Whole-exome sequencing in Russian children with non-type 1 diabetes mellitus reveals a wide spectrum of genetic variants in MODY-related and unrelated genes

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Introduction

Monogenic diabetes accounts for 1-6% of pediatric diabetes patients with the highest incidence among patients manifesting non-type 1 diabetes mellitus in childhood or adolescence (1). A large, clinically heterogeneous group of dominantly inherited disorders linked to primary β-cell dysfunction is classified as maturity onset diabetes in the young (MODY). To date, 13 genes causative of 13 types of MODY are known (2). MODY is typically diagnosed before 25 years of age; it is non-insulin dependent and its symptoms are usually mild. However, due to the variety of clinical forms caused by a wide spectrum of mutations in MODY-related genes, different treatment strategies are used: From appropriate diet and physical activity to oral and/or insulin therapy.

Abstract. The present study reports on the frequency and the spectrum of genetic variants causative of monogenic diabetes in Russian children with non-type 1 diabetes mellitus. The present study included 60 unrelated Russian children with non-type 1 diabetes mellitus diagnosed before the age of 18 years. Genetic variants were screened using whole-exome sequencing (WES) in a panel of 35 genes causative of maturity onset diabetes of the young (MODY) and transient or permanent neonatal diabetes. Verification of the WES results was performed using PCR-direct sequencing. A total of 38 genetic variants were identified in 33 out of 60 patients (55%). The majority of patients (27/33, 81.8%) had variants in MODY-related genes: GCK (n=19), HNF1A (n=2), PAX4 (n=1), ABCC8 (n=1), KCNJ11 (n=1), GCK+HNF1A (n=1), GCK+BLK (n=1) and GCK+BLK+WFS1 (n=1). A total of 6 patients (6/33, 18.2%) had variants in MODY-unrelated genes: GATA6 (n=1), WFS1 (n=3), EIF2AK3 (n=1) and SLC19A2 (n=1). A total of 15 out of 38 variants were novel, including GCK, HNF1A, BLK, WFS1, EIF2AK3 and SLC19A2. To summarize, the present study demonstrates a high frequency and a wide spectrum of genetic variants causative of monogenic diabetes in Russian children with non-type 1 diabetes mellitus. The spectrum includes previously known and novel variants in MODY-related and unrelated genes, with multiple variants in a number of patients. The prevalence of GCK variants indicates that diagnostics of monogenic diabetes in Russian children may begin with testing for MODY2. However, the remaining variants are present at low frequencies in 9 different genes, altogether amounting to ~50% of the cases and highlighting the efficiency of using WES in non-GCK-MODY cases.
Monogenic diabetes also includes a number of non-MODY transient or permanent neonatal forms occurring under 6 months of age. More than 20 genes are known to be related to congenital neonatal diabetes (3). Depending on the gene involved, neonatal diabetes may follow patterns of dominant or recessive inheritance and may be isolated or associated with a variety of syndromic features (4). However, due to a very early onset of diabetes, hyperglycemia is often diagnosed prior to other syndromic features. The treatment strategy for non-MODY neonatal diabetes depends on the specific genetic defect causing the diabetic phenotype.

Molecular genetic testing is highly recommended for patients suspected of monogenic diabetes as it allows tailoring treatments to specific etiological mechanisms. Up until recently, search for diabetes-related mutations was usually performed by Sanger sequencing and was therefore limited to only a few genes, leaving a considerable proportion of cases without a known cause. Moreover, a number of studies have demonstrated that frequencies of certain monogenic diabetes subtypes vary strongly among different populations (5), challenging the development of unified recommendations for the target gene choice. An efficient technology to detect previously known and to reveal novel mutations related to monogenic diabetes is next-generation sequencing. This technique allows for a rapid analysis of an unlimited number of genes and may provide valuable knowledge on the genetic variants causative of monogenic diabetes in different populations.

Here, using targeted whole-exome sequencing (WES), we studied the frequency and the spectrum of genetic variants causative of monogenic diabetes in a cohort of Russian children with non-type 1 diabetes mellitus.

Materials and methods

Study group. A total of 60 unrelated patients with diabetes and impaired glucose tolerance (pre-diabetes) were prospectively included in the study. All the patients were of Russian ethnicity and resided in Northwest Russia. In accordance with the guidelines of the American Diabetes Association (6), the diagnoses were based on plasma glucose criteria, either the fasting plasma glucose (FPG) and/or the 2-h plasma glucose (2-h PG) value after a 75-g oral glucose tolerance test (OGTT) and/or the HbA1C criteria. All the patients had an onset of diabetes before the age of 18 years and a detectable C-peptide secretion (or a detectable insulin level in the absence of insulin therapy) and were negative for insulin-, islet-cell-, tyrosinphosphatase IA2-, and glutamate decarboxylase-autoantibodies. The exclusion criterion was the presence of the already confirmed syndrome associated with impaired glucose metabolism (such as Prader-Willi syndrome). In 59 cases, family history was available, and in 41 of them, it was positive for diabetes. All the patients were referred to the study by their medical supervisors.

Sample preparation and whole-exome sequencing. Genomic DNA was extracted from whole blood by Magna Pure System (Roche) using the standard protocol. Exome DNA libraries were prepared from 100 ng DNA using TruSeq® Exome Sample Preparation kit (Illumina, Inc.), following the manufacturer's instructions. Libraries were sequenced on Illumina HiSeq 2500 in 2x100 PE mode. An average of 63.6 million sequencing reads were obtained for each sample, yielding ~50x mean coverage of CDS regions and an average of 89% of CDS bases covered at least 10x.

Bioinformatic analysis. Bioinformatic analysis of the WES data was done using a pipeline based on bwa v.0.7.12-r1044 aligner, Picard tools v.2.0.1, and Genome Analysis Tool kit (GATK) v.3.5 software with all the necessary preprocessing steps required by the GATK Best Practices workflow (https://software.broadinstitute.org/gatk/best-practices/) (7,8). Target enrichment metrics were collected using the Picard CalculateHsMetrics tool. Variant calling was done using GATK HaplotypeCaller in the cohort genotyping mode with 250 samples included into the cohort (samples with a similar ethnic background from St. Petersburg State University Biobank were used for cohort padding). Variants were filtered using variant quality score recalibration (VQSR) and annotated with SnvEff and SnvSift tools (version 4.2). Additional annotations included the following information: rsID of known variants from dbSNP (build 146), allele frequency (AF) from large sequencing consortia-1000 Genomes (9), Exome Aggregation Consortium (ExAC) (10), and ESP6500 (11); and pathogenicity predictions by Polyphen-2 (12), SIFT (13), PROVEAN (14) obtained from dbNSFP database (15) and by Human Sputing Finder (16) and DDIG (17). For additional prediction of protein stability changes caused by missence mutations with uncertain significance, I-Mutant 2.0 (18) was used. Variant ranking was done using a custom scoring metric. Reference minor allele presence in target genes was analyzed using RMA Hunter (19).

To check the possible presence of copy-number variants (CNVs), we analyzed the sequencing coverage across all targeted exons of interest. To this end, we calculated coverage for each interval using GATK, and then normalized the coverage matrix across samples and intervals. We then used z-score value of the normalized coverage to assess the statistical significance of the results.

Verification of the WES results and family analysis. Verification of the WES results in probands and subsequent family analyses were performed by PCR-direct sequencing. Specific primers were designed for verification of each case. The PCR products were purified with 5M NH4Ac and 96% ethanol and then with 70% ethanol, dried at 60°C, and dissolved in 10 µl of deionized water. After purification, the PCR products were sequenced using an ABI PRISM BigDyeTerminator 3.1 kit reagent (Applied Biosystems). Then, a capillary electrophoresis was performed in a GA3130xl Genetic Analyzer (Applied Biosystems). Sequences were analyzed using the Sequence Scanner software (Applied Biosystems).

Analysis of the GCK promoter for c.-71G>C genetic variant. A single-base substitution c.-71G>C in the GCK promoter is known to be linked to MODY2 phenotype (20). However, WES did not allow for analysis of the GCK promoter for c.-71G>C. For this reason, the GCK promoter was analyzed for c.-71G>C genetic variant by PCR-direct sequencing as described above with the use of Hae III endonuclease and the
following primers: F-5'-G ca TGG caG cTc Taa TGa caG G-3' and r-5'-caT ccT aGc cTG cTT ccc TGG-3'.

**Results**

Genetic variants causative of monogenic diabetes in Russian children with non-type 1 diabetes mellitus. Using whole-exome sequencing followed by PCR-direct sequencing, we identified the frequency and the spectrum of genetic variants causative of monogenic diabetes in 60 Russian children with non-type 1 diabetes mellitus. Genetic variants were screened for a total of 35 genes: 13 genes causative of MODY [HNF4A (MODY1), GCK (MODY2), HNF1A (MODY3), PDX1 (MODY4), HNF1B (MODY5), NEURODI (MODY6), KLF11 (MODY7), CEL (MODY8), PAX4 (MODY9), INS (MODY10), BLK (MODY11), ABCC8 (MODY12), and KCNJ11 (MODY13)] and 22 genes causative of transient or permanent neonatal diabetes, including the ones related to specific syndromes ([EIF2AK3, RFX6, WFS1, ZFP57, FOXP3, AKT2, PPARG, APPL1, PTFT1A, GATA4, GATA6, GLIS3, IER3P1, LMNA, NEUROG3, PAX6, PLAGL1, SLC19A2, SLC2A2, SH2B1, SERPINB4, and MADD]).

Overall, 33 out of 60 patients (55%) had genetic variants in the target genes (Table I; 21–40). For 12 patients, parents were available for genetic testing and origins of genetic variants were determined. In 11 cases, genetic variants had been inherited from the parents, and in one case, a *de novo* genetic variant was confirmed. Of 33 patients, 27 (81.8%) had genetic variants in MODY-related genes. The majority of these patients (19 out of 27) had genetic variants in GCK (MODY2). The spectrum of GCK genetic variants included 13 missense mutations, 3 nonsense mutations, 1 in-frame and 3 frameshift deletions, and 1 single-base substitution in the promoter. In two GCK mutation-positive patients, two genetic variants were present: Missense mutation along with a single-base substitution in the promoter (patient #27) and missense mutation along with a nonsense mutation (patient #78). The spectrum of the identified GCK genetic variants is shown in Fig. 1. Missense mutations in HNF1A (MODY3) were registered in two patients. The other MODY-related genetic variants included three cases of missense mutations: In PAX4 (MODY 9), in ABCC8 (MODY12), and in KCNJ11 (MODY 13).

The presence of genetic variants in different target genes was detected in three patients. In one of them, a GCK in-frame deletion was accompanied by an HNF1A missense mutation (patient #226). In another one, two missense mutations were present: In GCK and in BLK (patient #529). In the third patient (#662), a splicing defect in GCK and missense mutations in BLK and WFS1 were present.

Genetic variants causative of non-MODY monogenic diabetes were found in 6 out of 33 mutation-positive patients (18.2%). These included a nonsense mutation in GATA6, three cases of missense mutations in WFS1, one case of a homozygous EIF2AK3 nonsense mutation (patient #411), and one case of missense mutation and a frameshift deletion present in SLC19A2 (c.164delC and c.161C>A) (patient #432). The EIF2AK3 nonsense mutations had been inherited from consanguineous parents who were heterozygous carriers of the same mutation. The SLC19A2 mutations also appeared to have been inherited from the parents: C.164delC from the mother and c.161C>A from the father, indicating that both SLC19A2 alleles in patient #432 were affected.

Considering that monogenic diabetes may be associated with deletions and duplications, we analyzed the possible presence of CNVs in the target genes. We found no evidence for CNVs in the target genes in either sample. However, it should be noted that the limitations of WES technology do not allow for confident detection of small-scale CNVs.

**Relationship between genetic variants and diabetic phenotypes.** We analyzed the relationship of the detected genetic variants to the patients' diabetic phenotypes. Among the 38 detected genetic variants, 23 had been previously reported as linked to monogenic diabetes and 15 were novel ones (Table I). According to the American College of Medical Genetics and Genomics (ACMG) guidelines (41), most of the detected genetic variants (18 previously reported and 6 novel ones) were classified as pathogenic or likely pathogenic and thus were considered as causative of the diabetic phenotypes in the studied patients. However, the relationship of the detected KCNJ11 missense mutation to the diabetic phenotype was not apparent, because earlier it had been shown to be associated with hyperinsulinism (35), which was not present in patient #134.

Three previously reported and 9 novel genetic variants were classified as those of uncertain significance, and two genetic variants were likely benign (Table I). These variants included 12 missense mutations; for them, we performed an additional *in silico* analysis using I-Mutant 2.0 (18) (Table II). In all but one case, the *in silico* modeling attested to a decrease of protein stability, thus suggesting the pathogenic effect of the checked genetic variants. Of special interest were two novel WFS1 genetic variants, initially classified as likely benign. Patient #266 inherited the genetic variant from a non-diabetic mother, while patient #408 inherited the genetic variant from a mother with diabetes. Homozygous mutations in WFS1 lead to the development of Wolfram syndrome, an autosomal recessive disorder characterized by a list of clinical signs including a bilateral progressive optic atrophy, deafness, and diabetes mellitus (42). Heterozygous carriers of WFS1 mutations have been reported to have risk of early-onset diabetes mellitus (43). The latter cannot be excluded in our patients. However, an intriguing point is that the WFS1 genetic variant in patient #408, who inherited it from a diabetic mother, appeared to not decrease the protein stability according to I-Mutant, which makes its pathogenicity questionable.

**Clinical picture in patients with multiple genetic variants.** Finally, we analyzed the clinical picture in patients with more than one genetic variant in one or different target genes (Table III). A simultaneous presence of two GCK genetic variants in patient #27 raised the question of their location in one or both alleles. The parents were not available for analysis. The clinical picture was mild and typical for MODY2. It contrasted with the severe one usually reported in patients with both GCK alleles affected (44,45), suggesting that, in patient #27, both genetic variants were present in the same allele and thus had no accumulative effect. In patient #78, who was also a carrier of two GCK genetic variants, the clinical picture was typical for MODY2. As both genetic variants were inherited from
Moreover, only nonsense mutation c.199G>T seemed to be clinically significant, because the resulting stop-codon terminates translation before the c.766G>C site. The clinical picture in patient #226, who had genetic variants in GCK and HNF1A, was more typical for MODY2 than for MODY3:

Table I. Genetic variants identified in Russian children with non-type 1 diabetes mellitus.

| Patient number | Gene     | Nucleotide change (protein change) | Mutation type | Mutation origin | Pathogenicity according to ACMG (Refs.) |
|----------------|----------|------------------------------------|---------------|----------------|----------------------------------------|
| 59             | GCK      | c.772>T (p.Gly258Cys)              | Missense      | Unknown        | Likely pathogenic (21)                 |
| 62             | GCK      | c.930_931delGG (p.Asp311fs)        | Frameshift    | Unknown        | Pathogenic (22)                         |
| 83             | GCK      | c.930_931delGG (p.Asp311fs)        | Frameshift    | Unknown        | Pathogenic (22)                         |
| 95             | GCK      | c.130>G>A (p.Gly44Ser)             | Missense      | Father         | Likely pathogenic (23)                 |
| 167            | GCK      | c.128>G>A (p.Arg43His)             | Missense      | Mother         | Likely pathogenic (24)                 |
| 197            | GCK      | c.233>T>C (p.Leu77Pro)             | Missense      | Father         | Likely pathogenic (25)                 |
| 426            | GCK      | c.683>G>T (p.Thr228Met)            | Missense      | Mother         | Likely pathogenic (26)                 |
| 460            | GCK      | c.682>A>G (p.Thr228Ala)            | Missense      | Mother         | Likely pathogenic (21)                 |
| 580            | GCK      | c.775>G>A (p.Ala259Thr)            | Missense      | Unknown        | Likely pathogenic (27)                 |
| 663            | GCK      | c.1079>G>A (p.Ser360*)             | Nonsense      | Unknown        | Pathogenic (28)                         |
| 665            | GCK      | c.660>C>A (p.Cys220*)              | Nonsense      | Unknown        | Pathogenic (24)                         |
| 176            | GCK      | c.1349>G>T (p.Ala450Val)           | Missense      | Unknown        | Likely pathogenic (29)                 |
| 661            | GCK      | c.1349>G>T (p.Ala450Val)           | Missense      | Unknown        | Likely pathogenic (29)                 |
| 118            | GCK      | c.117_119delAAAG (p.Lys39del)      | In-frame      | Unknown        | Uncertain significance Novel           |
| 119            | GCK      | c.1346_1347delCG (p.Ala449fs)      | Frameshift    | Unknown        | Pathogenic Novel                        |
| 434            | GCK      | c.868>G>C (p.Glu290Gln)            | Missense      | Mother         | Uncertain significance Novel           |
| 578            | GCK      | c.1253>G>C (p.Ser418Thr)           | Missense      | Unknown        | Pathogenic Novel                        |
| 27             | GCK      | c.754>T>C (p.Cys252Arg)            | Missense      | Unknown        | Likely pathogenic (30)                 |
| 153            | HNF1A    | c.709>G>A (p.Asn237Asp)            | Missense      | Unknown        | Uncertain significance (32)            |
| 422            | HNF1A    | c.485>G>A (p.Leu162Arg)            | Missense      | Unknown        | Uncertain significance Novel           |
| 215            | PAX4      | c.574>C>A (p.Arg192Ser)            | Missense      | Unknown        | Uncertain significance (33)            |
| 114            | ABCC8     | c.4139>G>A (p.Arg1380His)          | Missense      | Unknown        | Likely pathogenic (34)                 |
| 134            | KCNJ11    | c.406>C>A (p.Arg136Ser)            | Missense      | Unknown        | Uncertain significance (35)            |
| 68             | GATA6     | c.1477>G>T (p.Arg493*)             | Nonsense      | De novo        | Pathogenic (36)                         |
| 266            | WFSI      | c.2452>C>T (p.Arg818Cys)           | Missense      | Mother         | Likely benign (37)                     |
| 408            | WFSI      | c.2327>A>T (p.Glu776Val)           | Missense      | Mother         | Likely benign (38)                     |
| 133            | WFSI      | c.1124>G>A (p.Arg375His)           | Missense      | Unknown        | Uncertain significance Novel           |
| 411            | EIF2AK3   | c.1912>C>T (p.Arg638*)             | Nonsense      | From parents   | Pathogenic Novel                       |
| 432            | SLC19A2   | c.164delC (p.Pro55fs)              | Frameshift    | Mother         | Pathogenic Novel                       |
| 226            | GCK      | c.543_545delCGT (p.Val182del)      | In-frame      | Unknown        | Uncertain significance Novel           |
|                | HNF1A    | c.92>G>A (p.Gly31Asp)              | Missense      | Unknown        | Likely pathogenic (39)                 |
| 529            | BLK       | c.939>G>C (p.Glu313Asp)            | Missense      | Unknown        | Uncertain significance Novel           |
| 662            | GCK      | c.1019+2>T>A (p.Pro55fs)           | Splicing      | Unknown        | Pathogenic Novel                       |
|                | BLK       | c.1148>G>A (p.Arg383Gln)           | Missense      | Unknown        | Uncertain significance Novel           |
|                | WFSI      | c.1957>C>T (p.Ala653Cys)           | Missense      | Unknown        | Likely pathogenic (40)                 |

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He had mild fasting and postprandial hyperglycemia, had no glucosuria, and was successfully being treated by a diet. Patient #411 had a homozygous \(\text{EIF2AK3}\) nonsense mutation, inherited from consanguineous parents and associated with Wolcott-Rallison syndrome, which, in turn, has been reported to be the most common genetic cause of permanent neonatal diabetes in consanguineous families (46). Patient #432 had two novel genetic variants affecting both \(\text{SLC19A2}\) alleles. \(\text{Homozygous mutations in SLC19A2}\) cause Rogers syndrome: Thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness (47). Among other clinical signs are congenital heart defects, retinal degeneration, ketonuria, dwarfism, and neurological symptomatology (42). Of note, patient #432 had only diabetes mellitus, retinal degeneration, ketonuria, and neurological symptomatology and thus did not manifest a typical clinical picture. Both patients #529 and #662 had typical clinical signs of \(\text{GCK-MODY}\) rather than \(\text{BLK-MODY}\), suggesting an absence of strong accumulation of the pathogenic effect of the detected genetic variants.

**Discussion**

In 1974, Tattersall reported on three families suffering from mild non-insulin dependent diabetes with Mendelian dominant inheritance (48). The disease was diagnosed in children and young adults and was later defined as maturity-onset type diabetes of young people (MODY) (49). The discovery of mutations in the genes encoding HNF4A (50), HNF1A (51), HNF1B (52), IPF (PDX1) (53), and GCK (54,55) as the causes of MODY provided evidence for genetic heterogeneity of familial diabetes. To date, MODY-causing mutations are identified in a total of 13 genes, and mutations in more than 20 genes are known to be associated with neonatal hyperglycemia (56). Because of such a variety of genetic causes, many cases of monogenic diabetes remain without a genetic diagnosis, and its frequency remains underestimated.

The development of high throughput sequencing became a milestone in the search for diabetes-related mutations. Allowing for simultaneous testing of an unlimited number of genes (i.e. of all known genetic etiology in monogenic diabetes), the method increased the mutation detection rate significantly (57). In our study, we detected genetic variants causative of monogenic diabetes and hyperglycemia-related syndromes in 33 out of 60 children (55%) with non-type 1 diabetes mellitus. This frequency is considerably higher than that detected by Sanger sequencing, which is usually restricted to the analysis of several MODY-related genes and confirms approximately 15% of the cases tested for MODY (58). The higher mutation detection rate in our study is achieved by increasing the number of genes tested and a thorough clinical selection of patients with possible monogenic diabetes. In this regard, one more advantage of WES should be mentioned: DNA sequencing data may be easily stored for further analysis of newly discovered candidate genes.

Ethnic differences play an important role in determining the epidemiology of monogenic diabetes, especially of MODY. Large population studies in European Caucasians showed a general trend of increased \(\text{HNF1A-MODY}\) frequency in Northern Europe, while \(\text{GCK-MODY}\) is prevalent in Southern European populations (5). Here, we report \(\text{GCK-MODY}\) in 19 and \(\text{HNF1A-MODY}\) in only 2 out of 27 MODY-positive Russian patients. These mutation rates appeared to be closer to those in Southern European populations than to those in Northern Europe residents. Our finding may indicate the population-specific frequency MODY types in Russian patients. The recently shown high prevalence of \(\text{GCK-MODY}\) cases among Russian patients with diabetes in pregnancy supports this suggestion (59). However, it should be also considered that our study was performed on children who developed diabetes before the age of 18 years. In the previous observations, it was noticed that the relative proportion of \(\text{GCK-MODY}\) is higher in cases ascertained through pediatric clinics, in contrast to \(\text{HNF1A-MODY}\), which predominates in cases from adult clinics (58,60). Thus, considering this information, our results are in good accordance with those reported in Spain, Italy, France, Germany, and the Czech Republic, where mostly...
pediatric cases were tested (25,61). The prevalence of GCK variants (57.6%) in our study suggests that genetic analysis in Russian children with suspected monogenic diabetes may start with testing for MODY2, which may not necessarily be performed by WES. However, other cases amount to 42.4% and are linked to 9 different genes, which attests to the efficiency of using WES for the search of genetic causes of diabetes in non-GCK-MODY cases.

Our results show that the spectrum of monogenic diabetes-related genetic variants in Russian children includes missense and nonsense mutations, in-frame and frameshift deletions, and a promoter mutation. Generally, these data do not contrast with results obtained in other populations, which also demonstrated a wide spectrum of mutations (62-64). Among genetic variants detected in our study, 60.5% had already been reported in diabetic patients and 39.5% were novel ones. On the one hand, these results point towards a significant recurrent variation within monogenic-diabetes-related genes. On the other hand, they suggest that, in spite of the multitude of monogenic diabetes studies, many variants still remain unidentified. Identification of novel genetic variants as well as accumulating data on previously known causes of monogenic diabetes is of high importance, both for fundamental understanding of the disease pathogenesis and for clinical practice.

Interpretation of genetic variants, especially novel ones, may be challenging. In this study, only 63.2% of the detected genetic variants (18 previously reported and 6 novel ones) were unambiguously considered as causative of the diabetes in the studied patients. The remaining 36.8% variants, including 9 novel ones, were initially classified as those of uncertain significance (n=12) or likely benign (n=2). Additional in silico predictions performed for missense mutations among these variants indicate that, with the exception of one variant, they all likely have an adverse effect on protein stability. Considering these results and the patients’ phenotypes, the assumption that the abovementioned variants may be causative of monogenic diabetes can be made. Importantly, the detected genetic variants are absent in non-diabetic Russian population resided in Northwest Russia (65). However, to make a strong conclusion on the pathogenic effect of each novel variant, more data are required, including functional characterization and reports of a specific genetic variant in multiple patients with similar phenotypes. The latter highlights the importance of our results for future studies of monogenic diabetes-related genetic variants.

Noteworthy, our analysis of the clinical picture in the patients simultaneously having BLK+GCK (patient #529 and #662) and GCK+HNF1A (patient #226) genetic variants suggests no accumulation of adverse effect: All these patients had a typical MODY2 phenotype. The most plausible explanation for this is the specific age of development of different MODY types. Patients suffering from GCK-MODY have an impaired glucose metabolism since birth (66). In contrast, carriers of HNF1A genetic variants may develop diabetes by the age of 35 years or even by the age of 55 years, although most of them have diabetes before 25 years of age (67). In the study by López-Garrido et al (68), the co-inheritance of GCK and HNF1A genetic variants was reported in two patients and was associated with a typical MODY3 phenotype in an adult patient and only impaired fasting glucose in a younger patient with the same genotype. In addition, HNF1A genetic variant detected in patient #226 in this study (c.92G>A) was previously reported in a diabetic proband and his non-diabetic sister of 43 years of age (69). Similarly, affected carriers of BLK genetic variants usually develop diabetes at the middle age (70). Thus, it is likely that patients #529, #662, and #226, who were all involved in our study before the age of 4 years, have not developed the clinical picture of HNF1A-MODY and BLK-MODY yet. The possibility of a late manifestation of HNF1A-MODY and BLK-MODY in the children who

| Patient number | Gene | Genetic variant (amino acid change) | Pathogenicity according to ACMG | Protein stability predicted by I-Mutant |
|----------------|------|-----------------------------------|---------------------------------|---------------------------------------|
| 434            | GCK  | c.868G>C (p.Glu290Gln)            | Uncertain significance          | Decrease                              |
| 153            | HNF1A| c.709A>G (p.Asn237Asp)            | Uncertain significance          | Decrease                              |
| 422            | HNF1A| c.485T>G (p.Leu162Arg)            | Uncertain significance          | Decrease                              |
| 215            | PAX4 | c.574C>A (p.Arg192Ser)            | Uncertain significance          | Decrease                              |
| 134            | KCNJ11| c.406C>A (p.Arg136Ser)           | Uncertain significance          | Decrease                              |
| 266            | WFS1 | c.2452C>T (p.Arg818Cys)           | Likely benign                   | Decrease                              |
| 408            | WFS1 | c.2327A>T (p.Glu776Val)           | Likely benign                   | Increase                              |
| 133            | WFS1 | c.1124G>A (p.Arg375His)           | Uncertain significance          | Decrease                              |
| 432            | SLC19A2| c.161C>A (p.Thr54Asn)            | Uncertain significance          | Decrease                              |
| 529            | BLK  | c.939G>C (p.Glu313Asp)            | Uncertain significance          | Decrease                              |
| 662            | BLK  | c.1148G>A (p.Arg383Gln)           | Uncertain significance          | Decrease                              |

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Table II. In silico prediction of increase/decrease in the protein stability caused by missense mutations with uncertain significance and by benign missense mutations.
already have GCK-MODY strongly suggests the necessity of their strict medical supervision in order to timely modify their therapy. Additional studies, including functional ones, on the pathogenicity of the novel BLK genetic variants detected in patients #529 and #662 will also facilitate the development of the most effective treatment strategies for them.

To summarize, our data show a high rate of genetic variants causative of monogenic diabetes in Russian children with non-type 1 diabetes mellitus. The use of a WES-based panel allowed us to identify a variety of previously known and novel genetic variants in MODY-related and unrelated genes, including multiple variants in a number of patients. The revealed variety is characterized by a prevalence of GCK genetic variants (MODY2) and also includes variants in HNF1A, PAX4, KCNJ11, BLK, ABCC8, GATA6, WFS1, EIF2AK3, and SLC19A2. These results, on the one hand, suggest that genetic analysis for monogenic diabetes in Russian children may start with testing for GCK variants, which may not necessarily be performed by WES. On the other hand, non-GCK variants are linked to 9 different genes, which attests to the efficiency of using WES while searching for genetic causes of diabetes in non-GCK-MODY cases. Notably, the detection of genetic variants in the genes linked to specific syndromes with recessive inheritance—WFS1, EIF2AK3, and SLC19A2—is essential for appropriate genetic counseling and family planning. Our study highlights the importance of using WES for monogenic diabetes testing and provides new information on the diabetes-related genetic variants in the Russian population.

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### Table III. Clinical characteristics of the patients with multiple genetic variants in monogenic diabetes-related genes.

| Patient number | Gene       | Nucleotide change          | Amino acid change       | Age at diagnosis (months) | Diabetic ketoacidosis | C-peptide (ng/ml) | HbA1C (%) | SDS BMI | Treatment                  |
|----------------|------------|---------------------------|-------------------------|---------------------------|-----------------------|-------------------|-----------|---------|---------------------------|
| 27             | GCK        | c.754T>C (p.Cys252Arg)    |                         | 3                         | No                    | 0.7               | 6         | -0.63   | Diet                      |
| 78             | GCK        | c.199G>T (p.Glu67*)       |                         | 39                        | No                    | 0.63              | 6.4       | +0.83   | Diet                      |
| 226            | GCK        | c.543_545delCGT (p.Val182del) |              | 36                        | No                    | 1.1               | 6         | -1.69   | Diet                      |
| 411            | E2F2AK3    | c.1912C>T (p.Arg638*)    |                         | 3                         | Ketonuria             | 0.2               | 9.2       | -0.72   | Insulin                   |
| 432            | SLC19A2    | c.164delC (p.Pro55fs)    |                         | 48                        | Ketonuria             | 1.1               | 5.3       | -1.0    | Insulin for a few days/diet |
| 529            | BLK        | c.939G>C (p.Glu313Asp)   |                         | 10                        | No                    | 0.43              | 6.7       | -0.46   | Diet                      |
| 662            | GCK        | c.1019+2T>A               |                         | 22                        | No                    | 1.1               | 6.82      | -1.32   | Diet                      |
|                | BLK        | c.1148G>A (p.Arg383Gln)  |                         |                           |                       |                   |           |         |                           |
|                | WFS1       | c.1957C>T (p.Arg653Cys)  |                         |                           |                       |                   |           |         |                           |

SdS BMI reference range: -1.5/+1.5; SdS, standard deviation score.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

OSG, EAS, MET, OAE, EBB and VSB designed the study. OSG, EAS, MET, OAE, ASG, Yan, deP, MaF, iVP, Tei, nYS, LAZ, LVT, OSB, ENS and EBB recruited the patients and performed experimental procedures. Y aB, aVP and r KS performed experimental procedures. Y aB, aVP and r KS designed the study. OSG, EAS, MET, OAE, ASG, Yan, deP, MaF, iVP, Tei, nYS, LAZ, LVT, OSB, ENS and EBB analyzed the data and performed statistical analysis. OAE wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology. All the patients/patients' representatives gave written informed consent to participate in the study. The study was performed in accordance with the Declaration of Helsinki.

Patient consent for publication

All the patients/patients' representatives gave written informed consent for publication of the study results.

Competing interests

The authors declare that they have no competing interests.

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