High resolution HLA genotyping with third generation sequencing technology-A multicentre study

Buhler, Stéphane; Nørgaard, Maja; Steffensen, Rudi; Kløve-Mogensen, Kirstine; Møller, Bjarne Kuno; Grossmann, Rebecca; Ferrari-Lacraz, Sylvie; Lehmann, Claudia

Published in:
HLA

DOI (link to publication from Publisher):
10.1111/tan.15632

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Received: 29 January 2024 | Revised: 4 July 2024 | Accepted: 19 July 2024

DOI: 10.1111/tan.15632

ORIGINAL ARTICLE

HLA Immune Response Genetics WILEY

High resolution HLA genotyping with third generation sequencing technology—A multicentre study

Stéphane Buhler[1] | **Maja Nørgaard**[2] | **Rudi Steffensen**[3] | **Kirstine Kløve-Mogensen**[3,4] | **Bjarne Kuno Møller**[2] | **Rebecca Grossmann**[5] | **Sylvie Ferrari-Lacraz**[1] | **Claudia Lehmann**[5]

[1]Transplantation Immunology Unit and National Reference Laboratory for Histocompatibility, Department of Diagnostic, Geneva University Hospitals, Geneva, Switzerland

[2]Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Denmark

[3]Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark

[4]Department of Clinical Medicine, Aalborg University, Aalborg, Denmark

[5]Laboratory for Transplantation Immunology, University Hospital Leipzig, Leipzig, Germany

Correspondence
Stéphane Buhler, Transplantation Immunology Unit and National Reference Laboratory for Histocompatibility, Department of Diagnostic, Geneva University Hospitals, Geneva, Switzerland.
Email: stephane.buhler@hcuge.ch

Claudia Lehmann, Laboratory for Transplantation Immunology, University Hospital Leipzig, Leipzig, Germany.
Email: c.lehmann@medizin.uni-leipzig.de

Molecular HLA typing techniques are currently undergoing a rapid evolution. While real-time PCR is established as the standard method in tissue typing laboratories regarding allocation of solid organs, next generation sequencing (NGS) for high-resolution HLA typing is becoming indispensable but is not yet suitable for deceased donors. By contrast, high-resolution typing is essential for stem cell transplantation and is increasingly required for questions relating to various disease associations. In this multicentre clinical study, the TGS technique using nanopore sequencing is investigated applying NanoTYPE™ kit and NanoTYPER™ software (Omixon Biocomputing Ltd., Budapest, Hungary) regarding the concordance of the results with NGS and its practicability in diagnostic laboratories. The results of 381 samples show a concordance of 99.58% for 11 HLA loci, *HLA-A*, *-B*, *-C*, *-DRB1*, *-DRB3*, *-DRB4*, *-DRB5*, *-DQA1*, *-DQB1*, *-DPA1* and *-DPB1*. The quality control (QC) data shows a very high quality of the sequencing performed in each laboratory, 34,926 (97.15%) QC values were returned as 'passed', 862 (2.4%) as 'inspect' and 162 (0.45%) as 'failed'. We show that an 'inspect' or 'failed' QC warning does not automatically lead to incorrect HLA typing. The advantages of nanopore sequencing are speed, flexibility, reusability of the flow cells and easy implementation in the

Abbreviations: CAR-T, chimeric antigen receptor; DAkkS, German accreditation body; DANAK, Danish accreditation fund; DZA, Deutscher Zellaustausch; EFI, European Federation for Immunogenetics; EPT, external proficiency testing; EQA, external quality assessment; HSCT, haematopoietic stem cell transplantation; NGS, next generation sequencing; ONT, Oxford Nanopore Technologies; QC, quality control; qPCR, quantitative real-time polymerase chain reaction; SAS, Swiss Accreditation Service; SOT, solid organ transplantation; TGS, third generation sequencing; UCLA, University of California, Los Angeles.

laboratory. There are challenges, such as exon coverage and the handling of large amounts of data. Finally, nanopore sequencing presents potential for applications in basic research within the field of epigenetics and genomics and holds significance for clinical concerns.

KEYWORDS

deceased donor typing, donor specific antibody determination, high-resolution typing, HLA-typing, nanopore sequencing, NanoTYPE, single sample HLA-typing, third generation sequencing

1 | INTRODUCTION

The HLA system located on the short arm of chromosome 6 contains the most polymorphic genes in humans. They are vital in ensuring successful transplantation outcomes by matching donors and recipients.[1] Accurate HLA typing, with high-resolution results, is crucial for allogeneic haematopoietic stem cell transplantation (HSCT), disease associations, pharmacogenetics and solid organ transplantation (SOT).[2–6] HLA matching improves graft outcomes, reduces rejection risks and prevents sensitization for future transplants.[7] High-resolution HLA typing enables epitope-level matching, enhances virtual cross-matching accuracy and identifies HLA alleles suitable for broadly sensitised recipients.[8–10] It may also reduce the reliance on heavy immunosuppression, leading to improved recipient quality of life.

Molecular HLA typing methods have now replaced serological typing techniques. Molecular technologies are currently undergoing a rapid technical evolution.[11–13] While high resolution HLA typing is required for HSCT, it is sufficient to perform a low-resolution HLA type on 11 loci (e.g., by quantitative real-time polymerase chain reaction [qPCR-SSP]) for kidney transplants. If allele specific donor antibodies are suspected post-transplant, some laboratories carry out high-resolution HLA typing for a more accurate diagnostic.

Next generation sequencing (NGS) has become the gold standard for routine high-resolution HLA typing although NGS has its limitations. The process is time-consuming, requires expensive equipment and can be challenging in phasing long distance polymorphic positions, causing ambiguities. Furthermore, NGS is not suitable for high-resolution HLA typing of deceased donors due to time constraints.

Oxford Nanopore Technologies (ONT) has introduced a new solution, nanopore sequencing, which can sequence long reads in real-time.[14] A flow cell contains 512 channels, with four nanopores per channel. Only one nanopore in each channel is measured at a time, allowing concurrent sequencing of up to 512 molecules.[15] When a DNA strand passes through a nanopore in the flow cell, each DNA base will change the electric field across the pore. Every DNA base has a unique obstruction of the current, and a basecaller converts the electrical signals into DNA sequences. The MinION platform is portable, cost-effective and offers uncomplicated flexible sample preparation and sequencing for patients and donors. This might prove beneficial for HLA typing of deceased organ donors.[16,17] Omixon has developed a commercial HLA typing kit for nanopore sequencing called NanoTYPE™, accompanied by the NanoTYPER™ software for data analysis and HLA genotype assignation.

Sequencing with nanopores for clinical HLA typing offers several advantages: (1) the portability of the MinION platform allows for its use in a variety of clinical settings, even smaller laboratories. (2) The protocol is clear and easy, facilitating its implementation in a laboratory. (3) Real-time data analysis has been shown to provide fast results, facilitating the clinical applications.[18,19] In addition, the sequencing generates long reads, enabling the accurate mapping of the highly polymorphic HLA loci.

This study evaluates the performance of nanopore sequencing applying the NanoTYPE™ kit and NanoTYPER™ software in a multicentre setting and discusses the advantages and disadvantages regarding implementation in routine diagnostics.

2 | METHODS

2.1 | Clinical study

The clinical study was initiated by the company Omixon to test the suitability of the NanoTYPE™ kit and NanoTYPER™ software (Omixon Biocomputing Ltd., Budapest, Hungary) in routine diagnostics in several HLA laboratories. The company's aim was to submit the data to obtain CE (*Conformité Européenne*) certification. All participating laboratories worked according to predefined study protocols (Figure 1A). In this study, HLA

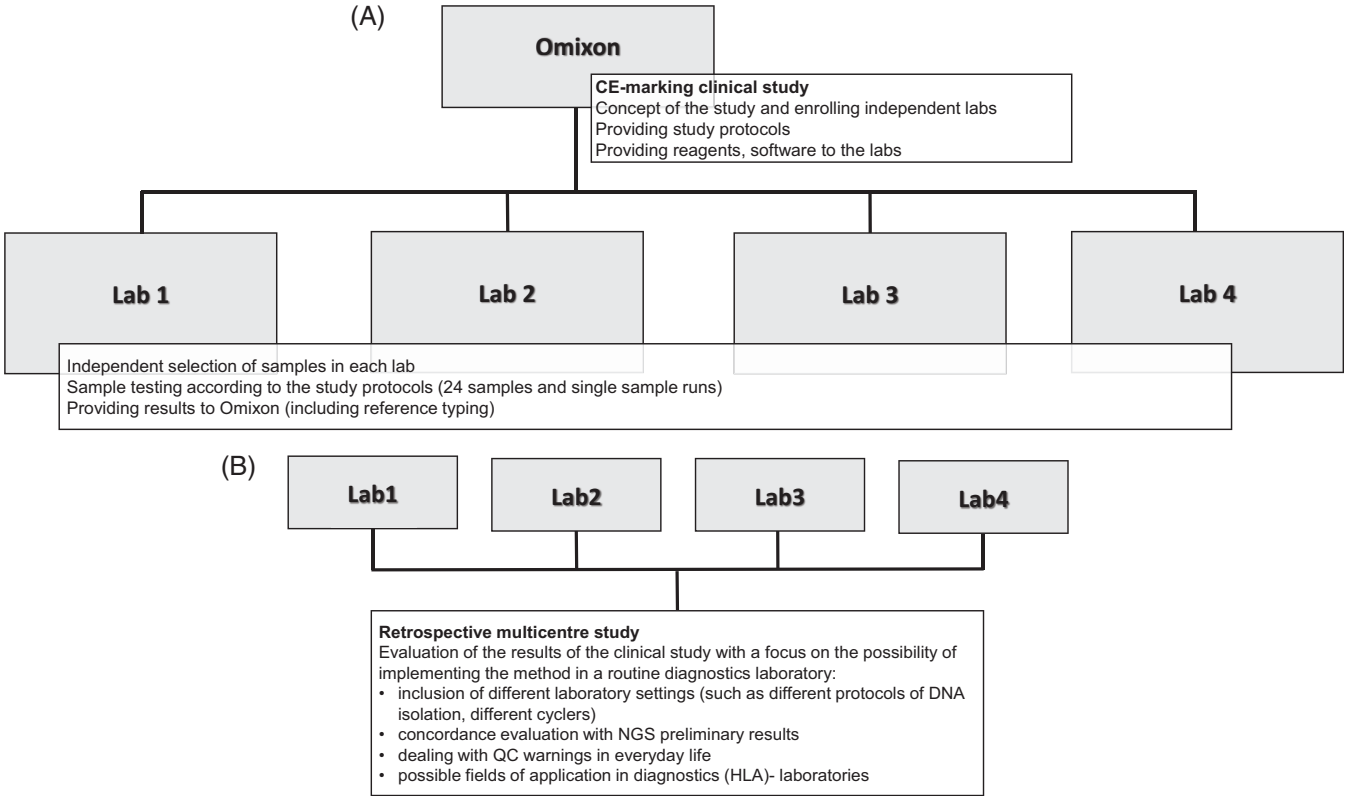

FIGURE 1 Study design (A) concept of the *Conformité Européenne* (CE)-marking clinical study and (B) independent laboratory study objectives of four participating laboratories. Primary and secondary objectives are indicated in the white boxes. NGS, next generation sequencing; QC, quality control.

genotyping of 11 HLA loci, *HLA-A*, *-B*, *-C*, *-DRB1*, *-DRB3*, *-DRB4*, *-DRB5*, *-DQA1*, *-DQB1*, *-DPA1* and *-DPB1*, was conducted on a total of 381 samples (including a few replicates) using third generation sequencing technology with the NanoTYPE™ kit (Omixon Biocomputing Ltd., Budapest, Hungary). This kit covers the entire gene for HLA class I loci *HLA-A*, *-B*, *-C* and for HLA class II loci *HLA-DQA1*, *-DQB1*, and *-DPA1*. Introns are not currently included in the bioinformatics analysis due to limitations in sequencing homopolymers and other repeated sequences, thus generating third field high resolution results. For some HLA class II loci only a part of the exons are covered (i.e., exons 2 to 4 for *HLA-DRB1*, *-DRB3* and *-DRB4*; exons 2 to 5 for *-DRB5* and *-DPB1*), so that a second to third field or G-group resolution can be achieved.[18,19] The best available resolution was always used in this study for the comparison of the results. This study was carried out across four different European laboratories (see Table 1). Three of the laboratories hold an EFI accreditation. One laboratory has additional accreditation from the Danish Accreditation Fund (DANAK), another is accredited according to German Accreditation Body (DAkkS), and one laboratory is accredited according to Swiss Accreditation Service (SAS).

2.2 | Sample preparation

The DNA samples selected by the individual laboratories were previously tested with high-resolution genotyping for 11 HLA loci using NGS, following the manufacturer's protocols. Homozygous or heterozygous samples or samples with homozygosity at individual loci could be selected. The genomic DNA extraction utilised different sample materials and different techniques as shown in Table 1. The samples were sourced from various origins, including patients and donors in the context of HSCT, SOT, diagnosis of disease (i.e., HLA associations) and registration of cord blood units. A total of 22 samples were sequenced in single runs and 359 samples underwent multiplexed sequencing with up to 24 samples per run.

2.3 | Sequencing applying NanoTYPE™

The Nanopore sequencing was carried out in accordance with specific performance evaluation protocols—namely Nanotype™ 24/11 and Nanotype™ 24/11 Single (Omixon Biocomputing Ltd., Budapest, Hungary). The sequencing was performed on the MinION device with R9.4 flow cells

TABLE 1 Overview of the materials and DNA isolation techniques of the individual laboratories.

Laboratory	Samples (*n*)	Sample material	DNA isolation technique	DNA isolation testkit	PCR amplification cycler	Mean starting DNA concentration (ng/µL/instrument)	Reference high resolution typing
Lab1	96 unique (102 total)	EDTA	Maxwell RSC instrument	Maxwell 16 Blood DNA Kit (Promega, USA)	Proflex	170/Qubit	HoloType HLA (Omixon Biocomputing Ltd., Budapest, Hungary) on MiSeq (Illumina, San Diego, CA, USA)
Lab2	102 unique	EDTA	Qiagen EZ1 Advanced XL	EZ1&2 DNA Blood 350 µL Kit (Qiagen, Hilden, Germany)	Veriti Thermal Cycler	48/Qubit	HoloType HLA (Omixon Biocomputing Ltd., Budapest, Hungary) on MiSeq (Illumina, San Diego, CA, USA)
Lab3	74 unique (76 total)	ACD, buccal swabs	Qiagen EZ1 Advanced XL	EZ1&2 DNA Blood 350 µL and DNA Tissue Kits (Qiagen, Hilden, Germany)	Veriti Thermal Cycler	86,7/NanoDrop	AllType™ NGS 11 Loci kit (One Lambda, West Hill, CA, USA) on an Ion S5 (Thermofisher Scientific) or on MiSeq (Illumina, San Diego, CA, USA)
Lab4	100 unique (101 total)	EDTA, buccal swabs	Manual protocol	QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany)	Applied Biosystems AB GeneAmp 9700	51/Qubit	AllType™ NGS 11 Loci (One Lambda, West Hills, CA, USA) or AlloSeq® Tx 17 (CareDx, San Francisco, CA, USA) on MiSeq (Illumina, San Diego, CA, USA)

(FLO-MIN106D), according to the specific performance evaluation protocols. At the time of study design, the R10 versions of ONT flow cells were not yet available. In addition, R10 versions and chemistry 14 are not yet in the Q-LINE products of ONT (i.e., providing locked-down technology, ISO 9001 certification, software and product support).[20] The manufacturer just announced the validation of these flow cells and chemistry for RUO but not yet for the upcoming CE-marked IVDR.

The workflow for HLA typing using the NanoType™ reagents involves genomic DNA and Master Mix preparation, HLA amplification, amplicon quantification and normalisation, library preparation, sequencing and ultimately the analysis of HLA sequencing data.[21,22] It is worth noting that all four participating laboratories strictly adhered to the specific performance evaluation protocol. The DNA samples (20 ng) went through a PCR for amplification for 2 h 35 min using an 11 loci multiprimer mix, following the amplification programme: 1 min at 94°C, 30 cycles of 98°C for 10 s, 68°C for 4 min 30 s. We used 200 ng of the amplicons for barcoding using the rapid library preparation kit (SQK-RBK110.96, Oxford Nanopore Technologies, Oxford, United Kingdom). The flow cells were washed after each run using a flow cell wash kit (EXP-WSH004, ONT) and were in general reused three times by all laboratories and in one laboratory up to seven times.

2.4 | Data analysis

The data were generated using the MinKNOW® (version 22.05.5, ONT) and the subsequent generation of FASTQ basecalling files. For the MinKNOW® software, we used the following setup: high accuracy basecalling mode, 5000 reads/gzip per FASTQ. The FASTQ files obtained from the MinKNOW® software were analysed using NanoTYPER™ software version 1.1.0 (Omixon Biocomputing Ltd., Budapest, Hungary), using the IPD-IMGT/HLA version 3.48.0_9 for HLA-alleles assignment.

Allele calls at a given locus for every sample are documented in NanoTYPER™ along with a set of quality control (QC) metrics. These metrics include overall summary, coverage, key exon imbalance, key exon minimum depth, key exon spot noise ratio, N50, other exon imbalance, other exon minimum depth, other exon spot noise ratio and PCR crossover ratio. For further details on the QC metrics readers are referred to the manufacturer's instructions and documentation. Each metric can have one of the following status labels: 'passed' (i.e., the value is within the acceptable threshold predefined for the metric), 'inspect' (i.e., the value is lower or higher than the threshold requiring verification) or 'failed' (i.e., the value is much lower or higher than the

threshold indicating a possible need for reanalysis or resequencing). The overall summary metric reflects the statuses of the other QC metrics, meaning that if one of them is tagged as 'inspect' or 'failed', the overall QC will also be tagged with the same status. In case, both 'inspect' and 'failed' statuses co-exist in two or more QC metrics for a given locus, the overall QC will be marked as 'failed'. As part of this study, we reviewed all QC metrics warnings that led to a non-concordant result with the reference typing. According to the manufacturer's recommendation, these warnings must be assessed in diagnostic routine. Authorised technical staff in the laboratory must then decide how to proceed and whether retesting is required.

The concordance of results with reference typing were compared based on allele calls, that is the number of correctly assigned allele(s) at each locus with possible scores of 0, 1 or 2 (i.e., two reflecting that both alleles assigned with NanoTYPER™ are concordant with the alleles expected from the reference typing). Alleles detected at the homozygous state were counted twice. For *HLA-DRB3*, *-DRB4 and -DRB5*, the expected presence or absence of allele(s) was also considered in the score.

3 | RESULTS

3.1 | QC metrics

In this study, a total of 381 samples including 372 unique samples and nine replicates underwent analysis by four participating laboratories, resulting in a total of 8382 allele calls (Table 2).

The QC metrics for the 8382 allele calls are presented for each laboratory in Figure S1 and per laboratory according to each locus in Figure 2. A total of 34,926 QC values were marked as 'passed' (97.15%), 862 as 'inspect' (2.4%) and 162 as 'failed' (0.45%), reflecting the very high quality of the sequencing conducted in each laboratory. If only the overall metric is considered as a global overview of the sequencing quality, 3152 were marked as 'passed' (87.68%), 369 as 'inspect' (10.26%) and 74 as 'failed' (2.06%). The most common deviations for the QC metrics were the coverage, key exon imbalance, other exon imbalance and PCR crossover ratio. HLA class II genes (especially *HLA-DRB1*, *-DQA1* and *-DQB1*) were exhibiting more deviations for several QC metrics than HLA class I genes and with very similar profiles observed across the four laboratories. Interestingly, laboratories 1 and 2 experienced more failures for coverage at *HLA-A*, while laboratory 3 exhibited more PCR crossover ratio deviations at the *HLA-C*, *-DQA1*, *DQB1* and *-DRB5* loci compared to the other laboratories. More warnings were also observed in laboratory 3 for *HLA-DRB3*, *-DRB4* and *-DRB5*.

Regarding the distribution of sequenced reads, the metric N50, which represents the median length of a set of sequences, had a mean value of 3131 (minimum of 1970 and maximum of 4800) across the four laboratories and some reads were up to 10 kb or more.

3.2 | Allele call comparisons, concordance and discrepancies

As evidenced by the high QC metrics reported above, 99.61% of the allele calls (8349 of 8382) were concordant with the reference typing previously conducted by each laboratory (Table 2). When considering the overall QC metric, it means that among the 12.32% of cases where a warning was issued (i.e., 74 tagged as 'failed' and 369 as 'inspect', see above), only a very tiny portion (i.e., two tagged as 'failed' and seven as 'inspect') led to a typing discrepancy (Table S1). In this study we identified 24 cases with one or two allele discrepancies reported at a specific locus, with various types of errors, which are outlined in detail in Table 3. It is worth noting that only one case was associated with an HLA class I locus (i.e., *HLA-B*), while all other discrepancies pertained to HLA class II loci. The most common reason for discordance with the reference typing was the identification of a potential novel allele according to NanoTYPER™, with two confirmed cases and five cases unconfirmed (Table 3). Additionally, one laboratory encountered five dropouts at the *HLA-DQB1* locus with no allele calls reported by NanoTYPER™. In four instances, NanoTYPER™ successfully identified an allele that was missing in the reference typing. Once it reported an unexpected second allele in homozygous samples. Five times, it failed to detect the second allele in expected heterozygous samples. On one occasion, it provided a typing result despite the absence of *HLA-DRB4* (low coverage probably due to a technical error or contamination), and once it led to an incorrect allele assignment at the third field level of resolution (i.e., changes involving a synonymous substitution). Looking back at the overall QC metric for these 24 discrepancies, two were tagged as 'failed', seven as 'inspect' (as already stated above), 10 as 'passed' and 5 had no metric associated due to the absence of sequencing reads (i.e., locus dropout). Regarding the 10 cases that did not lead to a warning, it concerned four of the five failures to detect the second allele in an expected heterozygote, the four cases where NanoTYPER™ correctly assigned a missing allele in the reference, one confirmed case of a novel allele and the wrong allele assignment at the third field level.

TABLE 2 Number of concordant allele calls and concordance of HLA typing between NanoTYPER™ and reference typing.

	Lab 1		Lab 2		Lab 3		Lab 4		Total	
Locus	**Allele calls (*n*)**	**Concordance (%)**	**Allele calls (*n*)**	**Concordance (%)**	**Allele calls (*n*)**	**Concordance (%)**	**Allele calls (*n*)**	**Concordance (%)**	**Allele calls (*n*)**	**Concordance (%)**
HLA-A	204/204	100.00	204/204	100.00	152/152	100.00	202/202	100.00	762/762	100.00
HLA-B	204/204	100.00	204/204	100.00	152/152	100.00	201/202	99.50	761/762	99.87
HLA-C	204/204	100.00	204/204	100.00	152/152	100.00	202/202	100.00	762/762	100.00
HLA-DPA1	204/204	100.00	204/204	100.00	152/152	100.00	202/202	100.00	762/762	100.00
HLA-DPB1	204/204	100.00	204/204	100.00	152/152	100.00	202/202	100.00	762/762	100.00
HLA-DQA1	204/204	100.00	204/204	100.00	151/152	99.34	201/202	99.50	760/762	99.74
HLA-DQB1	203/204	99.51	204/204	100.00	152/152	100.00	188/202	93.07	747/762	98.03
HLA-DRB1	204/204	100.00	204/204	100.00	152/152	100.00	202/202	100.00	762/762	100.00
HLA-DRB3	204/204	100.00	204/204	100.00	148/152	97.37	202/202	100.00	758/762	99.48
HLA-DRB4	200/204	98.04	197/204	96.57	152/152	100.00	202/202	100.00	751/762	98.56
HLA-DRB5	204/204	100.00	204/204	100.00	152/152	100.00	202/202	100.00	762/762	100.00
Total	2239/2244	99.78	2237/2244	99.69	1667/1672	99.70	2206/2222	99.28	8349/8382	99.61

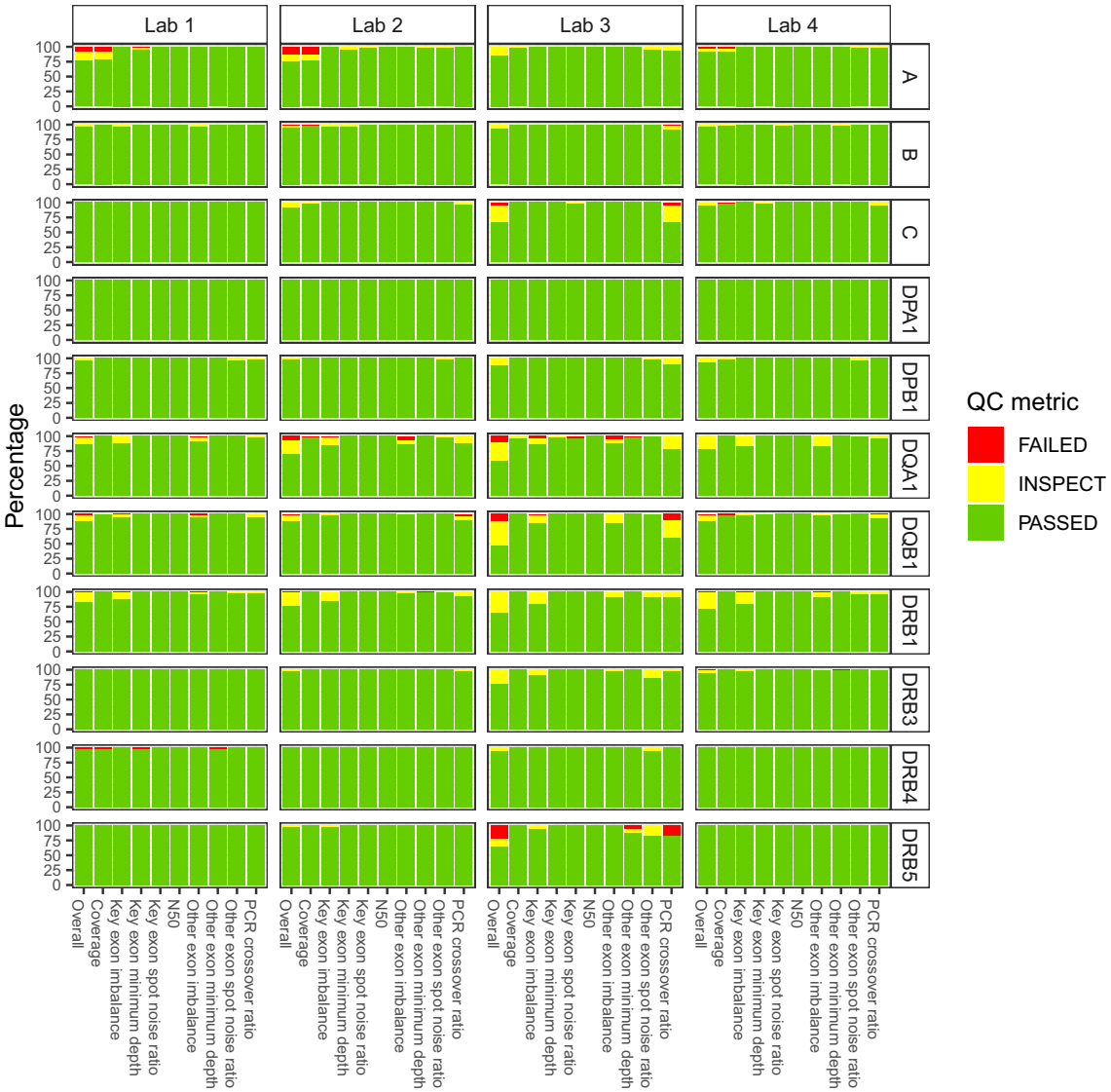

FIGURE 2 Quality control (QC) metrics of the nanopore sequencing in the four participating laboratories. The status for the 10 metrics (also see main text) is shown for each locus with a colour code: red: 'failed', yellow: 'inspect' and green: 'passed' and their percentage is reported on the *y*-axis.

3.3 | Re-using the flow cells

The flow cell for MinION is re-usable, which makes the method very effective in terms of costs. This study was not primarily designed to assess how often a flow cell can be re-used for HLA typing. However, all laboratories re-used flow cells in at least two consecutive sequencing runs. When re-using the flow cell, there might be some leftover DNA from the previous run. This is the 'carry-over' effect, meaning reads have been detected, that were not assigned to the barcodes used for that specific run. Even when using the flow cell for the first time, there is a 'carry-over' effect with 0.01%–0.05% of reads that are detected as belonging to barcodes that were not used. In three laboratories, the carry-over effect after first use ranged between 0.03% and 1%. In one laboratory, the carry over effect was up to 4%, which might be due to a lower efficiency of washing, although this remains marginal. Furthermore, one laboratory re-used a flow cell up to seven times with a 'carry-over' effect less than 0.19%. With each new run, new (unused) barcodes must be used, and should therefore not affect the reliability of the HLA typing, as only the reads tagged with the barcodes of interest (used barcodes) are considered for analysis. However, efficient wash is essential to prevent carry-over.

4 | DISCUSSION

4.1 | Concordance and discrepancies

This study involved the participation of four European laboratories, collectively examining 381 samples with the

TABLE 3 Results with discrepancies.

Discrepancies between NanoTYPER™ (TGS) and reference typing by NGS	*N* (cases)	Loci impacted	Further investigations and comments
Additional allele in TGS (expected homozygote in NGS)	1	HLA-DQA1 ($n = 1$)	Solved and corrected in upcoming release of NanoTYPER™
Correctly found an allele missing in NGS	4	HLA-DRB4 ($n = 4$)	
Locus dropout in TGS	5	HLA-DQB1 ($n = 5$)	Solved and corrected by re-testing with the NanoTYPE MONO™
Missed allele in TGS (expected heterozygote in NGS)	5	HLA-B ($n = 1$), HLA-DQB1 ($n = 4$)	Solved and corrected by re-testing with the NanoTYPE MONO™
Potential novel allele in TGS	7	HLA-DQA1 ($n = 1$), HLA-DQB1 ($n = 1$), HLA-DRB3 ($n = 4$) and HLA-DRB4 ($n = 1$)	Solved by re-analysis with upcoming release of NanoTYPER™. False potential novel alleles not confirmed due to artefacts and non-optimised read assignment using the algorithm in software version 1.1.0
Confirmed	2		
Not confirmed	5		
Unexpected locus coverage in TGS	1	HLA-DRB4 (n = 1)	Caused by very low coverage which was red flagged in NanoTYPER™. Probable contamination or technical error as repetition on the same PCR product didn't yield any DRB4 sequencing reads
Wrong allele assignment (third field) in TGS	1	HLA-DRB4 (n = 1)	
Total	24		

Abbreviation: NGS, next generation sequencing.

aim of validating a third-generation sequencing technology for long-range high resolution HLA typing. The results demonstrated that utilising the nanopore sequencing and the NanoTYPE™ kit achieved a concordance of 99.3%–99.8% with reference NGS typing across the four laboratories (see Table 2). Matern et al. showed a concordance of 98.5% for HLA class I in 2020 and a recent study by Devriese et al. reported a concordance of 99%.[12,18] Our study substantiates the robustness of the technology, as demonstrated through the utilisation of different sample materials and DNA isolation techniques (see Table 1). Moreover, the laboratories employed varying reference high-resolution typing methods to compare the nanopore sequencing results.

A total of 24 discrepancies between NanoTYPER™ and reference typing were identified (see Table 3). Most of these discrepancies are explicable. For instance, seven potential new alleles were detected, a common feature of HLA genes and the continuously growing IPD-IMGT/HLA database.[23] Of the seven potential alleles, two were confirmed and five were not. These potential novelties were artefacts due to the algorithm not performing well at assigning reads to the different *HLA-DRB* genes, creating unexpected low coverage or 'novelties' at specific nucleotide positions, an issue that is resolved in an upcoming version of NanoTYPER™.

Additionally, one laboratory experienced total dropouts at the *HLA-DQB1* locus in five samples, in particular *HLA-DQB1*03:01:01 + HLA-DQB1*03:02:01*. Although technical handling errors cannot be ruled out, these are test kit limitations known from the manufacturer: 'very rarely, allele dropouts may occur due to low amplification in some HLA class II alleles like *HLA-DQB1*03:01*, *HLA-DQB1*03:03*, *HLA-DQB1*04:02*, *HLA-DRB1*04* and *HLA-DRB1*07:01*'.[21] Dropouts at any locus can occur at any time and with any technology, whereby in routine such failures can be compensated and solved by repeating the analysis. Here we repeated the HLA typing of samples with dropouts using NanoTYPE MONO™ (Omixon Biocomputing Ltd., Budapest, Hungary) and found that they are in concordance with the expected results.

Discrepancies of more significance include missed alleles and additional alleles. In such cases, the analysis software presented warnings based on the expected linkage disequilibrium, along with recommendations to validate the homozygous results using an alternative HLA typing technology. Here we observed in one sample expected to be homozygous for *HLA-DQA1*05:05:01* an additional *HLA-DQA1*05:24* allele. We reanalysed the sequence data with a yet unreleased upgraded version of

the software, which provided results in concordance with the reference typing.

A more serious issue is the missing alleles, such as found at the *HLA-DQB1* locus. As already mentioned above, this failure could be caused by failed QC metrics or low amplification in specific alleles. As these discrepancies only occurred in laboratory 4, it cannot be ruled out that the PCR cycler used had an influence on the insufficient amplification. All samples with missed or additional alleles were re-tested with NanoTYPE MONO™ (Omixon Biocomputing Ltd., Budapest, Hungary) or re-analysed with the upcoming improved software version (2.1.0 beta) and are in concordance with the expected results. An optimization of the NanoTYPE™ kits, especially for HLA class II loci, is essential for clear results.

The conclusion regarding the 24 discrepancies observed is that these are mainly due to the known limitations of the NanoTYPE™ test or to the (as yet) non-optimised NanoTYPER™ software algorithm. Further investigations, for example, with NanoTYPE MONO™ or the upcoming improved NanoTYPE™ test and software version show that these errors are not expected to occur in future use of these products. A new beta version of the NanoTYPE™ kits was recently sent to the laboratories and is currently being tested.

As a simulation of interlaboratory exchange, we used two control samples provided by Omixon and the results were concordant between the four laboratories. Of note, a dropout at the *HLA-A* locus was detected in one laboratory (in concordance after re-testing with NanoTYPE MONO™) and two other laboratories experienced QC warnings (i.e., related to coverage failure) but could still assign the expected alleles. Thus, it is essential to note that these QC deviations do not necessarily lead to an incorrect HLA result or a missing allele assignment. This remains the task of each laboratory to assess and document in its procedures and quality assurance processes, which are the measures to undertake in case of warnings in the QC metrics of a specific routine analysis. For instance, a locus dropout would automatically result in re-testing in a diagnostic sample. Additionally, the laboratories have successfully participated in regular external proficiency testing (EPT). Notably, one laboratory successfully utilised nanopore based HLA typing in an INSTAND EQA (External Quality Assessment) (Program 444 for high-resolution HLA class I and II typing) for all five samples both for 2023 and 2024. Another laboratory passed the EPT program DZA 2023 (Deutscher Zellaustausch) for 10 samples. A third laboratory has successfully participated in the UCLA (University of California, Los Angeles) International cell exchange as EPT for HLA high-resolution typing by NGS with 12 passed samples for 2023.

4.2 | Implementation in a diagnostic laboratory

The nanopore technology is already known for its ease of integration into laboratory workflows and is distinguished by its robustness and reproducibility.[12] Nonetheless, technical staff without prior experience in NGS or TGS may require additional guidance and hands-on practice to confidently utilise nanopore sequencing and data interpretation at a diagnostic level. One of the four laboratories has already implemented the method in routine and two other laboratories are waiting for the authorisation for diagnostic use.

Furthermore, a key advantage for laboratory implementation is the minimal space requirements, low acquisition costs and the reusability of the flow cell. According to a preliminary assessment from this study, re-use of the flow cell up to three times in routine should generally be possible with good quality sequencing results, but depending on the number of samples analysed.[24,25]

4.3 | Possible applications of nanopore sequencing in the HLA laboratory

HLA typing using nanopore sequencing significantly surpasses the speed of traditional methods to achieve high resolution results. NGS typically takes up to three working days, with overnight sequencing and subsequent data analysis. Batch sequencing (e.g., 8, 16, 24 or 48 samples) is common with NGS as individual samples are not sequenced via NGS for economic reasons. Nanopore sequencing offers more flexibility, allowing a single sample to be sequenced and the data analysed within 1 day. The sample preparation and sequencing time is approximately 5 h. Sequencing using nanopores is also advantageous in terms of time for batch sequencing approaches (e.g., 24 samples); sample preparation can be carried out in 1 day, sequencing overnight and the data analysed the following day. The time frame for data analysis is strongly dependent on the available computer power. This increased speed makes long read sequencing feasible for HLA typing compared to more conventional methods.[9,11] Furthermore, owing to the more adaptable handling and re-usability of the flow cells, the protocol is not limited to processing predefined batches of samples in a routine HLA laboratory. It enables the preparation of individual samples, such as urgent samples containing any number between 2 and 24 samples in a multi-batch setup. The only constraint lies in the availability of active pores on the flow cell for the sequencing.

Following SOT, broadly sensitised recipients have developed antibodies against a wide range of HLA alleles.

Regular testing for HLA antibodies is conducted to detect donor-specific antibodies early. In this context, nanopore sequencing could help better determine the risk of allele specific antibodies, particularly when prompt clinical intervention is needed. This method also brings high-resolution HLA typing of deceased donors within reach. However, the current turnaround time of approximately 5 h from DNA to result is still too long for deceased donor HLA typing. A reduction in the processing time holds promise for identifying HLA alleles suitable for broadly sensitised recipients and potentially enhancing the transplantability of these recipients.

Additionally, the use of nanopore sequencing for HLA typing is applicable to all other conceivable requirements. This includes HLA typing for disease associations or in relation to treatments where high-resolution typing is necessary, for example, in cases of narcolepsy, coeliac disease, Abacavir therapy, autoimmune neutropenia or CAR-T cell therapy.[12,18,19,26–29] The verification of discordances or dropouts and single locus typing for disease associations at high-resolution using mono-locus tests, like NanoTYPE MONO™ could offer greater flexibility and time saving in the future.

4.4 | Limitations and challenges

The nanopore sequencing has shown significant improvements since 2015. Previously a high error rate of up to 10% was reported for HLA typing.[30] The total concordance of >99% observed in this study with the NanoTYPE™ kit is competing. Additionally, nanopore sequencing necessitates specialised bioinformatics tools and expertise in data analysis. The long-read sequencing results in larger output data, requiring a new approach for data storage and archiving in the future. However, the use of clouds and electronical data sharing is not yet fully implemented in every laboratory.

There are challenges related to the consumables required for nanopore sequencing. For an accredited, diagnostic laboratory, it is essential to minimise the number of individual reagent shipments to avoid additional effort and costs for shipment control. The short expiry date of the flow cells up to 12 weeks is a limiting factor in this regard.

The quality and quantity of extracted DNA are critical to ensure reliable and reproducible results (e.g., we encountered more difficulties with DNA extracted from buccal swabs, and some loci were more easily affected). As mentioned previously, enhancing the primer design for the *HLA-A* locus might help avoid the coverage QC metric warnings and contribute to improvements in overall data quality and accuracy. Furthermore, some HLA class II loci (*HLA-DRB1*, *-DRB3*, *-DRB4*, *-DRB5* and *-DPB1*) are only sequenced in key exons. Designing primers to cover all exons will significantly diminish the number of ambiguities, leading to clear three-field resolution and avoiding reporting of G-groups.

4.5 | Future perspectives

Despite these challenges, the potential of nanopore sequencing, as exemplified by the NanoTYPE™ kit for routine HLA typing, is highly promising. The release of NanoTYPE™ for diagnostic use is anticipated in the second quarter of 2024.

Currently the MinION and the GridION are available for HLA typing for larger workload and offers more flexible turnaround time for big donor registries or transplantation centres. Another ONT platform, PromethION, is particularly suitable for high-throughput projects and will be of interest for HLA typing in the future.

Results from this clinical study performed in four different European laboratories displayed the effectiveness and accuracy of NanoTYPE™ in a clinical setting, making it a valuable sequencing analysis for routine high-resolution HLA typing, even in laboratories with low throughput capabilities. By aligning HLA alleles at the epitope level for SOT, it will become feasible to enhance the compatibility, thus reducing the risk of rejection. Therefore, integrating NanoTYPE™ into routine matching strategies in laboratories of all sizes can be a proactive step toward advancing transplant procedures. HLA laboratories should prepare for the future by establishing the necessary computing power and identifying new storage capacities to accommodate a wide range of data volumes.

The nanopore technology presents many possibilities. On one hand, it offers potential applications in basic research within the field of epigenetics or genomics while, on the other hand, it holds significance for clinical concerns in cancer research, transplantation and microbiome research.[14] A recent study has highlighted the potential of third generation sequencing for haplotype-specific methylation calls.[31] Such DNA modification plays a critical role in signal transduction and gene expression and they may hold importance in transplantation.[18,19] For the field of transplantation, especially HLA typing, nanopore sequencing represents a profound change for the HLA world. Not only turnaround times will be minimised, but high-resolution HLA typing of deceased donors may also be possible, redefining allocation schemes.

AUTHOR CONTRIBUTIONS

All authors have generated their own data and results from their location for this study. A first version of the

article was produced as follows: introduction Maja Nørgaard, methods and discussion Claudia Lehmann, results Stéphane Buhler. Stéphane Buhler performed the bioinformatic and statistical analysis. Rudi Steffensen, Kirstine Kløve-Mogensen, Bjarne Kuno Møller, Sylvie Ferrari-Lacraz and Rebecca Grossmann were involved in the critical review, discussion, edited the manuscript and approved the final version.

ACKNOWLEDGEMENTS

The authors are thankful to all technicians not named in the authorship of this paper who performed the hands-on work in the laboratory. Open access funding provided by Universite de Geneve.

CONFLICT OF INTEREST STATEMENT

This clinical study was initiated and organised by Omixon Biocomputing Ltd., Budapest, Hungary and the test kits provided without charge to all participating laboratories. Omixon had no influence on the writing of the article, which only reflects the experiences obtained by the authors. The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Stéphane Buhler https://orcid.org/0000-0001-6675-5287
Kirstine Kløve-Mogensen https://orcid.org/0000-0003-4095-8291
Claudia Lehmann https://orcid.org/0000-0002-2791-764X

REFERENCES

1. Klein J, Sato A. The HLA system. First of two parts. *N Engl J Med*. 2000;343:702-709. doi:10.1056/NEJM200009073431006
2. Chung W-H, Hung S-I, Hong H-S, et al. Medical genetics: a marker for Stevens–Johnson syndrome. *Nature*. 2004;428:486. doi:10.1038/428486a
3. Daly AK, Donaldson PT, Bhatnagar P, et al. *HLA-B*5701* genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet*. 2009;41:816-819. doi:10.1038/ng.379
4. Hung S-I, Chung W-H, Liou L-B, et al. *HLA-B*5801* allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc Natl Acad Sci U S A*. 2005;102:4134-4139. doi:10.1073/pnas.0409500102
5. Mosaad YM. Clinical role of human leukocyte antigen in health and disease. *Scand J Immunol*. 2015;82:283-306. doi:10.1111/sji.12329
6. Shear NH, Milpied B, Bruynzeel DP, Phillips EJ. A review of drug patch testing and implications for HIV clinicians. *Aids*. 2008;22:999-1007. doi:10.1097/QAD.0b013e3282f7cb60
7. Hariharan S, Israni AK, Danovitch G. Long-term survival after kidney transplantation. *N Engl J Med*. 2021;385:729-743. doi:10.1056/NEJMra2014530
8. Duquesnoy RJ. Antibody-reactive epitope determination with HLAMatchmaker and its clinical applications. *Tissue Antigens*. 2011;77:525-534. doi:10.1111/j.1399-0039.2011.01646.x
9. Bhaskaran MC, Heidt S, Muthukumar T. Principles of virtual crossmatch testing for kidney transplantation. *Kidney Int Rep*. 2022;7:1179-1188. doi:10.1016/j.ekir.2022.03.006
10. Duus Weinreich I, Andersson T, Birna Andrésdóttir M, et al. Scandiatransplant exchange program (STEP): development and results from an international kidney exchange program. *Transplant Direct*. 2023;9:e1549. doi:10.1097/TXD.0000000000001549
11. Baxter-Lowe LA. The changing landscape of HLA typing: understanding how and when HLA typing data can be used with confidence from bench to bedside. *Hum Immunol*. 2021;82:466-477. doi:10.1016/j.humimm.2021.04.011
12. Matern BM, Olieslagers TI, Groeneweg M, et al. Long-read nanopore sequencing validated for human leukocyte antigen class I typing in routine diagnostics. *J Mol Diagn*. 2020;22:912-919. doi:10.1016/j.jmoldx.2020.04.001
13. Bravo-Egana V, Sanders H, Chitnis N. New challenges, new opportunities: next generation sequencing and its place in the advancement of HLA typing. *Hum Immunol*. 2021;82:478-487. doi:10.1016/j.humimm.2021.01.010
14. Pugh J. The current state of nanopore sequencing. *Methods Mol Biol*. 2023;2632:3-14. doi:10.1007/978-1-0716-2996-3_1
15. Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. *Nat Biotechnol*. 2021;39:1348-1365. doi:10.1038/s41587-021-01108-x
16. Mosbruger TL, Dinou A, Duke JL, 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*. 2020;81:413-422. doi:10.1016/j.humimm.2020.06.004
17. de Santis D, Truong L, Martinez P, D'Orsogna L. Rapid high-resolution HLA genotyping by MinION oxford nanopore sequencing for deceased donor organ allocation. *HLA*. 2020;96:141-162. doi:10.1111/tan.13901
18. Devriese M, Da Silva S, Le Mene M, et al. Two-field resolution on-call HLA typing for deceased donors using nanopore sequencing. *HLA*. 2024;103:e15441. doi:10.1111/tan.15441
19. Devriese M, Rouquie J, Da Silva S, et al. Single locus HLA sequencing with the nanopore technology for HLA disease association diagnosis. *HLA*. 2024;103:e15424. doi:10.1111/tan.15424
20. Oxford Nanopore Technologies. Q-Line|Oxford Nanopore technologies. https://nanoporetech.com/products/sequence/qline. Accessed May 29, 2024.
21. Omixon|NGS for HLA. NanoTYPE RUO downloads—Omixon|NGS for HLA. https://www.omixon.com/my-omixon/products/nanotype/nanotype-ruo-downloads/. Accessed Jan 01, 2024.
22. Nanopore store: Training. https://store.nanoporetech.com/eu/catalog/?id=21. Accessed Jan 01, 2024.
23. Services EW. IPD-IMGT/HLA database. https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/growth/. Accessed Jan 17, 2024.

24. Oxford Nanopore Technologies. Nanopore sequencing 101 Q&A. https://nanoporetech.com/events/nanopore-sequencing-101-online-event-qa. Accessed Jan 18, 2024.
25. Lipworth S, Pickford H, Sanderson N, et al. Optimized use of Oxford Nanopore flowcells for hybrid assemblies. *Microb Genom*. 2020;6(11). doi:10.1099/mgen.0.000453
26. Nathan P, Hassel JC, Rutkowski P, et al. Overall survival benefit with tebentafusp in metastatic uveal melanoma. *N Engl J Med*. 2021;385:1196-1206. doi:10.1056/NEJMoa2103485
27. Damato BE, Dukes J, Goodall H, Carvajal RD. Tebentafusp: T cell redirection for the treatment of metastatic uveal melanoma. *Cancers (Basel)*. 2019;11:971. doi:10.3390/cancers11070971
28. Anukul N, Jenjaroenpun P, Sirikul C, et al. Ultrarapid and high-resolution HLA class I typing using transposase-based nanopore sequencing applied in pharmacogenetic testing. *Front Genet*. 2023;14:1213457. doi:10.3389/fgene.2023.1213457
29. Kløve-Mogensen K, Haunstrup TM, Masmas TN, et al. High-resolution HLA genotyping identifies risk alleles in both class I and II for primary autoimmune neutropenia in early childhood in a Danish cohort. *HLA*. 2024;103:e15429. doi:10.1111/tan.15429
30. Liu C. A long road/read to rapid high-resolution HLA typing: the nanopore perspective. *Hum Immunol*. 2021;82:488-495. doi:10.1016/j.humimm.2020.04.009
31. Kolmogorov M, Billingsley KJ, Mastoras M, et al. Scalable nanopore sequencing of human genomes provides a comprehensive view of haplotype-resolved variation and methylation. *Nat Methods*. 2023;20:1483-1492. doi:10.1038/s41592-023-01993-x

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Buhler S, Nørgaard M, Steffensen R, et al. High resolution HLA genotyping with third generation sequencing technology—A multicentre study. *HLA*. 2024; 104(2):e15632. doi:10.1111/tan.15632