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RESEARCH

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Analysis of the *CYP21A2* gene pathogenic variants in CAH patients from Surgut using next-generation sequencing (NGS)

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Abstract

Background 21-hydroxylase deficiency is present in 90–95% of cases of congenital adrenal hyperplasia (CAH). Eleven major pathogenic variants account for 93% of all identified variants in the *CYP21A2* gene in various clinical forms of the disease. Each population has its own range of significant pathogenic variants. We aimed to study the frequency of pathogenic variants in the *CYP21A2* gene using NGS technology and real-time PCR in Surgut patients with different clinical forms of CAH. NGS was performed on 70 patients with salt-wasting and non-classical clinical forms of 21-hydroxylase deficiency, verified by direct Sanger sequencing and PCR–RFLP analysis.

Results Eleven different pathogenic variants were found in 68.57% (48/70) of patients. Among 92.86% (13/14) of patients with salt-wasting CAH, variants were found to be homozygous, with *CYP21A2* gene deletion as the most frequent mutation (46.4% or 13/28 alleles). In the group with non-classical CAH, pathogenic variants were identified only in 60.71% (34/56) of patients. V282L was discovered to be the most common variant in heterozygous carriers (45.45%, 15/33). NGS method identified 2 variants that were not determined by the standard method for major mutations detection: p.C170* and p.W22X, accounting for 3% of all known pathogenic variants.

Conclusion Our data make it possible to clarify the specific spectrum of *CYP21A2* gene pathogenic variants in CAH patients from Surgut. The NGS method allows for the identification of rare pathogenic variants (3%) in the *CYP21A2* gene that are not included in the conventional PCR–RFLP analysis.

Keywords CAH, *CYP21A2* gene, Mutation, Pathogenic variants, p.C170*, p.W22X

Background

Congenital adrenal hyperplasia is the main cause of adrenal hyperandrogenism. The disease was first described by the Italian anatomist Luigi De Crecchio in 1865 [1]. Clinical manifestations of the disease vary widely and include adrenal insufficiency, genital ambiguity, infertility, growth retardation, hypertension, and increased risk of metabolic syndrome in adolescence and adulthood. The severity and clinical features of CAH vary depending on the enzymatic defect and residual enzymatic activity [2]. Currently, 7 forms of CAH are described: 21-hydroxylase deficiency (classical and non-classical forms), 11β-hydroxylase deficiency (classical and non-classical forms), 3β-hydroxysteroid dehydrogenase deficiency, 17α-hydroxylase deficiency (with and without 17,20-lyase deficiency), 20,22-desmolase deficiency, StAR-proterin deficiency (so-called lipoid adrenal hyperplasia), and P450-oxidoreductase deficiency [3]. Cortisol

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deficiency occurs in all forms of CAH. By a negative feedback mechanism, lack of cortisol leads to increased secretion of corticotropin-releasing hormone and ACTH and subsequently to adrenal cortex hyperplasia. Enzymatic defect in any form of CAH can be partial or complete, resulting in a wide range of clinical manifestations [4]. The degree of masculinization of patients with CAH at birth varies from a slightly hypertrophied clitoris to a completely male genital structure (Prader scale 2–5) [5]. The conversion of progesterone to 11-deoxycorticosterone and 17-OH-progesterone to 11-deoxycortisol is disrupted. There are cases when the disease is not diagnosed in time, additional examination and diagnosis is required at a later age, including through genetic testing.

Different degrees of enzymatic activity determine the severity of the disease and correspond to three clinical forms of CAH: salt-wasting form, simple virilizing form, and non-classical form [6]. It is accepted to distinguish the classical form of the disease with a severe 21-hydroxylase deficiency, which is manifested by intrauterine virilization (male-type development of external genitalia), and the non-classical form with a moderately pronounced enzymatic defect, which manifests in the postnatal period. The classical form is divided into a simple viril form (SV, about 25% of cases) and a salt-wasting form (SW, more than 75% of cases), in which the aldosterone synthesis is impaired [7–9]. Life-threatening salt-wasting crises may occur in newborns with SW. Excessive production of androgens in female individuals at the early stages of intrauterine development (from 7 to 8 weeks) already causes the development of external genitalia rudiments according to the male type. The degree of virilization depends on the severity of the enzymatic defect. Simple virile form (SV) CAH has almost the same clinical manifestations as SW form, except for salt loss symptoms [10, 11]. The non-classical form (NC) does not lead to adrenal insufficiency and includes various symptoms of postnatal androgen excess (premature pubarche, hirsutism, acne, menstrual irregularities, miscarriage, infertility) [12].

CAH is quite common and ranges from 1:5,000 to 1:67,000 newborns. According to data obtained from mass examinations (almost 6.5 million newborns worldwide), the average incidence of the disease is 1 per 15,000 newborns [13–15]. Thus, early diagnosis and adequate treatment are of great importance for both mild and severe cases of CAH.

Disturbances in the 21-hydroxylase enzyme function occur due to the presence of pathogenic variants in the *CYP21A2* gene, which encodes this enzyme. The 21-hydroxylase gene was mapped to the short arm of chromosome 6 (6p21.3). The *CYP21A1P* pseudogene, which is 98% homologous to the *CYP21A2* sequence but does not carry functional load due to inactivating mutations, was also identified in this locus. To date, more than 200 mutations in the *CYP21A2* gene have been identified [16–18]. Molecular genetic testing for CAH caused by 21-hydroxylase deficiency is feasible, offered worldwide, and important for differential diagnosis, carrier identification, adequate genetic counseling, and family planning. The major pathogenic variants identified (almost 90%) are as following: delA2/LGC, P31L, I2splice(c.293-13C > G), del8bp in exon 3, I173N, ClusterE6, V282L, Q319X, R357W, and P454S [19]. In this work, NGS method was used in conjunction with PCR–RFLP analysis to identify mutations in the *CYP21A2* gene. The ever-increasing availability of NGS, decreasing in cost over time and with short turnaround times, suggests that molecular genetic testing is a more cost-effective practical complement to modern primary diagnostic laboratory testing based on steroid intermediate metabolite assays. Knowledge of the specific spectrum of pathogenic variants for each population allows for a more efficient approach to the preconception diagnostics of CAH.

Subjects and methods

The aim

To determine the spectrum of pathogenic variants in the *CYP21A2* gene in Surgut patients with SW and NC CAH using a combination of NGS and real-time PCR approaches.

Research subjects

The study included blood samples obtained from 70 patients with 21-hydroxylase deficiency. Patient groups were formed on the basis of clinical and laboratory data. The 1st group included 14 patients with salt-wasting form of CAH in the compensation stage; the 2nd group included 56 patients with non-classical form of CAH. Samples were provided by Surgut State University in 2020–2022. The identification of the relevant clinical and pathogenic variants of 21-hydroxylase deficiency was carried out by specialists on the basis of the clinical picture of the disease and laboratory data.

DNA extraction

Genomic DNA was isolated using a salting out procedure for extracting DNA from human nucleated cells. This method involves salting out of cellular proteins by dehydration and precipitation with a saturated NaCl solution [20].

Genotyping

NGS was carried out using Ion Torrent S5 instrument (Thermo Fisher Scientific, USA). Preamplification of the *CYP21A2* gene target fragment (3.2 kb) was performed using the forward gene-specific primer A2F and the

reverse general primer-Zrev [16, 21]. Library construction involved fragmentation of the preamplified target fragment, end repair, and ligation of barcoded adapters, followed by final amplification of the library according to the manufacturer's recommendations (Thermo Fisher Scientific, USA). Library quantification was performed using Qubit 2.0 fluorimeter (Invitrogen, USA) and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.). The SAMtools package [22] was applied for the primary data analysis. The identification of genetic variants was carried out using the Torrent Variant Caller 4.4 software module (Thermo Fisher Scientific). The interpretation of genetic variants pathogenicity was based on the analysis of information from dbSNP databases (Database of Single Nucleotide Polymorphisms, www.ncbi.nlm.nih.gov), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) and data from articles. The identified variants were confirmed with PCR–RFLP analysis and/or Sanger sequencing. The latter was performed using an automatic sequencer ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's recommendations. The results of NGS genotyping were confirmed in all cases. The previously described 2-step PCR technique was used to analyze major deletions and verify pathogenic variants that were identified by NGS. The *CYP21A2* gene products obtained at the first stage of PCR were used as a template at the second stage of amplification. In this case, special modified primers were used to create specific restriction sites designed to detect studied mutations [23, 24]. Enzymatic digestion of amplified DNA fragments was carried out in accordance with the manufacturer's recommendations (Sibenzyme LLC). Oligonucleotide sequences, reaction conditions, and restriction endonucleases were described in earlier works [25]. The programmable thermal cycler MC2 from DNA Technology (Russia) was used for DNA fragments' amplification. The separation of PCR products and enzymatic hydrolysis products was carried out in 7.5% non-denaturing polyacrylamide gel. The gel was stained in aqueous solution of ethidium bromide (0.5 μg/ml) to visualize the results obtained; the electrophoretic separation of DNA fragments was recorded in transmitted ultraviolet light (wavelength 380 nm) on a Macrovue transilluminator (LKB, UK). The PCR product detection system with a TaqMan probe (real-time PCR) was used to determine the gene dose [26]. Probes and primers were synthesized by Synthol (Russia). Real-time PCR was performed using Rotorgene 3000 instrument (Corbett Life Science, Australia).

Statistical analysis

Statistical analysis (average value, standard deviation) was performed using GraphPad InStat**.**

Results

Analysis of *CYP21A2* gene mutations was performed in 70 patients with SW or NC forms of 21-hydroxylase deficiency. Pathogenic variants were identified in 96.4% of alleles (27/28) in 14 patients with the salt-wasting form of the disease. One patient was a heterozygous carrier of the gene deletion with the second variant undetermined. Gene deletion was the most common variant, found in 13 of 28 alleles (46.42%); also were identified i2splice (14.29%; 4/28), Q319X (14.29%; 4/28), R357W (7.15%; 2/28), W22X (3.56%; 1/28). Conversion variants were found in two patients with one allele simultaneously including 2 mutations, i2splice and P454S (7.15%; 2/28) (Table 1). In one case a mild variant P454S (3.56%; 1/28) was detected. The W22X variant (*CYP21A2*:c.66G > A, chr6-32006265, p.Trp22*, NM_000500.9dbSNP, rs756302021) refers to rare variants and is not detected by the standard PCR–RFLP analysis method for the most common mutations. To verify NGS data, a system was created to detect this substitution using the Pst1 (CTGCA^G) restriction endonuclease (Fig. 1).

Absolute values of potassium, sodium, 17OHP, and identified pathogenic variants in the *CYP21A2* gene in the group of patients with SW CAH are presented in Table 1. Mean potassium and sodium levels are within the reference values for each indicator, but the mean sodium level leans toward the lower limit of reference. The level of 17OHP in this group significantly exceeds the reference values.

In the group of 56 patients with NC CAH, pathogenic variants were identified in 34 individuals (60.7%). In one case (1.78%) mutations were detected in heterozygous compound and in 33 cases (58.9%) in heterozygous state. The V282L variant was the most common change identified in the gene in heterozygous carriers (45.45%, 15/33 patients). Additionally, the following were identified: Q319X (27.3%, 9/33), delA2 (12.1%, 4/33), R357W (3.03%, 1/33), P31L (3.03%, 1/33), i2splice (3.03%, 1/33), P454S (3.03%, 1/33), and conversion including exons 4 to 8 (3.03%, 1/33), i.e. almost the entire spectrum of major pathogenic variants in the CYP21A2 gene. In the compound heterozygote, variants V282L and C170X (*CYP21A2*:c.510C > A, chr6-32007195, p.Cys170*, NM_000500.9) are described. This variant according to https://varsome.com/ was classified as probably pathogenic, leading to the translation termination. It was confirmed by direct Sanger sequencing. No description of this option was found in the analyzed literature. Its frequency in other populations is also unknown.

Absolute values of potassium, sodium, 17OHP, and the presence of pathogenic variants in the *CYP21A2* gene in the group of patients with NC CAH are presented in

Table 1 Absolute values of potassium, sodium, 17OHP, and identified pathogenic variants in the *CYP21A2* gene in patients with SW form

Patients (date of birth)	Potassium (with absolute value 3.5–5.5 mmol/l)	Sodium (with absolute value 135–155 nm/l)	17-hydroxyprogesterone at screening (with absolute value < 5 nm/l)	Pathogenic variants
2012	4.30	137	–	delA2/delA2
2018	7.50	129	600	delA2/delA2
2018	5.67	129	333	delA2/delA2
2017	5.87	136	197.2	delA2/i2splice
2017	5.6	142	143.9	R357W/R357W
2019	5.39	137	882	i2splice/i2splice,P454S
2015	4.42	130	307.3	i2splice/delA2
2016	5.52	141	480.3	i2splice,P454S/P454S
2012	4.64	140	81.1	Q319X/Q319X
2016	4.7	140	893	Q319X/Q319X
2011	4.28	136	712	i2splice/W22X
2002	3.71	140	–	delA2/delA2
2013	5.3	141	–	wt/delA2
2015	4.77	136	533	delA2/delA2
Average value	5.12 ± 0.9	136.71 ± 4.5	469.35 ± 283.8	

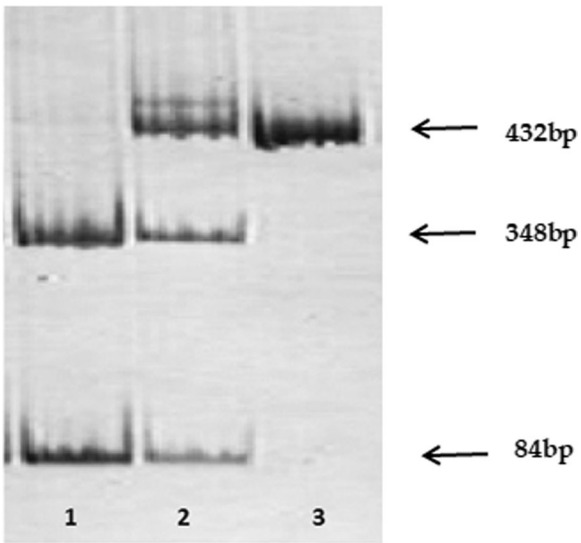

Fig. 1 Electropherogram of the W22X variant detection system (rs756302021). The amplified fragment has a length of 432 bp and has the restriction site for Pst1 in healthy individuals. Track 1. Two fragments are visualized in the wild type sample (348 bp + 84 bp). Track 2. Sample with the W22X variant in heterozygous state: the restriction site is disrupted and the fragment of 432 bp identified. Track 3. The control sample without Pst1 enzyme exposure

Table 2. The indicators were found to be heterogeneous and therefore with a wide range of values. This table presents the results for patients over one year of age in order to eliminate the possibility of distortion of the 17OHP level due to the prematurity of the child.

Table 2 Absolute values of potassium, sodium, 17OHP, and the presence of pathogenic variants in the *CYP21A2* gene in patients with NC form

Potassium (with absolute value 3.5–5.5 mmol/l)		Sodium (with absolute value 135-155 nm/l)		17-hydroxyprogesterone at screening (with absolute value < 5 nm/l)	
No mutations	Mutation presence	No mutations	Mutation presence	No mutations	Mutation presence
4,95 ± 0,56	4.52 ± 0.42	137.87 ± 3.30	140.41 ± 2.48	6.8 ± 7.34	10.46 ± 20.03

Discussion

We analyzed a range of the *CYP21A2* gene pathogenic variants in 70 patients with two clinical forms of CAH, SW and NC. The neonatal screening of 14 SW patients showed a high level of 17OHP, with an average value of 469.35 nm/l (Table 1), whereas the reference value is less than 5 nm/l. As it is known, the residual percentage of 21-hydroxylase enzyme activity is correlated with various clinical manifestations of CAH, as the 21-hydroxylase deficiency results in the accumulation of precursor hormones, including 17OHP. High level of 17OHP is associated with the most severe clinical forms, such as SW. Moreover, due to the early manifestation of symptoms of the disease, such as salt imbalance, increased 17OHP levels, and male-type external genitalia development, most patients are diagnosed in the first months of their life. In the analyzed group of SW CAH patients, the diagnosis was made in the first year of life and based on the results of neonatal screening for most patients. In 93% (13/14) of

cases, the pathogenic variants were found to be homozygous. Gene deletion (46.4%) resulting in protein synthesis with complete loss of functional activity is the most common variant in the studied group of SW patients, while in some other populations i2splice and I173N were noted as major variants in the SW form [27, 28]. We detected Q319X and i2splice with equal frequency (14.81%). We also identified alleles (7.41%) with several pathogenic variants, including several exons, which is typical of the 21-hydroxylase gene. These changes are the result of large-scale gene conversion, including the gene and pseudogene. Interestingly, variants associated with higher residual activity of the 21-hydroxylase enzyme and typical of NC CAH may also occur in more severe classical forms. We identified a P454S mutation in the compound position with the allele carrying 2 variants: i2splice and P454S. The P454S variant results in a protein that retains 20–50% of activity and typical of NC CAH. Although the phenotype was to be consistent with a milder mutation, in this case the patient was found to have a high 17OHP at screening (480 nm/l), followed by level of 411 nm/l in repeated venous blood sampling. Similar cases were noted in other studies [29] in which compound heterozygous carriers with the "mild" variant V282L in combination with some "severe" variants were described for SW. In such a discrepancy between the effect of a pathogenic variant and the clinical manifestation, additional molecular mechanisms involved in the implementation of genetic information at the phenotypic level are possible. A wide spectrum of the *CYP21A2* gene's pathogenic variants was identified in NC patients, ranging from complete impairment of the 21-hydroxylase protein function, such as gene deletion, to mutations typical of the enzyme with residual activity of 50% (V282L). The V282L variant occurred in 47.0% of patients. In other studies, however, the most common mutation in non-classical form were P454S [29], P31L [30], and V282L [14]. The value of 17-OHP value for the non-classical form is considered to exceed 30 nm/l. And usually, pathogenic variants are detected in most patients [31]. But in our study, the average value of 17OHP for this group was 15.39 nm/l, which is above the upper limit of the benchmark, but is not enough to make a diagnosis of NC. And in almost all cases, the pathogenic variants identified were presented in a heterozygous state (58.9%). These results may be due to the fact that patients with unclear clinical symptoms are more often referred to molecular genetic testing in order to confirm the diagnosis at the genetic level. On the one hand, it can be assumed that in patients with suspected NC who have identified pathogenic variants in the heterozygous state, the diagnosis of CAH could be excluded. Since CAH has an autosomal recessive mode of inheritance, in heterozygous carriers, a normal allele encodes an enzyme with full functional activity that supports normal physiological function. In this case, symptoms of CAH should not appear clinically [32]. However, some studies have previously noted that the presence of a heterozygous pathogenic variant in the *CYP21A2* gene may also cause clinical manifestations associated with androgen excess [33]. This phenomenon was explained by the dominant-negative effect of certain gene mutations, such as I173N, cluster E6, and V282L [34, 35]. This effect suggests that the final activity of the enzyme may depend on the pathogenic variant present in one allele. In this case, a decrease in enzyme activity may occur to a greater than expected extent for these mutations and results in an increase in androgens secretion by the adrenal glands and corresponding clinical manifestations of NC. Coexpression of the wild-type enzyme with the mutant enzyme in the presence of variants V281L, I172N, or V237E may have resulted in a lower enzymatic activity than expected. And these pathogenic variants in the heterozygous state may negatively affect the enzymatic activity of the wild-type enzyme [33]. It can be assumed that there is a link between the heterozygous carriage of pathogenic variants in the 21-hydroxylase gene and mild non-classical phenotype.

It was difficult to properly assess the effectiveness of using NGS in the present study due to a small sample and unclear diagnosis in patients with presumed non-classical form of CAH. NGS analysis in combination with real-time PCR helped to quickly and fully identify the entire possible spectrum of *CYP21A2* gene pathogenic variants in all patients with CAH. The use of this method, in conjunction with the analysis of deletions in the *CYP21A2* gene, made it possible to verify that patients with NC in this sample were indeed mainly heterozygous carriers of pathogenic variants. A rare variant not included in the list of major mutations was discovered only in one NC patient (1.8%). This study revealed that the concordance rates of severe genotypes with their phenotypes were good, while those of the milder genotypes were poor, as well as in selected studies [36].

Thus, the Surgut patients with SW and NC CAH are characterized by a wide range of identified pathogenic variants of the 21-hydroxylase gene, which includes 11 individual variants (Table 3). The use of the NGS method in molecular diagnosis of 21-hydroxylase deficiency helps to identify variants in the *CYP21A2* gene as fully and quickly as possible and plays an important role in the diagnosis of individual patients, both with SW (7% or 1/14) and NC (1.8% or 1/56) forms of CAH.

Table 3 *CYP21A2* gene major pathogenic variants in Surgut patients with SW and NC form of the CAH

dbSNP	GRch38.p12 NC_000006.12	Complementary DNA NM_000500.9	Protein NP_000491.4	Residual activity enzyme %	CAH form	
					Non-classical form (NC) 35 alleles with mutation	Salt-wasting form (SW) 27 alleles with mutation
rs9378251	chr6:32038514	c.92C > T	P31L	30–60	2.86% (1/35)	–
rs6467	chr6:32039081	c.293–13C > G	I2splice	< 2	2.86% (1/35)	14–81% (4/27)
rs387906510	chr6:32039133–32039140	c.332_339del	Gly111fs	0	–	–
rs6475	chr6: 32039426	c.518T > A	I173N	3–7	–	–
rs12530380	chr6: 32039810	c.713T > A	V238E		–	–
rs6471	chr6: 32040110	c.844G > T	V282L	20–50	45.71% (16/35)	–
rs267606756	chr6:32040182	c.923dup	L308FfsTer6	0	–	–
rs7755898	chr6:32040421	c.955C > T	Q319X	0	25.70% (9/35)	14.81% (4/27)
rs7769409	chr6: 32040535	c.1069C > T	R357W	2	2.86% (1/35)	7.41% (2/27)
rs6445	chr6: 3204100	c.1360C > T	P454S	20–50	2.86% (1/35)	3.7% (1/27)
Del30kb				0	11.43% (4/35)	48.2 (13/27)
Conversion (4–8 exons)			I173N, V238E, V282L, Q319X	?	2.86% (1/35)	–
Conversion (2intron and -10exon)			i2splice, P454Ser	?	–	7.41% (2/27)
no	chr6: 32,039,418	c.510C > A	p.C170*	?	2,86% (1/35)	–
rs756302021	chr6: 32,038,488	c.66G > A	p.Trp22Ter	?	–	3.7% (1/27)

Conclusion

We analyzed the spectrum of the *CYP21A2* gene mutations in 70 patients with different clinical manifestations of CAH: SW and NC. Our results indicate a heterogeneous range of mutations among Surgut patients from different clinical subgroups. Pathogenic variants of varying severity may differentially contribute to the phenotypic variation of different clinical conditions. The spectrum of pathogenic variants varies depending on the clinical form of CAH and has certain population characteristics. The NGS method allows for the identification of rare pathogenic variants in the *CYP21A2* gene that are not included in the conventional PCR–RFLP analysis. Knowledge of the characteristics of the CAH-associated mutation range for each population allows for a more conscious approach to family planning and prevention of an increase in the frequency of this pathology in certain populations.

Abbreviations

CAH	Congenital adrenal hyperplasia
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
NGS	Next generation sequencing
ACTH	Adrenocorticotropic hormone
17OHP	17-Hydroxyprogesterone
SW	Salt-wasting form
SV	Simple virile form
NC	Non-classical form
DNA	Deoxyribonucleic acid

Author contributions

Conceptualization, N.O.; methodology, N.O.; software, E.V., O.T, M.Dan; validation, I.S.; formal analysis, N.O.; investigation, N.O.; resources, M.Don.; data curation, N.O. and O.G; writing—original draft preparation, N.O.; writing—review and editing, Y.N.; supervision, A.G.; project administration, Y.N.; funding acquisition, A.G. All authors have read and agreed to the published version of the manuscript."

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Availability of data and materials

Detailed genotyping data are available on request from the corresponding author. The raw data are not publicly available due to privacy or ethical restrictions.

Declarations

Ethics approval and consent to participate

"The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of D.O. Ott Institute of Obstetrics, Gynecology, and Reproductology (protocol #130 from 16 July 2020)."

Informed consent

Informed consent was obtained from all subjects involved in the study.

Competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results".

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