of the anorexic hormones that control eating behavior are highly associated with the presence of metabolically unhealthy obesity in children.

Реферат. Значення однонуклеотидних варіантів генів анорексигенних гормонів при ожирінні в дітей. Нікуліна А.О. *Індукувана ожирінням дисрегуляція гіпоталамічних нейронів не в повному обсязі усувається відновленням маси тіла, тому найактуальнішим завданням сучасної прецизійної медицини є прогнозування траєкторії розвитку метаболічних порушень, пов'язаних з ожирінням у дітей. Метою дослідження було визначити рівень асоціації однонуклеотидних варіантів генів, що детермінують харчову поведінку – нейронального ростового регулятора 1 типу; гена, асоційованого з жировою масою та ожирінням; глюкагоноподібного рецептора 1 типу; греліну; лептинового рецептора; холецистокініну з розвитком метаболічно нездорового ожиріння. Обстежено 252 дитини з ожирінням віком 6-18 років. Основну групу (n=152) представили діти з метаболічно нездоровим ожирінням (МНО) згідно з критеріями Identification and prevention of Dietary – and lifestyle-induced health EFfects In Children and infantS 2014 criteria. Контрольну групу (n=100) склали діти з метаболічно здоровим ожирінням (МЗО). Усім дітям було проведене загальноклінічне, імунобіохімічне дослідження в лабораторії (Synevo, Україна). Проведено повногеномне секвенування (CeGat, Німеччина) в 31 дитини основної та 21 дитини контрольної групи. Статичний аналіз: дисперсійний аналіз ANOVA, метод оцінки розкиду даних, ROC-аналіз, метод перевірки статистичних гіпотез. Рівень асоціації однонуклеотидних варіантів генів анорексигенних гормонів з МНО, що переважав поріг, який приймали 75% наявних даних, відповідно у порядку зростання: лептинового рецептора (leptin receptor – LEPR) rs1137101 (40,38%), глюкагоноподібного рецептора 1 типу (Glucagon-like peptide-1 receptor – GLP1R) rs1126476 (40,38%), GLP1R rs2235868 (42,31%), GLP1R rs1042044 (42,31%), LEPR rs3790435 (48,08%), холецистокініну (cholecystokinin – CCK) rs754635 (50%), LEPR rs2186248 (55,76%), GLP1R rs6918287 (55,76%). Генотипи гена GLP1R, такі як CC rs10305421, детермінують інсулінорезистентність (F=5,6); GA/AA rs3765468 – метазапалення (F=5,8); AA rs6918287 – базальну гіперглікемію (F=6,3) та тригліцеридемію (F=51,3), p<0,05. Високоасоційованими з наявністю метаболічно нездорового ожиріння в дітей є однонуклеотидні варіанти гена GLP1R rs6918287, LEPR rs2186248, CCK rs754635 анорексигенних гормонів, що контролюють харчову поведінку.*

Globalization of the modern standard of living has led to significant changes in eating behavior and physical activity. These changes determine the disruption of energy homeostasis and cognitive-emotional control over body weight regulation under the influence of genetic, epigenetic mechanisms in connection with environmental and socio-cultural trends and lead to the pandemic prevalence of excess body weight and obesity [1]. Over the last four decades, the population of children and adolescents with obesity worldwide has increased by 10-12 times, and it is believed that the increase in obesity may lead to a decrease in life expectancy in the future. If current trends continue, by 2030, about 60% of the world's population will have excess weight or obesity.

Many Genome-Wide Association Studies (GWAS) have proven the genetic determination of an imbalance between energy intake and expenditure localized in more than 400 loci of the human genome as a 40-60% negative contribution to impaired carbohydrate tolerance, diabetes mellitus type 2 (T2DM) [2, 3, 4]. The most unfavorable course of obesity is metabolically unhealthy obesity (MUO), which runs as a "diseasome" of metabolically-associated insulin-resistant diseases and is diagnosed, according to various data, in the range of 0.3–26.4% among children worldwide [5].

The main representatives involved in the regulation of energy consumption are genes: Ghrelin *(GHRL),* Leptin/Leptin receptor *(LEP/LEPR),* Fat mass and obesity associated gene *(FTO),* Glucagon-like peptide-1 receptor *(GLP1R),* Cholecystokinin *(CCK),* Neuronal growth regulator 1 *(NEGR1)* [6, 7]. Given that obesity-induced dysregulation of hypothalamic neurons is not completely eliminated by restoring body weight [8, 9], therefore, the most urgent task of modern precision medicine is to predict the probability of MUO and personalize the prevention of the development trajectory of various comorbid metabolic disorders associated with obesity in children.

The aim of this study: to determine the level of association of single-nucleotide variants of genes that determine eating behavior – Neuronal growth regulator 1, Fat mass and obesity associated gene, Glucagon-like peptide-1 receptor, Ghrelin, Leptin receptor, Cholecystokinin, in the development of metabolically unhealthy obesity.

MATERIALS AND METHODS OF RESEARCH

The study was approved by the commission on biomedical ethics of the DSMU (protocol No. 4 dated 02.09.2020) and was conducted in accordance with the written consent of the parents or guardians of the children and in accordance with the principles of bioethics set forth in the Helsinki Declaration "Ethical Principles of Medical Research Involving Humans" and "General Declaration on Bioethics and Human Rights (UNESCO)". Time of data collection: October 2020 - February 2023.

Research design: observational, analytical, longitudinal, cohort study. Inclusion criteria: children with polygenic obesity (Body Mass Index (BMI)≥97th percentile) aged 6-18 years. Exclusion criteria: children with monogenic and/or syndromic obesity, pregnant women. There were examined 252 obese children aged 6-18 years. The main group (n=152) consisted of children with MUO. The control group (n=100) was formed by children with metabolically healthy obesity (MHO). For inclusion in the main observation group, the presence of abdominal obesity and two of the presented criteria were taken into account:

1. Fasting blood glucose ≥5.6 mmol/l [10] and/or according to the recommendations of the study Identification and prevention of Dietary- and Lifestyle-induced Health Effects in Children and Infants 2014, the level of basal insulinemia that exceeded the 90th percentile [11, 12];

2. High-density lipoprotein cholesterol (HDL-C) ≤ 1.03 mmol/L or less than the 10th percentile for age [13];

3. Triglycerides (TG) ≥1.7 mmol/L or more than the 90th percentile of the age norm;

4. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) above the 90th percentile for age, sex, and height [14]. Abdominal obesity was defined according to the consensus of the International Diabetes Federation based on the excess of waist circumference (WC) above the 90th percentile for children aged 6-15 years and for children aged 16-18 years, more than 94 cm for boys and more than 80 cm for girls [15].

Interleukin (IL)-1β was detected by an immunochemiluminescent assay (IHLA). Analyzer and test system: Immulite (Siemens AG), Germany. The reference value of IL-1β level was 0-5 pg/ml. IL-6 was determined by enzyme-linked immunosorbent assay (ELISA) using a Cobas 6000/Cobas 8000 kit provided by Roche Diagnostics (Switzerland). The reference value of the IL-6 level was 1.5-7.0 pg/ml.

The sample population studied by the method of whole-genome sequencing (NGS, Illumina CSPro®, CeGat, Germany) consisted of 31 children of the main and 21 children of the control group and was qualitatively homogeneous in relation to the general population. The average amount of DNA in the samples is 0.875 μg. Library preparation: 50 ng amount used. Library preparation kit: Twist Human Core Exome plus Kit (Twist Bioscience). Sequencing parameters: NovaSeq 6000; 2 x 100 bp.

Bioinformatic analysis – demultiplexing of sequencing reads was performed using Illumina bcl2fastq (version 2.20). Trimming of adapters is done using Skewer version 0.2.2 [16]. DNA-Seq: trimmed raw reads were aligned to the human reference genome (hg19-cegat) using the Burrows-Wheeler Aligner, BWA – mem version 0.7.17-cegat [17]. ABRA version 2.18 and GenotypeHarmonizer v.1.4.20 were used to locally restructure reads in target regions to improve more accurate detection of indels in the genome after mutagenesis [18, 19]. Clinical and functional variants were used for annotation ClinVar Version 20200316 [20], InterVar gnomeAd Version 3.0 [21] и dbNSFP Version v4 [22] and catalog database annotations GWAS [23]. The reference sequence was obtained from the RefSeq database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/RefSeq/) [24].

Statistical processing of the research results was carried out using Microsoft Excel (Office Home Business 2KB4Y-6H9DB-BM47K-749PV-PG3KT) and the STATISTICA 6.1 software product (StatSoftInc, No. AGAR909E415822FA) using parametric or non-parametric methods, depending on the compatibility of their distribution with the Gaussian curve using the Shapiro-Wilk test. Quantitative data are presented using indicators of variation statistics. Categorical variables are presented using frequencies and percentages. Differences between polymorphisms were tested using the Kruskal-Wallis test and a post-test when a statistically significant difference was found. Deviations from Hardy-Weinberg equilibrium expectations were determined using the criterion $\chi 2$.

In addition, to investigate whether any of the studied SNVs were associated with an increased risk of MUO, one-way analysis of variance ANOVA, a method for estimating data dispersion, ROC-analysis were performed in MedCalc® statistical software version 17.4 (MedCalc Software Ltd), statistical hypothesis testing method (in the Python software package version 3.8.10 in the Visual Studio Code integrated development environment version 1.81.1). Only significant relationships ($p<0.05$) were considered [25].

RESULTS AND DISCUSSION

On the basis of clinical and paraclinical examination of children, we formed observation groups for obesity phenotypes, which are presented in Table 1.

Table 1

Clinical and paraclinical examination data of children with different phenotypes of obesity

Indicator	Metabolically unhealthy obesity (n=152), M±m	Metabolically healthy obesity (n=100), M±m	Probability, p
BMI in percentiles	99.54±0.21	98.74±0.29	0.12
The presence of extreme obesity stage 2 (120-139% over the 95th percentile), %	19±3.92	16.1±3.68	0.06
The presence of extreme obesity stage 3 (over 140% of the 95th percentile), %	32.3±4.66	0	0.00001
Waist circumference in percentiles	96.65±0.42	93.38±0.82	0.0004
Systolic blood pressure in percentiles	83.77±3.05	71.38±3.96	0.014
Diastolic blood pressure in percentiles	87.48±2.75	66.33±4.09	0.0006
High-density lipoprotein cholesterol in percentiles	30.83±4.04	32.81±2.79	0.68
Triglycerides in percentiles	87.7±2.28	80.33±3.63	0.04
Fasting blood glucose level, mmol/L	4.15± 0.37	3.36±0.48	0.2
Basal insulin, μU/ml	29.47±1.14	12.53±1.44	0.00001
Interleukin-6, pg/ml	3.4±0.82	1.04±0.22	0.007
Interleukin-1β, pg/ml	3.6±0.63	1.78±0.17	0.008

Significant differences among children in the comparison groups were characterized by the presence of a greater proportion of children with the MUO phenotype, who had extreme obesity stage 3, abdominal obesity, increased SBP/DBP, triglyceridemia, hyperglycemia, basal insulinemia, and increased levels of pro-inflammatory cytokines in blood serum. A molecular genetic study was performed in randomly selected 52 children with different phenotypes of obesity and had the following characteristics: the size of the exome was 36.5 Mb; average overall coverage (12 GB) ≥100x; on target indicators ≥70%; more than 20X coverage – 96%; >30X coverage – 95%. We identified 36 single-nucleotide variants (SNVs) of *NEGR1, FTO, GLP1R, GHRL, LEPR, CCK* genes associated with "diseasome" formation of metabolically unhealthy obesity, T2DM and cardiovascular complications.

SNVs of genes of anorexic hormones that control eating behavior were highly associated with MUO, the level of association of which exceeded the threshold that accepted 75% of the available data, respectively in ascending order: *LEPR* rs1137101 (40.38%), *GLP1R* rs1126476 (40.38%), *GLP1R* rs2235868 (42.31%), *GLP1R* rs1042044 (42.31%), *LEPR* rs3790435 (48.08%), *CCK* rs754635 (50%), *LEPR* rs2186248 (55.76%), *GLP1R* rs6918287 (55.76%), Figure.

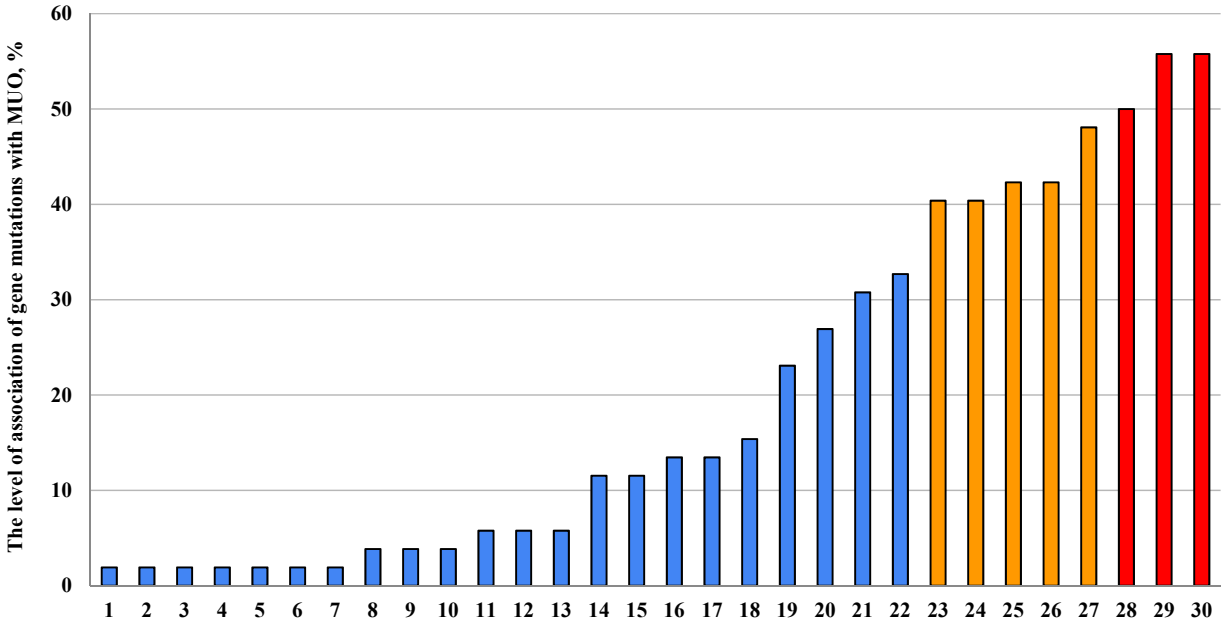
ROC-analysis using the logistic regression method was performed to compare the effectiveness of several different methods of binary classification within the task of correctly predicting metabolically healthy/unhealthy types of obesity with the available list of gene mutations and demonstrated the following parameters: Accuracy – 0.69%; Sensitivity – 0.75% Specificity – 0.63%; AUC (area under the ROC-curve) – 0.66%.

We identified 14 SNVs of the *GLP1R* gene: six synonymous SNVs (rs6918287, rs1126476, rs2235868, rs3765468, rs61754624, rs1472308929) and eight non-synonymous SNVs, of which six variants (rs6923761, rs1042044, rs10305420, rs10305421, rs10305492, rs10305493) resulting from missense mutations and two variants associated with point mutations in the intronic region (rs761386, rs10305457).

The highest coefficient of pathogenic influence, calculated as combined annotation dependent depletion (CADD), was noted for SNVs with missense mutations: rs10305493 (CADD=26.1); rs10305421 (CADD=25); rs10305492 (CADD=22.5) of the *GLP1R* gene.

It is interesting that the frequency of synonymous SNVs of the *GLP1R* gene is statistically significantly higher among children with MUO than among children with MHO, respectively, in the presence of rs1126476 – in 68% compared to 48% of cases, and in rs2235868 – in 71% in comparison in 52% of cases,

p<0.05. Key criteria for MUO determined by certain SNVs *GLP1R* are meta-inflammation, impaired fasting glucose, insulin resistance, and dyslipidemia. Meta-inflammation is associated with the GA/AA rs3765468 genotype, the clinical indicator of which is an increase in the level of IL-6 in blood serum 5.8 times higher (F=5.8; p<0.05) than in carriers of the GG rs3765468 genotype. Impaired fasting glycemia in the form of basal hyperglycemia is associated with the AA rs6918287 genotype, which was diagnosed 6.3 times more often than in probands with the GG rs6918287 genotype (F=6.3; p<0.05). Insulin resistance is associated with the CC rs10305421 genotype, the clinical indicator of which is the basal insulin level, which was 5.6 times higher than in individuals with the TT rs10305421 genotype (F=5.6; p<0.05). Dyslipidemia, the clinical indicator of which is an increase in the level of TG in the blood serum, is associated with the AA rs6918287 genotype and was diagnosed 51.3 times more often than with the GG rs6918287 genotype (F=51.3; p<0.05).



Notes: 1 – LEPR rs1359482195; 2 – FTO rs1080312; 3 – FTO rs542356043; 4 – GLP1R rs10305421; 5 – GLP1R rs1472308929; 6 – GLP1R rs10305493; 7 – GHRL rs139684563; 8 – LEPR rs13306520; 9 – FTO rs 2287142; 10 – GHRL rs696217; 11 – LEPR rs1805134; 12 – FTO rs17823223; 13 – GLP1R rs3765468; 14 – GHRL rs696217; 15 – GHRL rs4684677; 16 – LEPR rs70940803; 17 – GLP1R rs10305457; 18 – LEPR rs1805096; 19 – LEPR rs1805094; 20 – GLP1R rs6923761; 21 – GLP1R rs10305420; 22 – LEPR rs1137100; 23 – LEPR rs1137101; 24 – GLP1R rs1126476; 25 – GLP1R rs2235868; 26 – GLP1R rs1042044; 27 – LEPR rs3790435; 28 – CCK rs754635; 29 – LEPR rs2186248; 30 – GLP1R rs6918287.

List of SNVs of *FTO, GLP1R, GHRL, LEPR, CCK* genes, which are highly associated with the presence of MUO in a child (values outside the 75% quantile are highlighted in orange, values outside the 90% quantile are highlighted in red)

The contribution of SNV genes *LEPR, CCK, GLP-1* (anorexigenic hormones) and *GHRL* (orexigenic hormone) controlling eating behavior was considered in our previous works [26, 27] and works of other researchers [4, 23, 28, 29, 30]. So, A.C. Rupp et al. [28] demonstrated in a mouse model that GABAergic Glp1r-expressing LepRb neurons (LepRbGlp1r), which express more Lepr than other LepRb cell populations, play a crucial role in the inhibition of food intake by leptin and *GLP1R* agonists, even in positive energy balance. Thus, it is the genetically determined disruption of the interaction between orexigenic hormones such as ghrelin and anorexigenic hormones such as leptin, cholecystokinin, and GLP-1 with hypothalamic centers and feeding centers of the central nervous system that is responsible for excessive adipose tissue mass and the development of obesity. Similar to our study, S. Steinsbekk et al. [31] demonstrated the polygenic influence of 32 SNVs of 19 genes selected for genotyping based on data from GWAS libraries, showing the highest contribution of SNV rs2568958 of the *NEGR1* gene to the increase in BMI in 652 preschool children. At the same time, the study of *LEPR, CCK*, and *GLP1R* genes, which control the synthesis of anorexic hormones, was not involved in this study.

They also showed that a standard increase in the genetic risk score was associated with a 0.22 point increase in BMI at baseline age of 4 years (p=0.008) and concluded that early intervention is needed for precise prevention of this disease. Unlike our predecessors, we used a risk calculation of MUO predictors, based on a genome-wide molecular genetic study based on bioinformatic and statistical methods, identifying 36 SNVs, among which the only SNV of the *NEGR1* gene (rs1413368) was also represented, but its association level with the MUO did not exceed the threshold that accepted 75% of the available data. Only one of the *GLP1R* gene SNVs identified by us (rs61754624) is described according to the ClinVar database as "likely benign" [30], which coincided with the results of our study. The clinical significance of the other 13 SNVs of the *GLP1R* gene identified by us was currently unknown and is being described by us for the first time.

The originality of the presented research lies in the fact that we have proven a high association with MUO, namely SNV rs754635 *CCK*; rs2186248 *LEPR*; rs6918287 *GLP1R*, with a threshold prevalence that accepted 90% of the available data and an association level of more than 50%.

Various mutations in genes that control energy homeostasis lead to structural changes in receptors that differentially redistribute excitation, loading some signaling pathways, or reducing the activity of others, which causes a variety of predominant clinical manifestations: meta-inflammation, insulin resistance, impaired fasting glycemia, dyslipidemia, or all at the same time. Among the various molecular systems involved in the regulation of energy balance and eating behavior, the most important are GLP-1/GLP-1R axis dysfunctions caused by SNV of the *GLP1R* gene located on the short arm of chromosome 6 and associated with the development of obesity and comorbid metabolic disorders.

CONCLUSIONS

1. Single-nucleotide variants of genes GLP1R rs6918287, LEPR rs2186248, CCK rs754635 of the anorexic hormones that control eating behavior are highly associated with the presence of metabolically unhealthy obesity in children.
2. GLP1R gene variants in children with a metabolically healthy obese phenotype determine the level of inflammatory status (GA/AA SNV rs3765468), carbohydrate tolerance (AA rs6918287), insulin resistance (CC rs10305421), and serum lipid spectrum of blood atherogenicity (AA rs6918287) and thus, lead to its transformation into metabolically unhealthy obesity.
3. From the point of view of determining the genotype of single-nucleotide variants of the GLP1R gene, it is possible to predict the probability of metabolically unhealthy obesity and personalize the development trajectory of practically various metabolic disorders in obese children and the possibility of using the target signal in other drugs affecting the glucagon-like peptide type 1 pathway.

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Conflict of interests. The authors declare no conflict of interest.

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