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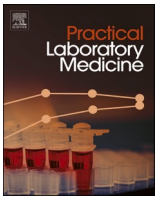

Registered Report Stage II

Implementation of long-read sequencing for routine molecular diagnosis of familial mediterranean fever

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ABSTRACT

Background: Long-read sequencing technology, widely used in research, is proving useful in clinical diagnosis, especially for infectious diseases. Despite recent advances, it hasn't been routinely applied to constitutional human diseases. Long-read sequencing detects intronic variants and phases variants, crucial for identifying recessive diseases.

Methods: We integrated long-read sequencing into the clinical diagnostic workflow for the MEFV gene, responsible for familial Mediterranean fever (FMF), using a Nanopore-based workflow. This involved long-range PCR amplification, native barcoding kit library preparation, GridION sequencing, and in-house bioinformatics. We compared this new workflow against our validated method using 39 patient samples and 3 samples from an external quality assessment scheme to ensure compliance with ISO15189 standards.

Results: Our evaluation demonstrated excellent performance, meeting ISO15189 requirements for reproducibility, repeatability, sensitivity, and specificity. Since October 2022, 150 patient samples were successfully analyzed with no failures. Among these samples, we identified 13 heterozygous carriers of likely pathogenic (LP) or pathogenic (P) variants, 1 patient with a homozygous LP/P variant in MEFV, and 4 patients with compound heterozygous variants.

Conclusion: This study represents the first integration of long-read sequencing for FMF clinical diagnosis, achieving 100 % sensitivity and specificity. Our findings highlight its potential to identify pathogenic variants without parental segregation analysis, offering faster, cost-effective, and accurate clinical diagnosis. This successful implementation lays the groundwork for future applications in other constitutional human diseases, advancing precision medicine.

1. Introduction

In the rapidly evolving landscape of genomic diagnosis and precision medicine, long-read sequencing has emerged as a transformative technology with profound implications for understanding the intricacies of the human genome. Unlike traditional short-read sequencing methods, long-read sequencing provides the unique ability to decipher extended stretches of DNA, offering unprecedented insights into complex genomic structures, repetitive regions, and elusive variant. Rapid advances in long reads sequencing by Nanopore technologies have led to substantial improvements but still suffered of lack of data quality (reads PHRED quality around Q20) [1]. In the context of clinical genetics, long read sequencing is a promising technology used up to now mainly for research and

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infectious pathogens sequencing [2]. However, the advantages of Nanopore sequencing, such as its simplicity, customizability, cost-effectiveness, and ability to generate long reads enabling phasing and detection of intronic variants, could also prove highly valuable in the diagnosis of constitutional diseases.

To further explore the ability of a Nanopore workflows to reach application in clinical setting for constitutional diseases, we choose as a proof of concept the analysis of the *MEFV* gene. Pathogenic variants within this gene are responsible of Familial Mediterranean Fever, a recessive disease with a high prevalence (1:500 to 1:1000 in endemic countries), characterized by recurrent outbreaks of fever associated with inflammatory manifestations [3]. In addition to its medical interest, the *MEFV* gene represents a textbook case for testing long read sequencing. Indeed, its size of about 14 kb allows it to be amplified by a single long-range PCR and to be fully included in a single sequencing read.

An analytical validation of a this Nanopore workflow for SNV in the *MEFV* gene have already been made [4], but does not make use of all the advantages of long reads sequencing. Indeed, in this study, short fragments (<1000 pb) have been sequenced by a Nanopore device, limiting the ability to ascertain variant phasing—an essential piece of information, especially for recessive diseases like FMF.

Here we present an optimized Nanopore based workflow which takes advantages of long read sequencing and tackles the low-quality data using deep sequencing and an optimized bioinformatic pipeline.

We believe such a workflow is an improvement in comparison to our actual short read sequencing routine protocol. Indeed, for the patient and the clinician, the advantage of this approach lies in the possibility of highlighting the phase of the variations (cis or trans) without requiring parental sequencing, simplifying care channel. Also, whereas no deep intronic variant have been related to FMF [5], sequencing the whole *MEFV* gene is an opportunity to discover pathogenic intronic variant. In fact, up to 25 % of individuals with FMF have only one *MEFV* pathogenic variant, suggesting an unrevealed second hit [6].

2. Material & methods

2.1. Sample collection

39 samples from patients that were drawn for routine *MEFV* assessment were included into this study after the diagnostic routine testing of Eurofins Biomnis was performed.

3 samples from the European Molecular Genetics Quality Network (EMQN), Hereditary Recurrent Fevers 2020 (HFR) and systemic autoinflammatory diseases 2021 and 2022 (SAID) schemes, were also used as standard.

After the initial validation of the method, we swapped the *MEFV* routine diagnosis protocol from short to long read sequencing and monitored the outcome of 150 patient samples.

The study was performed in accordance with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all patients regarding de-identified clinical and personal patient data collection, analysis, and publication. The Eurofins Biomnis laboratory is accredited with the national GPRD (General Data Protection Regulation), protecting patients' personal data. Furthermore, our laboratory adheres to the stringent requirements of the ISO 15189 accreditation (https://www.iso.org/fr/standard/76677.html), which we have received from the competent national authorities, including ARS (Agence Régionale de Santé) and COFRAC (Comité Français d'Accréditation). This accreditation confirms our commitment to upholding the highest standards of quality in medical laboratory practices, including the handling of personal health information and patient samples.

2.2. Gold standard short reads assay

The previous routine diagnostic workflow includes DNA isolation, polymerase chain reaction (PCR) amplification of selected targets within *MEFV* and sequencing. DNA isolation from EDTA whole blood samples was performed on QIAsymphony instruments (QIAGEN) using QIAsymphony DSP DNA mini Kit CE-IVD. PCR amplification of the 10 exons of *MEFV* was performed with KAPA 2G FAST MULTIPLEX PCR KIT and primers list in Supplemental Table 1. Libraries were prepared with the Nextera XT index Kit (ILLUMINA®) and allowed to multiplex 24 samples. Sequencing was performed on a MiSeq® sequencer with paired end reads of 150bp. Bioinformatic analysis was performed with the proprietary software (VariantStudio). This workflow is ISO15189 accredited, to fulfill the French requirement for diagnosis testing.

2.3. Nanopore based workflow

Long Range PCR amplification of the *MEFV* whole gene was performed using target specific primers as previously described [7]. Briefly, we performed LR-PCR with LongAmp Hot Start Taq 2X Master Mix (New England Bio Labs) and the following primers: 5′-TCC TCT GAA CCT GTA AGA GAA CAC AGC-3′ and 5′-ATC CAT GGT GTG TCA GTA CAT GTC TTC-3′. The cycling conditions performed on a GeneAmp PCR System 9700 (Applied Biosystems) were as follows: 94 °C for 30 s; 30 cycles at 94 °C for 20 s, and 65 °C for 12.5 min and a final elongation step of 10 min at 65 °C.

The theoretical product size of LR-PCR was 13,866 bp, and the GC content was 51.5 %. This range was verified using Tape Station (ROCHE).

2.4. Library preparation

To be able to determine phase of variants, we chose to prepare libraries from the long-range PCR products. The nanopore libraries

were prepared following the protocol for Native Barcoding Kit 96 (SQK-NBD112.96) (Oxford Nanopore Technologies).

The DNA was repaired with NEB Next Ultra II End Repair/dA-tailing Module (New England Bio Labs) and prepared for adapter attachment. Ligation of Native barcodes to the DNA ends allows to pool up to 96 samples. Purification steps allow the selection of long fragments. Adapters and barcodes are ligated to the amplicons, prior to quantification and pooling. The libraries were sequenced with a Minion Flow cell (R9.4.1) on a GridION device for 8 h.

Table 1
List of reported variants found with the previous and Nanopore workflow, for 39 clinical samples.

Sample	Previous workflow		Nanopore workflow	
	Variant reported	Genotype	Variant reported	Genotype
1	c.124C > T; p. (Arg42Trp): VUS	0/1	c.124C > T; p. (Arg42Trp): VUS	1|0
2	c.2080A > G; p. (Met694Val): P	1/1	c.2080A > G; p. (Met694Val): P	1|1
3	c.2177 T > C; p. (Val726Ala): P	0/1	c.2177 T > C; p. (Val726Ala): P	1|0
	c.1437C > G; p. (Phe479Leu): LP	0/1	c.1437C > G; p. (Phe479Leu): LP	0|1
	c.501G > C; p. (Glu167Asp): LP	0/1	c.501G > C; p. (Glu167Asp): LP	0|1
4	c.2080A > G; p. (Met694Val): P	0/1	c.2080A > G; p. (Met694Val): P	1|0
	c.1437C > G; p. (Phe479Leu): LP	0/1	c.1437C > G; p. (Phe479Leu): LP	0|1
	c.501G > C; p. (Glu167Asp): LP	0/1	c.501G > C; p. (Glu167Asp): LP	0|1
5	c.2177 T > C; p. (Val726Ala): P	0/1	c.2177 T > C; p. (Val726Ala): P	1|0
	c.2082G > A; p. (Met694Ile): P	0/1	c.2082G > A; p. (Met694Ile): P	0|1
6	c.1772 T > C; p. (Ile591 Thr): VUS	0/1	c.1772 T > C; p. (Ile591 Thr): VUS	0|1
7	/		/	
8	/		/	
9	c.2282G > A; p. (Arg761His): LP	0/1	c.2282G > A; p. (Arg761His): LP	0|1
	c.2082G > A; p. (Met694Ile): P	0/1	c.2082G > A; p. (Met694Ile): P	1|0
10	/		/	
11	c.2177 T > C; p. (Val726Ala): P	0/1	c.2177 T > C; p. (Val726Ala): P	1|0
	c.2040G > C; p. (Met680Ile): P	0/1	c.2040G > C; p. (Met680Ile): P	0|1
12	/		/	
13	/		/	
14	c.2082G > A; p. (Met694Ile): P	0/1	c.2082G > A; p. (Met694Ile): P	1|0
	c.2230G > T	0/1	c.2230G > T	0|1
15	c.1223G > A	0/1	c.1223G > A	1|0
	c.1105C > T	0/1	c.1105C > T	1|0
16	c.2080A > G; p. (Met694Val): P	1/1	c.2080A > G; p. (Met694Val): P	1|1
17	c.1772 T > C; p. (Ile591 Thr): VUS	0/1	c.1772 T > C; p. (Ile591 Thr): VUS	1|0
18	c.442G > C; p. (Glu148 Gln): VUS	0/1	c.442G > C; p. (Glu148 Gln): VUS	1|0
19	c.1223G > A; p. (Arg408 Gln): VUS	0/1	c.1223G > A; p. (Arg408 Gln): VUS	0|1
	c.1105C > T; p. (Pro369Ser); VUS	0/1	c.1105C > T; p. (Pro369Ser); VUS	0|1
	c.442G > C; p. (Glu148 Gln): VUS	0/1	c.442G > C; p. (Glu148 Gln): VUS	1|0
20	c.1223G > A; p. (Arg408 Gln): VUS	0/1	c.1223G > A; p. (Arg408 Gln): VUS	1|0
	c.1105C > T; p. (Pro369Ser); VUS	0/1	c.1105C > T; p. (Pro369Ser); VUS	1|0
	c.442G > C; p. (Glu148 Gln): VUS	0/1	c.442G > C; p. (Glu148 Gln): VUS	1|0
21	c.800C > T; p. (Thr267Ile): LP	0/1	c.800C > T; p. (Thr267Ile): LP	0|1
22	/		/	
23	c.2177 T > C; p. (Val726Ala): P	0/1	c.2177 T > C; p. (Val726Ala): P	1|0
24	c.2082G > A; p. (Met694Ile): P	0/1	c.2082G > A; p. (Met694Ile): P	0|1
25	c.2080A > G; p. (Met694Val): P	1/1	c.2080A > G; p. (Met694Val): P	1|1
26	c.2282G > A; p. (Arg761His): LP	0/1	c.2282G > A; p. (Arg761His): LP	1|0
	c.2080A > G; p. (Met694Val): P	0/1	c.2080A > G; p. (Met694Val): P	0|1
27	/		/	
28	c.442G > C; p. (Glu148 Gln): VUS	0/1	c.442G > C; p. (Glu148 Gln): VUS	1|0
29	c.2177 T > C; p. (Val726Ala): P	0/1	c.2177 T > C; p. (Val726Ala): P	0|1
30	c.2038A > C; p. (Met680Leu): LP	0/1	c.2038A > C; p. (Met680Leu): LP	1|0
31	c.2003G > C; p. (Gly668Ala): VUS	0/1	c.2003G > C; p. (Gly668Ala): VUS	0|1
	c.1223G > A; p. (Arg408 Gln): VUS	0/1	c.1223G > A; p. (Arg408 Gln): VUS	1|0
	c.1105C > T; p. (Pro369Ser): VUS	0/1	c.1105C > T; p. (Pro369Ser): VUS	1|0
32	c.2082G > A; p. (Met694Ile): P	0/1	c.2082G > A; p. (Met694Ile): P	1|0
33	c.442G > C; p. (Glu148 Gln): VUS	0/1	c.442G > C; p. (Glu148 Gln): VUS	0|1
34	c.2177 T > C; p. (Val726Ala): P	0/1	c.2177 T > C; p. (Val726Ala): P	0|1
	c.2080A > G; p. (Met694Val): P	0/1	c.2080A > G; p. (Met694Val): P	1|0
35	c.2080A > G; p. (Met694Val): P	0/1	c.2080A > G; p. (Met694Val): P	0|1
36	c.2080A > G; p. (Met694Val): P	0/1	c.2080A > G; p. (Met694Val): P	0|1
37	/		/	
38	c.2080A > G; p. (Met694Val): P	1/1	c.2080A > G; p. (Met694Val): P	1|1
39	/		/	

The variants are described with transcript NM_000243.3.
VUS: Variant of Uncertain Significance/LP: Likely Pathogenic/P: Pathogenic.

2.5. Bio-informatics analysis

Base-calling was carried out on the local computer of the GridION device, in real time using MinKnow software (Oxford Nanopore Technologies, version 22.10.5).

After subsampling of 1000 reads with PHRED score quality >7 using Seqtk [8], and quality control check with Nanoplot [9], the reads were mapped against reference genome GRCh37 using minimap2 [10]. Variant calling was performed using Clair3 [11]. As this tool down-sample data to obtain a depth of 150X max, we set a threshold of 300X for QC. SNPeff [12] and SIFT [13] were run to annotate the VCF with relevant database (ClinVar [14], GnomAD [15] and Infevers [16]) and to create a human-readable file for interpretation.

For interpretation, after validation of QC metrics, the variants referred in Infevers [16] as pathogenic, likely pathogenic or variant of uncertain significance were retained, and interpreted regarding their phase.

2.6. Results comparison

Method comparison was done in Microsoft Excel. After importing the data sets, Nanopore based workflow variant calls were compared to our gold standard method for genomic position, nucleotide change, zygosity, amino acid position, and amino acid change.

3. Results

3.1. Robustness of the method

To date, 10 runs have been performed in the laboratory, including 192 samples. For practical reasons, we decided to perform 8h00 overnight sequencing. This has the consequence of generating for each sample an excessive number of reads (14,033 on average) that require a huge amount of computing resources for analysis. After several optimization rounds, we decided to reduce by a random selection of thousand Q7 reads. At the end of the Nanopore workflow, the mean depth across *MEFV* gene is thus close to 1000X (900X ± 38x) and only 3 samples have mean depth across *MEFV* gene <300X. those 3 samples were rerun and depth >900X were obtain.

3.1.1. Intrarun repeatability

One sample was analyzed 5 times in the same run. After removing low quality variants (QUAL according to Clair3 <10), we analyzed the remaining variants for each replicate. All the 33 variants (intronic and exonic) were found for each barcode, with a similar VAF, QUAL and depth (Supplemental Table 2)

3.1.2. Interrun reproductibility

2 samples were analyzed in 2 different runs. After removing low quality variants (QUAL<10), we analyzed the remaining variants for each replicate. All the variants (35 and 17) were found in the different run, with a similar VAF, QUAL and depth (Supplemental Table 3)

3.2. Validation cohort

3.2.1. 39 samples were included in this cohort

We first focused on the 31 variants of medical interest found with our gold standard technique. In this cohort, 18 samples had no reported variant, 9 were carrier of 1 VUS/LP/P heterozygous variant, 6 were carrier of 2 VUS/LP/P heterozygous variants, 2 were carrier of 3 VUS/LP/P heterozygous variants and 4 were carrier of 1 VUS/LP/P homozygous variant. Of note, the previous technique cannot determine the phase of some heterozygous variants, because they were not close enough to be included in the same amplicon).

3.2.2. With the nanopore workflow, we obtained the same results, with phase determination (Table 1)

We then looked at all the 476 variants discovered with previous technique (194 homozygous and 282 reported as heterozygous). Among those variants, only 55 were not detected by the Nanopore workflow. It consist of 3 frequent, not pathogenic variants: NM_000243.2:c.*267G > A, NM_000243.2:c.*245G > A (3′UTR variants) and NC_000016.10:g.3256757C > T (upstream gene variant), which are not covered by the LR-PCR design. Except those 55 variants, the 421 variants in his cohort were found. Quality of variant calling was higher than 10 (10.84–42.05, mean: 25.14). Zygosity was identical, defined for Nanopore sequencing as

Table 2
List of discordant variants between the previous and Nanopore workflow.

Sample	Previous workflow				Nanopore workflow			
	Variant	VAF	Depth	Genotype	Variant	VAF	Depth	Genotype
14	c.605G > A	93,4	995	hom	c.605G > A	0.5175	999	0|1
14	c.306 T > C	95	2353	hom	c.306 T > C	0.5375	999	0|1
15	c.605G > A	96,3	109	hom	c.605G > A	0.5311	996	1|0
15	c.495C > A	96,2	213	hom	c.495C > A	0.5733	996	1|0

heterozygous if VAF <65 % and homozygous if VAF >65 %, except for 2 samples (Table 2). Those 2 samples were homozygous for 3 variants in exon 2 with short-read workflow, whereas long-reads workflow detected those variants at VAF near 50 %. No other differences were found. Looking at BAM file (Fig. 1), it seems that those 2 samples carrying a frequent multi-nucleotide variant (MNV) ([GRCh37]chr16:g.3303817CC > AA), at heterozygous state in the sequence sequencing where the *MEFV*-Exon2-For primer hybridize for the Illumina multiplex assay. Nanopore sequencing emphasize that the allele carrying this MNV is WT for variants in exon 2. The hypothesis is a selective amplification of the mutant allele with the multiplex PCR, resulting in artefactual homozygous call.

To explore such bias in the presented workflow, we check polymorphism in GnomAD [15]. A polymorphism is reported in the left primer used in the present protocol ([GRCh37] chr16:3,306,757 > T, 7 % in African ancestry individuals). To verify the absence of allele drop-out, 2 samples carrying this SNP at heterozygous were processed according the present protocol. The presence of heterozygous variants for both samples allow to conclude in the absence of allele dropout for samples carrying this SNP.

3.3. Reference samples

To further validate this method, we analyze samples from external quality assessment (EQA). The expected results for those samples were the same than the results from our gold standard workflow, and would give a 'Satisfactory' result on EQA scheme Table 3 (Conclusion of EQA 2022were not available when writing this study).

3.4. Prospective cohort

Since the routine adoption of the Nanopore workflow in October 2022, 8 batch were performed including 150 patients. The results are the following.

- 109 patients were negative for causative variants in *MEFV* gene
- 29 patients were heterozygous carrier of a LP/P variant in *MEFV* gene
- 4 patients were homozygous for a LP/P variant in *MEFV* gene
- 8 patients were compound heterozygous for 2 LP/P variants in *MEFV* gene. Of note, with the previous method, segregation analyses in parents would have been required.

One of the advantages of the long-read workflow is the ability to sequence the whole *MEFV* gene, including introns. Within the whole cohort, 180 variants were identified. Across all them, one variant is predicted to affect splicing according SpliceAI (Illumina) > 0.2 and Spip [17]. NM_000243.3 (MEFV):c.1223G > A; p. Arg408Gln is predicted to alter splicing by creation of a new splice site + Alter ESR by Spip at 85.91 % [79.27 %–91.06 %]; and to lead to acceptor loss by SpliceAI (score 0.23). This variant has already been studied [18] and reported several times as Benign or VUS in ClinVar [14] and Infevers [16], but his effect on splicing wasn't evaluated to date. Unfortunately, no intronic variant were predicted to alter splicing according to Splice AI and Spip.

4. Discussion

We aimed to prove that long read based workflow is a valuable tool for routine diagnosis of constitutional human disease. The value of long red sequencing have been shown for repeat expansion analysis in neurological disorder [19], or highly similar sequence like *TMEM231*, leading to Meckel–Gruber syndrome (MKS) [20] or the HLA locus if the fiels of transplantation [21]. Compared to short-read sequencing, it allows the phasing of variant, a step required when studying recessive disease, and to detected intronic

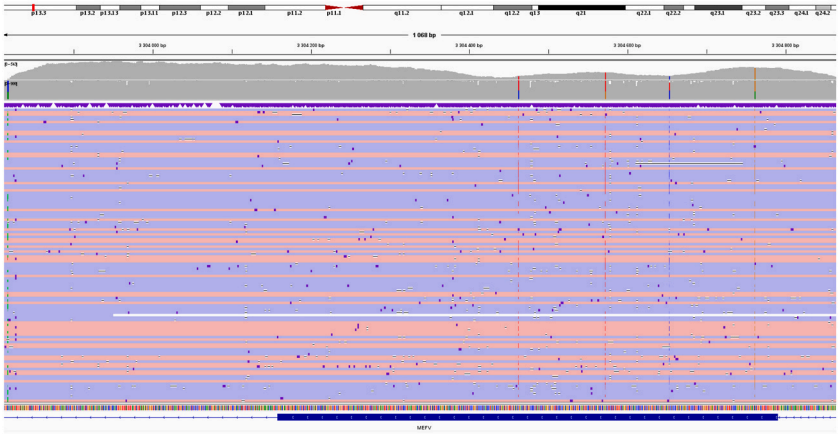

Fig. 1. IGV view of the BAM file for sample 15. The upper track is the coverage from previous workflow (short-reads). The middle and lower tracks are the coverage and the reads from Nanopore workflow.

Table 3
Results of EQA Schemes with both workflows.

	Illumina	Scheme result	Nanopore
EMQN HRF 2020 - Case 2	c.1105C > T ()1223G > A ()2080A > G p.Pro369Ser()Arg408 Gln ()Met694Val	Satisfactory	c. [1105C > T; 1223G > A]; [2080A > G] p. [Pro369Ser; Arg408 Gln]; [Met694Val]
EMQN SAID 2021 - Case 1	c. [442G > C]; [442G > C]()1105C > T ()1223G > A p. [Glu148 Gln; [Glu148 Gln]()Pro369Ser()Arg408 Gln	Satisfactory	c. [442G > C; 1105C > T; 1223G > A]; [442G > C] p. [Glu148 Gln; Pro369Ser; Arg408 Gln]; [Glu148 Gln]
EMQN SAID 2022 - Case 1	c. [2082G > A]; [2082G > A] p. [Met694Ile]; [Met694Ile]	NA	c. [2082G > A]; [2082G > A] p. [Met694Ile]; [Met694Ile]

variant, classically not explored with targeted short read sequencing. We choose to demonstrate this statement with the replacement of our short reads technique for *MEFV* testing, as this gene is responsible for a recessive disease (Familial Mediterranean fever, OMIM #249100) and is amplifiable by long range PCR (gene size: 14,607 bases).

We demonstrate the analytical and clinical validation on a cohort of 39 patients and 3 standard samples, leading to the same conclusions and a recall of 100 % for medical interest variant. Requirements for ISO15189 certifications are proven. As expected, we emphasized less bias due to PCR: the previous technique required 10 PCR fragments versus 1 with the long-read workflow. An allele drop out effect was observed with the previous technique in one amplicon for 2 patients due to a frequent MNV in the hybridization zone of primers. Even if a frequent SNP is present in the left primer of the present protocol, it doesn't lead to PCR bias.

As this Nanopore workflow allow the detection of intronic variants, we looked for causative variants of Mediterranean fever in intronic regions of *MEFV* gene, beside no intronic variant have been reported to date [5]. The analysis on 150 samples do not reveals intronic variant reaching likely pathogenic or pathogenic level of ACMG classification. For such rare and intronic variant, functional studies will be needed for characterization of pathogenicity, requiring collaboration between clinical and research labs [22].

A big advantage of long read sequencing is the ability to determine the phase of heterozygous variant, without need for parents' sample. Within 150 patients referred for *MEFV* testing, 8 were compound heterozygous for a causative variant. Using short-reads strategy, parents' sample would have been required to demonstrate the inheritance of the variants. Hence, we propose an organizational and time effective solution.

Regarding cost, this long-reads strategy is cheaper than the previous workflow based on Illumina reagents. The reagents are cheaper with this workflow, mainly due to the flow cell cost difference. Also, we save one day of technician time with the Nanopore method compared to Illumina. Of note, this workflow is also cheaper than Sanger sequencing approach. Indeed, 10 reactions will be needed to cover the 10 exons containing VUS of (likely) pathogenic variants [16]. However, the targeted screening of the six most prevalent variants (M694V, V726A, M680I, M694I, V694I, E148Q) - representing 80 % of all cases [23] – is cheaper, but miss 20 % of the diagnosis.

Switching to this Nanopore technology have limitations. The reagents used for now are not accurate to detect small indels [24]. However, to date, no such variant have been reported to be responsible of Mediterranean fever [14,16]. Pacific Bioscience, a long-read sequencing alternative, would probably offer better accuracy but would reduce the cost effectiveness, mainly because of the instrument cost. Secondly as the kit we used here was not meant to deliver diagnosis kit, we faced kit maintenance issues. By September 2023, the chemistry kits version 12 as well as the flow cells version 9 will no longer be distributed. Those reagents are replaced by chemistry version 14 and flow cells version 10. This switch involves the modification of the wet lab and the dry lab process. As the presented workflow was accredited following ISO15189 rules by the COFRAC, a demonstration of non-regression will be required to use those new reagents in clinical routine diagnosis. This step is time and money consuming and was not consider for cost evaluation. According to Oxford Nanopore Technologies, the new clinical division is now offering kits with a longer lifetime to answer this issue.

5. Conclusion

In conclusion, our study represents a pivotal step toward integrating long-read sequencing into clinical diagnosis for constitutional human diseases. The demonstrated benefits of this approach in identifying pathogenic variants and determining variant phasing without parental sequencing present a compelling case for its widespread adoption in precision medicine. The successful implementation of long-read sequencing for FMF diagnosis not only enhances patient care in this disorder but also holds promise for broader applications in the diagnostic assessment of genetic disorders in the future.

Ethics approval and consent to participate

The study was performed in accordance with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all patients regarding de-identified clinical and personal patient data collection, analysis, and publication.

Consent for publication

Not applicable.

Availability of data and materials

Raw genetics data are available upon request to the corresponding author. All data produced in the present work are contained in the manuscript.

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Authors' information (optional)

All the authors are employed by Eurofins Biomnis, a private medical biology laboratory.

CRediT authorship contribution statement

X. Vanhoye: Writing – original draft, Investigation. **P. Mouty:** Formal analysis. **S. Mouty:** Methodology. **N. Bargues:** Software. **N. Couprie:** Investigation. **E. Fayolle:** Methodology. **V. Géromel:** Investigation. **M. Taoudi:** Formal analysis. **L. Raymond:** Writing – review & editing, Supervision. **J.-F. Taly:** Writing – review & editing, Software.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Xavier Vanhoye reports a relationship with Eurofins Biomnis that includes: employment. Pascal Mouty reports a relationship with Eurofins Biomnis that includes: employment. Sandrine Mouty reports a relationship with Eurofins Biomnis that includes: employment. Nicolas Bargues reports a relationship with Eurofins Biomnis that includes: employment. Nicole Couprie reports a relationship with Eurofins Biomnis that includes: employment. Evelyne Fayolle reports a relationship with Eurofins Biomnis that includes: employment. Vanna Geromel reports a relationship with Eurofins Biomnis that includes: employment. Mohamed Taoudi reports a relationship with Eurofins Biomnis that includes: employment. Laure Raymond reports a relationship with Eurofins Biomnis that includes: employment. Jean-Francois Taly reports a relationship with Eurofins Biomnis that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2024.e00423.

References

[1] Y. Wang, Y. Zhao, A. Bollas, Y. Wang, K.F. Au, Nanopore sequencing technology, bioinformatics and applications, Nat. Biotechnol. 39 (2021) 1348–1365.
[2] L.L. Zhang, C. Zhang, J.P. Peng, Application of nanopore sequencing technology in the clinical diagnosis of infectious diseases, Biomed Environ Sci BES 35 (2022) 381–392.
[3] A. Tufan, H.J. Lachmann, Familial Mediterranean fever, from pathogenesis to treatment: a contemporary review, Turk. J. Med. Sci. 50 (2020) 1591–1610.
[4] J. Schmidt, S. Berghaus, F. Blessing, H. Herbeck, J. Blessing, P. Schierack, et al., Genotyping of familial Mediterranean fever gene (MEFV)-Single nucleotide polymorphism-Comparison of Nanopore with conventional Sanger sequencing, PLoS One 17 (2022) e0265622.
[5] M.G. Booty, J.J. Chae, S.L. Masters, E.F. Remmers, B. Barham, J.M. Lee, et al., Familial Mediterranean fever with a single MEFV mutation: where is the second hit? Arthritis Rheum. 60 (2009) 1851–1861.
[6] M. Shohat, Familial Mediterranean Fever, University of Washington, Seattle, 2016.
[7] T. Ishige, S. Itoga, K. Kawasaki, E. Utsuno, M. Beppu, S. Sawai, et al., Evaluation of analytical factors associated with targeted MEFV gene sequencing using long-range PCR/massively parallel sequencing of whole blood DNA for molecular diagnosis of Familial Mediterranean fever, Clin. Chim. Acta 495 (2019) 562–569.
[8] Li H. Lh3/seqtk, 2022.
[9] W. De Coster, S. D'Hert, D.T. Schultz, M. Cruts, C. Van Broeckhoven, NanoPack: visualizing and processing long-read sequencing data, Bioinformatics 34 (2018) 2666–2669.
[10] H. Li, New strategies to improve minimap2 alignment accuracy, Bioinformatics 37 (2021) 4572–4574.

[11] Z. Zheng, S. Li, J. Su, A.W.-S. Leung, T.-W. Lam, R. Luo, Symphonizing pileup and full-alignment for deep learning-based long-read variant calling, Nat Comput Sci. 2 (2022) 797–803.
[12] P. Cingolani, A. Platts, L.L. Wang, M. Coon, T. Nguyen, L. Wang, et al., A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3, Fly 6 (2012) 80–92.
[13] P. Cingolani, V.M. Patel, M. Coon, T. Nguyen, S.J. Land, D.M. Ruden, et al., Using Drosophila melanogaster as a model for genotoxic chemical mutational studies with a new program, SnpSift, Front. Genet. 3 (2012) 35.
[14] M.J. Landrum, J.M. Lee, M. Benson, G.R. Brown, C. Chao, S. Chitipiralla, et al., ClinVar: improving access to variant interpretations and supporting evidence, Nucleic Acids Res. 46 (2018) D1062–D1067.
[15] K.J. Karczewski, L.C. Francioli, G. Tiao, B.B. Cummings, J. Alföldi, Q. Wang, et al., The mutational constraint spectrum quantified from variation in 141,456 humans, Nature 581 (2020) 434–443.
[16] Infevers: an online database for autoinflammatory mutations. https://infevers.umai-montpellier.fr/. (Accessed 28 December 2022).
[17] R. Leman, B. Parfait, D. Vidaud, E. Girodon, L. Pacot, G. Le Gac, et al., SPiP: splicing Prediction Pipeline, a machine learning tool for massive detection of exonic and intronic variant effects on mRNA splicing, Hum. Mutat. 43 (2022) 2308–2323.
[18] J. Ryan, S. Masters, M. Booty, N. Habal, J. Alexander, B. Barham, et al., Clinical features and functional significance of the P369S/R408Q variant in pyrin, the familial Mediterranean fever protein, Ann. Rheum. Dis. 69 (2010) 1383–1388.
[19] S. Miyatake, E. Koshimizu, A. Fujita, H. Doi, M. Okubo, T. Wada, et al., Rapid and comprehensive diagnostic method for repeat expansion diseases using nanopore sequencing, NPJ Genomic Med 7 (2022) 62.
[20] C.M. Watson, P. Dean, N. Camm, J. Bates, I.M. Carr, C.A. Gardiner, et al., Long-read nanopore sequencing resolves a TMEM231 gene conversion event causing Meckel–Gruber syndrome, Hum. Mutat. 41 (2020) 525–531.
[21] T.L. Mosbruger, A. Dinou, J.L. Duke, D. Ferriola, H. Mehler, I. Pagkrati, et al., Utilizing nanopore sequencing technology for the rapid and comprehensive characterization of eleven HLA loci; addressing the need for deceased donor expedited HLA typing, Hum. Immunol. 81 (2020) 413–422.
[22] S. Grandemange, I. Aksentijevich, I. Jeru, A. Gul, I. Touitou, The regulation of MEFV expression and its role in health and familial Mediterranean fever, Gene Immun. 12 (2011) 497–503.
[23] MEFV - an overview | ScienceDirect Topics. https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/mefv. Accessed 27 December 2023.
[24] S.D. Goenka, J.E. Gorzynski, K. Shafin, D.G. Fisk, T. Pesout, T.D. Jensen, et al., Accelerated identification of disease-causing variants with ultra-rapid nanopore genome sequencing, Nat. Biotechnol. 40 (2022) 1035–1041.