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Oxford Nanopore long-read sequencing with CRISPR/Cas9-mediated target selection for accurate characterization of copy number variants in the LDLR gene

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ABSTRACT
Introduction: Familial hypercholesterolemia (FH) affects around 1 in 250 people. Most FH cases are caused by pathogenic LDLR variants, with copy number variations (CNVs) accounting for about 10 %. However, short-read gene panel sequencing and multiplex ligation-dependent probe amplification (MLPA) are limited in the specification of CNV breakpoints and the identification of complex structural variants (SVs).
Materials and methods: We designed crRNAs for Cas9-mediated target selection of LDLR and performed long-read sequencing (LRS) on an Oxford Nanopore MinION device using high-molecular-weight (HMW) DNA or DNA from standard purification. After establishing the LRS approach, we characterized two known LDLR CNVs and tested two individuals with strong clinical evidence of FH but no pathogenic variant in short-read gene panel sequencing.
Results: Complete coverage of LDLR was achieved for both HMW DNA and DNA from standard purification. LRS allowed us to specify CNV breakpoints and showed that the known LDLR deletion is 19.2 kb in size encompassing exons 1–2 and the 5'-untranslated and promoter regions. Furthermore, LRS verified the in tandem localization of a large LDLR duplication covering exons 4–8. Both CNVs were classified as loss-of-function. Moreover, breakpoint information enabled confirmatory analysis by PCR and Sanger sequencing for both CNVs. No SVs were detected in two apparently mutation-negative FH probands using our approach.
Conclusions: Nanopore LRS with CRISPR/Cas9-mediated target selection allows for accurate characterization of CNVs and can therefore serve as a complementary method to short-read sequencing-based FH diagnostics by facilitating variant interpretation and enabling cost-effective PCR-based variant confirmation in subsequent familiar analyses.

1. Introduction

Familial hypercholesterolemia (FH, OMIM: #143890, #144010, #603776, #603813) is an inherited metabolic disease with a prevalence of about 1:250 that is associated with high plasma concentrations of low-density lipoprotein cholesterol (LDL-C) and leads to an increased risk of premature atherosclerosis and coronary heart disease (Akioyamen et al., 2017; Hopkins et al., 2011). The US MEDPED, the UK Simon Broome or the Dutch Lipid Clinic Network (DLCN) criteria can be applied for clinical FH diagnosis. The latter are recommended for use in Europe and incorporate plasma LDL-C concentrations, physical signs, as well as the personal and family history of a patient. They are based on a weighted point system and can help to decide when to recommend genetic analysis for an affected person (Nordestgaard et al., 2013).

In most FH cases, autosomal-dominant inherited pathogenic variants can be found in the LDLR, APOB, and PCSK9 genes. The rare autosomal-recessive form is due to biallelic changes in LDLRAP1. About 90% of the FH-causing variants described to date are localized in LDLR with missense and frameshift variants being the most common alterations (Leigh et al., 2017). However, a large number of unique copy number variations (CNVs) are also known and collectively account for about 10% of pathogenic LDLR variants (Concolino et al., 2023; Goldmann et al., 2010; Hori et al., 2022; Iacocca and Hegele, 2018; Iacocca et al., 2017; Rutkowska et al., 2022; Taylor et al., 2009). The LDLR locus is particularly susceptible to CNV formation because it contains many Alu repeat elements mediating homogeneous recombination events (Amsellem et al., 2002; Goldmann et al., 2010).

Standard methods commonly used to detect CNVs in genetic testing for FH and other well-known Mendelian disorders are multiplex ligation-dependent probe amplification (MLPA) and short-read gene panel sequencing (Iacocca et al., 2017; Taylor et al., 2009). However, these methods are limited in precise determination of breakpoints and detection of complex structural variants (SVs). These limitations can be overcome by long-read sequencing (LRS) platforms like PacBio or Oxford Nanopore, which have been shown to be powerful tools for SV detection (Miller et al., 2021). Different strategies have been applied to enrich regions of interest in targeted LRS approaches. Besides amplification- or hybridization-based (Watson et al., 2022; Yamaguchi et al., 2021) and computational approaches (Miller et al., 2021), Cas9-mediated selection allows fast, flexible and amplification-free LRS of target regions (Gilpatrick et al., 2020).

LRS not only allows the determination of CNV breakpoints but may also help to explain some of the missing heritability in FH patients. Notably, in up to 60 % of patients that are clinically suspected to have FH no causal variants can be identified in one of the FH-associated genes with standard diagnostic procedures (Talmud et al., 2013; Taylor et al., 2010). To date, polygenic inheritance of common, LDL-Cincreasing single nucleotide polymorphism (SNPs) (Talmud et al., 2013), deep-intronic variants (Reeskamp et al., 2018), rare alterations in the STAP1 gene (Fouchier et al., 2014) or phenocopies (Medeiros et al., 2024) have been described as possible explanations for these cases. However, large balanced rearrangements such as translocations and inversions could be another cause that may be missed by standard diagnostic techniques.

In this study, we used Cas9-mediated Nanopore sequencing for the precise characterization of two large CNVs in the LDLR gene, thus facilitating better variant interpretation and enabling cost-effective PCR-based assays for familiar analyses. We additionally characterized a cohort of 21 FH probands who had been analyzed by standard short-read next-generation sequencing (NGS) and applied our LRS approach to two mutation-negative probands with strong clinical evidence of FH.

2. Materials and methods

2.1. Probands and ethical considerations

We characterized 21 probands with the clinical diagnosis “possible FH”, “probable FH” or “definite FH” (=3 points or higher according to the DLCN criteria) that were referred to FH gene panel testing with standard short-read sequencing at the Institute of Human Genetics Greifswald. DNA samples from two mutation-negative “probable FH” probands from this cohort and from two additional individuals with

previously identified LDLR CNVs (exons 1–2 deletion and exons 4–8 duplication) (Rutkowska et al., 2022) were subjected to long-read sequencing of the LDLR gene. All probands gave written informed consent for genetic analyses and the use of the clinical data. The work was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

2.2. NGS gene panel analysis

Genomic DNA for NGS gene panel analysis was isolated using the NucleoSpin Blood L Kit (Macherey-Nagel). Hybridization capture-based target enrichment and library preparation was performed using an Agilent SureSelectQXT custom enrichment kit (Panel ID: 3152261, Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced on an Illumina MisSeq platform with 2x150 cycles (Illumina, San Diego, CA, USA). FASTQ files were generated with the MiSeq Reporter Software (Illumina). The SeqNext module of the Sequence Pilot v5.0.0 software (JSI Medical Systems, Ettenheim, Germany) was used for read mapping, alignment, and variant calling, as well as read depth-based detection of CNVs. Target regions were defined as all exons (±20 bp) of LDLR (including promoter region), APOB, PCSK9, and LDLRAP1.

2.3. Nanopore sequencing

CrRNA design, library preparation with CRISPR/Cas9-mediated target selection, and Nanopore sequencing were performed as described before (Skowronek et al., 2022). CrRNA sequences are listed in Table S1 and were purchased from Integrated DNA Technologies (Coralville, IA, USA). Genomic DNA was isolated either by standard spin column purification using the NucleoSpin Blood L Kit (Macherey-Nagel, Düren, Germany) or by glass bead-based extraction of high-molecular-weight (HMW) genomic DNA using the Monarch HMW DNA Extraction Kit (New England Biolabs, Ipswich, MA, USA). The extracted DNA samples were used for target selection in a single reaction and subsequent Nanopore sequencing on a MinION device (Oxford Nanopore Technologies, Oxford, United Kingdom) equipped with R9.4.1 flow cells (Oxford Nanopore Technologies). Guppy or Dorado integrated in the MinKNOW software version 22.03.6 or 23.07.15 (Oxford Nanopore Technologies) were used for live basecalling. Bioinformatic data analyses and SV calling with the cuteSV and Sniffels2 tools were performed as described before (Skowronek et al., 2022). LRS data were further visualized with the Integrative Genomics Viewer (IGV) 2.9.2 (Robinson et al., 2011) to design variant-specific PCR.

2.4. Multiplex ligation-dependent probe amplification, PCR, and Sanger sequencing

The SALSA MLPA Probemix P062 LDLR was used according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands) for the two individuals with known LDLR CNVs. Fragment analysis was performed on a 3500 Series Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). The Coffalyser.Net software (MRC-

Holland) was employed to analyze the data. PCR with variant-specific primers (Integrated DNA Technologies) using the Taq DNA-Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) or the OneTaq DNA Polymerase (New England Biolabs) with GC-Rich Buffer was performed for breakpoint confirmation. Primer sequences are available upon request. Amplification products were analyzed on a 1.5 % agarose gel that was imaged on a ChemiDoc XRS+ system (BioRad, Hercules, CA, USA). Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) was carried out on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific) and sequencing data were evaluated with the Chromas software v2.6.6 (Technelysium, Brisbane, Australia).

3. Results

3.1. Cas9-mediated targeted long-read sequencing approach for the LDLR gene

For Cas9-mediated target enrichment of the genomic region of LDLR, we designed two pairs of crRNAs that are located upstream and downstream of the gene, representing a dual-cut excision approach (Table S1, crRNAs I + II + IV + V). We tested our approach using HMW DNA or
DNA from standard purification. Nanopore sequencing on a MinION device achieved complete coverage of the LDLR gene including the promoter region for both conditions (Fig. 1). In the approach with HMW DNA, 1,489

of 200,041 total mapped reads were assigned to the target region (on-target ratio: 0.74%), whereas the on-target ratio was 0.54% for the approach with DNA from spin column purification (584/108,862 reads). This corresponds to an enrichment of 454-fold vs. 188-fold (Table S2). The mean sequencing coverage was considerably higher using HMW DNA (381x) compared to DNA from standard purification (35.7x) with mean read lengths of 30.08 kb and 11.14 kb, respectively.

3.2. Precise determination of the size and breakpoints of large LDLRCNVs using long-read sequencing

To test our Cas9-mediated LRS approach, we sequenced two samples with known CNVs in the LDLR gene that were previously identified with MLPA (Fig. 2A,D) (Rutkowska et al., 2022). In the first case, MLPA identified a heterozygous duplication of LDLR exons 4–8 (Fig. 2A). Using our dual-cut excision approach, Nanopore sequencing with a mean read length of 21.05 kb detected approximately 8 kb-sized duplication sequences and thus verified the in tandem localization of the variant with breakpoints located in intron 3 and intron 8 of LDLR (Fig. 2B, Table S3).

Fig. 1. Targeted long-read sequencing of the LDLR gene. Schematic illustration of the LDLR gene and target selection using CRISPR/Cas9 in a dual-cut excision approach. Green arrows indicate crRNA cleavage sites and sequencing directions. IGV plots show sequencing results using high-molecular-weight DNA or DNA from standard purification as input. Grey bars show sequencing coverage (cov) with mean values of 381x or 35.7x inside the LDLR gene. Pink and purple lines represent sequencing reads.

A

B

D

E

C

F

Fig. 2. Precise characterization of large CNVs in the LDLR gene using CRISPR/Cas9-mediated Nanopore sequencing. LRS allowed exact breakpoint determination for a heterozygous exons 4–8 duplication (A–C) and a heterozygous exons 1–2 deletion (D–F) in the LDLR gene that were previously identified with MLPA (A,D). The upstream MLPA probe binds in the neighbouring SMARCA4 gene. (B) LRS data showed approximately 8 kb-sized in tandem duplication of exons 4–8 of LDLR. (C) Duplication-specific PCR detected a 374 bp band in the sample of the index proband (P) and its absence in healthy control (C) and negative control (-) samples. Sanger sequencing revealed the exact breakpoints in intron 8 and intron 3 of LDLR. (E) LRS data showing a 19.2 kb large deletion, spanning exons 1–2 and the 5'-unstrated and promoter regions of LDLR. (F) Deletion-specific PCR detected a 404 bp band in the sample of the index proband (P) and its absence in healthy control (C) and negative control (-) samples. Sanger sequencing revealed the exact deletion breakpoints. Read data of Nanopore sequencing were inspected in IGV. Primer locations for variant-specific PCR are depicted schematically (B,E; green and red arrows). For breakpoint positions, the hg19 genomic coordinates are given.

Visual inspection of the long-read data in the IGV as well as the results of the bioinformatic SV detection tools allowed refinement of the breakpoints (Table S4) and the design of variant-specific primers. PCR and Sanger sequencing validated the exact breakpoints of the 8117 bp duplication (Fig. 2C). The breakpoints were located in an AluSx1 (intron 8) and a MER83 element (intron 3).

In the second case, MLPA detected a heterozygous deletion in LDLR encompassing the first two coding exons as well as the 5'-unstranslated and promoter regions of LDLR (Fig. 2D). As this deletion is located at the gene boundary, we did not apply our dual-cut excision approach, but used a single crRNA located downstream of exon 14 for targeted LRS (Table S1, crRNA III). Nanopore sequencing with a mean read length of 22.9 kb revealed the deletion to be 19.2 kb in size (Fig. 2E, Table S3) and enabled refining of the breakpoints (Table S4) that were located in an AluY (upstream) and an AluSx1 element (intron 2). LRS allowed the design of specific PCR primers to confirm the deletion by PCR and verify the exact breakpoints by Sanger

sequencing (Fig. 2F).

3.3. Clinical and genetic characterization of FH probands referred to short-read gene panel analysis

Next, we clinically characterized a cohort of 21 FH probands who had been referred to standard short-read sequencing of the LDLR, APOB, PCSK9, and LDLRAP1 genes to identify candidates for complementary LRS analysis. The clinical characteristics of the cohort are summarized in Table 1. The probands were between the ages of 36 and 78 years. 70 % of the probands reported a positive family history according to the DLCN criteria. Similarly, 70 % had a positive personal history, while none of the probands presented with characteristic physical symptoms like an arcus corneae or tendon xanthomas. An elevated mean LDL-C value of

Table 1
Basic characteristics of the FH proband cohort.

sex (n = 21)	male: 66.7 female: 33.3	age [years] (n = 21)	55.4 (±10.6)
positive personal history* (n = 20)	70	BMI [kg/m^2] (n = 20)	27.2 (±4.7)
physical symptoms* (n = 20)	0	total cholesterol [mmol/l] (n = 20)	5.96 (±1.76)
positive family history* (n = 20)	70	LDL-C [mmol/l] (n = 21)	6.15 (±1.84)
nicotine consumption (n = 15)	curently: 13.3	HDL-C [mmol/l] (n = 20)	1.27 (±0.32)
	formerly: 60		
arterial hypertension (n = 21)	66.7	triglycerides [mmol/l] (n = 20)	2.14 (±1.59)
diabetes mellitus (n = 21)	4.8		
obesity (n = 21)	19.1		

SD: standard deviation. BMI: body mass index. *according to DLCN criteria.

A

B

Fig. 3. Gene panel testing in a FH proband cohort. (A) Summary of DLCN clinical diagnosis and mutation detection rates in 21 FH probands referred to short-read gene panel testing. Probands without a pathogenic variant in NGS but with strong clinical evidence of FH (DLCN score above 5) were selected for Cas9-mediated LRS of LDLR (highlighted in red). DLCNS - Dutch Lipid Clinic Network Score. (B) Schematic representation of (likely) pathogenic variants that were identified with gene panel sequencing in FH probands. The functional domains of the LDLR and ApoB-100 proteins are illustrated. Figure created with biorender.com.

6.15 mmol/l was observed for the cohort. Arterial hypertension was one of the most frequently observed concomitant diseases (66.7%), while diabetes mellitus and obesity (BMI ⩾30 kg/m 2) were only diagnosed in 4.8 % and 19.1 % of the probands, respectively. Before molecular genetic diagnostics were initiated, one proband was clinically diagnosed with “definite FH”, eight probands with “probable FH’, and twelve probands with “possible FH” according to the DLCN criteria (Fig. 3A). Standard short-read sequencing detected pathogenic or likely pathogenic variants according to the ACMG criteria in eight probands (Table S5). This represents mutation detection rates of 100 % (definite FH, 1/1), 75 % (probable FH, 6/8), and 8.3 % (possible FH, 1/12). Most of the (likely) pathogenic variants were located in the LDLR gene. For two probands, the most common pathogenic variant c.10580G>A [p. (Arg3527Gln)] in the APOB gene was identified (Fig. 3B, Table S5). Bialleic variants or (likely) pathogenic alterations in the PCSK9 or LDLRAPI genes were not identified.

All variants, including the previously described large CNVs, were submitted to the ClinVar database (Submission ID: SUB15045022).

3.4. Long-read sequencing for two mutation-negative “probable FH” probands

To see whether hard-to-detect SVs in LDLR could be identified in apparently mutation-negative probands with strong clinical evidence of FH (DLCN score >5), we applied our LRS approach to the two probands with

a clinical diagnosis of “probable FH” but without a pathogenic variant in standard short-read sequencing analysis (Fig. 3A). DNA from standard purification was used for LRS, since blood samples for HMW DNA isolation were not available. Complete coverage of the gene was achieved for both cases. A high mean sequencing coverage of 111x was achieved for the first proband (Fig. 51A, Table S2). However, no large SV was detected after visual inspection of the read data in the IGV and analysis with the tools cuteSV and Sniffels2. For the second proband, no SV was detected either. However, although the entire stored DNA sample was already used here, the DNA input was apparently insufficient. Therefore, the mean sequencing coverage was low (6.4x) and does not exclude the possibility of a false negative result (Fig. S1B, Table S2).

4. Discussion

In this study, we successfully used amplification-free, Cas9-mediated target selection and Nanopore LRS to completely cover the genomic region of the LDLR gene and to precisely determine breakpoints of known CNVs. As a complementary analysis to short-read sequencing-based FH diagnostics, our LRS approach can overcome limitations of gene panel sequencing and MLPA in SV detection or breakpoint specification and allows for faster and more cost-effective predictive analyses by allele-specific PCR.

LRS of LDLR on the MinION or PacBio platform after long-range PCR amplification has been successfully used to detect pathogenic SNVs or CNVs as well as in phasing approaches to confirm in trans configuration of variants (Chaudhry et al., 2023; Nazarenko et al., 2023; Soufi et al., 2022). However, amplification-based approaches have several limitations, such as incomplete coverage of the gene, inability to amplify large and complex regions or unsuccessful phasing due to insufficient segment size and overlap (Nazarenko et al., 2023; Soufi et al., 2022). Amplification-free CRISPR/Cas9-mediated target selection for LRS, on the other hand, can overcome these limitations and has recently been applied by Xu et al. for Nanopore sequencing of the LDLR and PCSK9 genes (Xu et al., 2024), demonstrating for the first time its potential value in FH diagnostics. Using this approach, they achieved mean coverages of LDLR in the range of 13-277x and were able to detect pathogenic SNVs, identify a novel large deletion and rule out a false positive deletion caused by amplification error (Xu et al., 2024). Independently, we here also established Cas9-mediated target selection and Nanopore sequencing of the LDLR gene. By adding more cases to the still limited number of LRS analyses described so far in the FH context, our study contributes to establishing Cas9-mediated LRS as a complementary

amplification-free method in FH diagnostics.

Our dual-cut excision approach achieved complete coverage of LDLR including the promoter region and similar sequencing depths compared to the study of Xu et al. for both HMW DNA (381x) and DNA from standard purification (6.4x to 111x). A mean target coverage of ⩾30x was achieved for all but one case (Table S2, Fig. S1B). Although the use of high-quality HMW DNA resulted in a higher sequencing depth and is recommended to ensure best performance of LRS, these results show that DNA from standard purification may also be used to achieve sufficient sequencing results. This is particularly relevant, as HMW DNA extraction is costly, and new blood samples may not always be available. However, our data also illustrate that DNA input and quality are important factors that limit the LRS efficiency. Since a DNA input of 5 g is recommended for optimal coverage in Cas9-mediated LRS in current Oxford Nanopore protocols, the low coverage of 6.4x in one case is most likely due to the low DNA input of 1.48 g which could not be increased due to limited available sample amount (Table S2). While on-target coverages below 30x may still be sufficient for confirmatory analyses of known CNVs (de Boer et al., 2024; Iyer et al., 2022; Skowronek et al., 2022; Stangl et al., 2020), higher coverages would be needed for screening approaches.

Our Cas9-mediated LRS approach could reliably reidentify known CNVs that were previously identified by MLPA. LRS confirmed in tandem localization and thus the pathogenicity of the duplication of exons 4 to 8 in the LDLR gene. This variant has been frequently described in the literature for FH patients (Chmara et al., 2010; Goldmann et al., 2010; Madar et al., 2022). Notably, the breakpoints determined in our study

match the breakpoint positions as determined in a recent study for this duplication (Konecna et al., 2024). In the case harboring a deletion of exons 1–2 of LDLR, MLPA analysis was already able to narrow down the extent of the variant, which encompasses the 5'UTR and promoter regions of LDLR. However, breakpoint information from Cas9-mediated LRS enabled precise determination of the variant size as well as design of a variant-specific PCR assay which can be a cost-effective option in subsequent familiar analyses. Additionally, breakpoint information by LRS can shed light on the mechanisms of CNV formation. Three out of four breakpoints for the two CNVs analyzed here were located in Alu repeats. This reflects the susceptibility of LDLR to CNV formation by non-allelic homogeneous recombination mediated by Alu repeats that can be found with a particular high frequency in the locus (Amsellem et al., 2002; Goldmann et al., 2010). While it has been proposed that the duplication rearrangement of exons 4 to 8 could have also been initiated by the pairing of two Alu repeats (Konecna et al., 2024), the breakpoint in intron 3 is in fact located in MER83 which belongs to the long terminal repeats (LTRs) retrotransposons.

Even using LRS, no SVs were detected in our mutation-negative subjects with “probable FH”. Therefore, alternative explanations such as a polygenic cause or phenocopies must be considered in these cases. For example, familiar combined hyperlipidemia with elevated triglyceride levels and elevated Lp(a) (Ellis et al., 2016) could be a possible differential diagnosis for these two probands.

One limitation of our study is the small sample size. Therefore, future studies with larger cohort of mutation-negative probands with high clinical evidence of FH will need to determine whether amplification-free LRS can be used as a screening tool to identify complex SVs, and thus elucidate some of the missing heritability in mutation-negative FH patients. Another limitation is that we did not apply our LRS approach to mutation-negative probands with "possible FH" (DLCN score 3–5). However, some guidelines do not even recommend standard short-read sequencing in these cases (National Institute for Health and Care Excellence, 2019; Nordestgaard et al., 2013), as the mutation detection rate is rather low (Grenkowitz et al., 2016; Rieck et al., 2020). Although this does not exclude the existence of SVs in these patients, the benefit-cost ratio of targeted LRS in this group appears to be unfavorable.

5. Conclusions

In summary, Cas9-mediated Nanopore LRS is a powerful tool for accurate CNV characterization. As a complementary technology in FH diagnostics for selected cases, LRS thus allows for better variant interpretation and PCR-based predictive analyses, while further studies will have to establish its value in screening approaches to clarify genetically unsolved cases.

CRediT authorship contribution statement

Robin A. Pilz: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. Dariush Skow-ronek: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. Loisa D. Bonde: Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. Tadeusz Kahuzewski: Writing – review & editing, Resources. Ole J. Schamuhn: Writing – review & editing, Methodology, Raila Busch: Writing – review & editing, Resources, Investigation. Agnizszka Gach: Writing – review & editing, Visualization, Resources, Investigation, Data curation. Mathias Rath: Writing – review & editing, Supervision, Data curation, Conceptualization. Elisabeth Steinhagen-Thiessen: Writing – review & editing, Resources, Investigation. Ute Felbor: Writing – review & editing, Supervision, Conceptualization.

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Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmg.2025.105003.

Data availability

All relevant data supporting the results of this study are included in the article and its supplementary material. The sequencing data are not publicly available due to data protection restrictions.

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