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*Editorial*

# Multiplex ligation-dependent probe amplification – a short overview

Valeriu Moldovan[1], Elena Moldovan[2*]

*1. Center for Advanced Medical and Pharmaceutical Research, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, România*
*2. Pediatric Intensive Care Unit, Cardiovascular and Transplant Emergency Institute of Târgu Mureș, România*

## Abstract

*Multiplex Ligation-dependent Probe Amplification is a technique proposed for the detection of deletions or duplications that may lead to copy number variations in genomic DNA, mainly due to its higher resolution, and shorter overall diagnosis time, when compared with techniques traditionally used, namely karyotyping, fluorescence in situ hybridization, and array comparative genomic hybridization. Multiplex Ligation-dependent Probe Amplification is a fast (about 2 days), useful and cost-effective technique, being suitable for the diagnosis of hereditary conditions caused by complete or partial gene deletions or duplications, as these conditions are either more difficult or impossible to be diagnosed by other techniques, such as PCR, Real-Time PCR, or sequencing (Sanger or Next Generation). Due to its numerous advantages over conventional cytogenetic analysis techniques, Multiplex Ligation-dependent Probe Amplification could be used in the near future as the main technique for the molecular investigation of genetic conditions caused by copy number variations, in both rare and complex genetic disorders.*

***Keywords:*** *MLPA; copy number variation; CNV; karyotyping; cytogenetic analysis*

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## Introduction

Copy number variation (CNV) is a type of structural abnormality that is ubiquitous in eukaryotic genomes, being represented by large Deoxyribonucleic acid (DNA) duplications and deletions. The human genome shows 5%-10% CNVs in normal individuals. Numerous studies are focusing on the distribution and functional significance of CNVs as well as its causative mechanisms. Traditionally, conventional cytogenetic analysis (karyotyping) and fluorescence in situ hybridization (FISH) were used to obtain important diagnostic and prognostic information for patients harboring CNVs, but, in recent years, the Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland, Amsterdam, The Netherlands) technique was proposed for the detection of deletions and/or duplications that may lead to copy number alterations in genomic DNA, mainly due to its higher resolution

***Corresponding author**: Elena Moldovan, Pediatric Intensive Care Unit, Cardiovascular and Transplant Emergency Institute of Târgu Mureș, România. E-mail: bitirelena@gmail.com

[single gene anomalies are too short to be identified by FISH or karyotyping (1, 2)], cost-effectiveness (1), and possibly even as an alternative technique to array comparative genomic hybridization (array CGH) (2, 3).

MLPA is a fast [about 2 days overall diagnosis time (4)] and cost-effective technique, being useful in the investigation of various human diseases caused by DNA copy number aberrations. MLPA is a simple and robust method based on multiplex Polymerase Chain Reaction (PCR), allowing the simultaneous investigation of CNVs in up to 60 (3) different genomic DNA sequences (e.g. exons in a gene of interest), being able to distinguish between sequences that differ by only one nucleotide (4).

The vast majority of genetic diseases are either polygenic or caused by point mutations, and most of them will not be detected by MLPA. On the other hand, a relatively small percentage of hereditary conditions are caused by (partial) gene deletions or duplications, the MLPA technique having indication in their diagnosis, as they are either more difficult or impossible to be diagnosed by other techniques, such as PCR, Real-Time PCR or sequencing (Sanger or Next Generation-NGS) (3-6).

## Applications

To date, there are over 400 (4) different SALSA MLPA probemixes offered by MRC-Holland, targeting numerous human genes, with diagnostic significance in various medical fields. The number of probes contained in each MLPA probemix (usually 30-60), coverage, and density within the genomic region vary according to the investigated condition. Although the majority of SALSA MLPA probemixes are for research use only (RUO), there is an increasing number of probemixes already certified as In Vitro Diagnostic (IVD) tools within the European Union (4).

Malignancy diagnosis has immediate benefits, since MLPA can be used, for example, in the investigation of Breast cancer type-1 *BRCA1* and type-2 *BRCA2* genes to assess hereditary cancer risk (6), study of Epidermal growth factor receptor *EGFR* in lung cancer (7), while for the diagnosis of breast and/or ovarian cancer *BRCA1*-associated RING domain-1 *BARD1* CNVs were explored (8).

Blood cancer investigation is another possible application for these probemixes, and positive results have emerged when used, for example, as a first-intention technique in the investigation of aneuploidies in pre-B acute lymphoblastic leukemia (ALL) patients (9), and the assessment of large numbers of samples with acute myeloid leukemia (AML) (10, 11), as seen in a couple of recently published studies.

Other authors reported using the technique for the diagnosis of degenerative diseases, like Duchenne and Becker muscular dystrophy (12), as well as other genetic diseases, such as 22q11.2 deletion syndrome, associated with a congenital heart disease (13) and Williams-Beuren syndrome (14).

Sex development disorders determined by Sex-determining region Y *SRY,* SRY-Box Transcription Factor-9 *SOX9,* Nuclear Receptor Subfamily-0 Group-B Member-1 *DAX-1,* Splicing Factor-1 *SF-1,* and Wingless-Type MMTV Integration Site Family, Member-4 *WNT4* deletions and/or duplications have also been successfully diagnosed using this method (15), while other authors have reported using the technique in the prenatal diagnosis of the most common aneuploidies on chromosomes X, Y, and 13, 18, 21, from amniotic fluid that was uncultured or from chorionic villi (1).

Recently, other uses for MLPA have been published, for instance, origin determination of small supernumerary marker chromosomes (sSMC) previously detected by cytogenetic investigation (16), and identification of a heterozygous muta-

tion, namely p.Pro250Arg (c.749C>G) in the Fibroblast growth factor receptor-3 *FGFR3* gene, in a case of intellectual disability with craniosynostosis (the mutation being later confirmed by Sanger sequencing) (17).

## Samples

DNA isolates can come from a variety of sources, including the most commonly used human fresh tissues (blood, buccal swabs, tissue biopsies), fresh frozen tissues, cell cultures, but also formalin-fixed paraffin-embedded (FFPE) tissue, although the accuracy of the results is highly dependent on fixation conditions, as well as the nucleic acid isolation method used.

Additionally, from our experience, all reference DNA samples and investigated samples should be isolated using the same extraction method and possibly even the same isolation kit, in order to avoid possible inter-sample variations caused by the different characteristics of the purification method.

The recommended DNA amount for MLPA reaction is 50-100 ng (4) and all DNA samples should contain (or be directly eluted in) 5-10mM Tris-Ethylenediaminetetraacetic acid (TE) buffer and its pH should be between 8.0 and 8.5 so as to avoid depurination, a chemical reaction at DNA level, which may appear during the denaturation process.

## Principle of the technique

During the MLPA reaction, the target DNA sample does not get amplified, but instead the ligated probes are exponentially amplified during the PCR reaction. This is possible because each MLPA probe is made up of two "half-probe" oligonucleotides that hybridize to their target sequences (3) next to the sequence of interest, following the denaturation of DNA. Afterwards, only when the two "half-probes" are both hybridized, they will be ligated together and be further amplified using a single pair of PCR primers (one primer is fluorescently labeled for further detection purposes in a capillary electrophoresis analyzer). The resulting amplicons have to be further separated by capillary electrophoresis, followed by data analysis with different third-party software or Coffalyser.Net (4).

MLPA can be used as a relative quantitation technique whereas just the probes that are ligated are amplified throughout the PCR reaction, the number of probe products of ligation being in direct proportion to the number of target sequences in the original unamplified sample.

An advantage of MLPA is that the elimination of unbound probes is not necessary, because probes that are not ligated contain just half of the future primer sequence, and therefore they cannot be exponentially amplified during the mandatory PCR steps (5).

Typically, the MLPA assay targets genes that are coding proteins, as their copy number variations will likely influence the protein level and thus may lead to phenotype changes. Unfortunately, sequence changes (single nucleotide polymorphism-SNPs, indels, and point mutations) in the target sequence can compromise the ability of an MLPA probe to recognize it and thus be the source of false results, as these changes can ultimately prevent probe hybridization. Similarly, SNPs present within the ligation site will prevent the ligation of the probes falsely indicating a deletion of that region (5). Moreover, the accuracy of the results is also conditioned by the MLPA probe specificity as it will generate a non-specific signal when alternative target sites exist in the genome.

## MLPA reaction

MLPA reactions can be set up in a molecular laboratory equipped with a genetic analyzer (necessary for capillary electrophoresis), the workflow

(4) being straightforward for anybody working in molecular biology. Besides the investigated samples and the reference DNA specimens, the use of a "no DNA template" control within each run is encouraged (5), to test for MLPA reagents, electrophoresis reagents or capillaries possible contamination (4). The addition of a positive DNA sample will further facilitate the final copy number variation interpretation, but it is not mandatory.

Table 1 summarizes the MLPA one-tube protocol for the detection and quantification of short DNA sequences, using a thermocycler with a heated lid (99-105°C).

**Table 1. Summary of MLPA one-tube protocol (4)**

| PCR Program | Action | Reagents for one sample [should be multiplied by n (x n)] [1] |
|---|---|---|
| **Denaturation (First day)** | | |
| 98°C, 5 minutes | Denaturate the samples | 5µl DNA sample (or 5µl reference DNA, or 5µl "no DNA" template) |
| 25°C, ∞ (infinity) | Bring samples to room temperature | |
| Pause | Samples can be removed from the thermocycler | |
| **Hybridization (First day)** | | |
| 95°C, 1 minute | Sample denaturation | + 1.5µl (x n) recommended buffer |
| 60°C, 16–20 hours | Hybridize the half-probes to their DNA targets | + 1.5µl (x n) recommended probemix |
| 54°C, ∞ | Adjust the temperature for the next step | |
| Pause at 54°C | Do not remove samples from the thermocycler! Pipet ligation reagents while samples are at 54°C in the thermocycler! | |
| **Ligation (Second day)** | | |
| 54°C, 15 minutes | Ligate the adjacent hybridized half-probes | + 25µl (x n) Ultrapure water |
| 98°C, 5 minutes | Heat inactivation of the Ligase-65 enzyme | + 3µl (x n) Ligase Buffer A |
| 20°C, ∞ | Bring samples to room temperature | + 3µl (x n) Ligase Buffer B |
| Pause | Samples may be removed and at room temperature the reagents for the next steps can be added | + 1µl (x n) Ligase-65 enzyme |
| **Amplification (Second day)** | | |
| 35 cycles of:<br>95°C, 30 seconds<br>60°C, 30 seconds<br>72°C, 1 minute | Amplify ligated MLPA probes only | + 7.5µl (x n) Ultrapure water<br>+ 2µl (x n) recommended PCR primer mix<br>+ 0.5µl (x n) recommended Polymerase |
| 72°C, 20 minutes | Final extension of PCR amplicons | |
| 15°C, ∞ | Cooldown samples before removing from the thermocycler | |
| **Preparation for Capillary electrophoresis (Second day or Third day)** | | |
| 86°C, 3 minutes<br>4°C, 2 minutes | Preparing the injection mixture for electrophoresis | + PCR amplicons from previous step[2]<br>+ Molecular size standard[2]<br>+ Highly deionized formamide[2]<br>+ Molecular grade water or mineral oil (necessary for some systems)[2] |

[1] - n represents the total number of reactions (investigated samples, reference DNA, "no DNA template" control, positive DNA sample) plus an excess of 10% to 15% (to compensate for liquid loss on tips during pipetting); [2] - different quantities have to be added per sample, according to the instrument manufacturer's recommendations. For example, several ABI systems (3130xl, 3500xl, 3730xl, 3130, 3500, 3730) compatible with MLPA IVD (4) certified probemixes require 0.7µl of PCR amplicons, 0.2µl of GS500 (ThermoScientific, USA) size standard [or 0.3µl of 500ROX (ThermoScientific, USA) size standard], and 9µl of highly deionized formamide for every reaction.

MLPA amplicon separation shall be executed in denaturing condition settings on a standard capillary electrophoresis DNA analyzer (4) with fragment analysis software. The specific run parameters (such as injection time, injection volume, run voltage, etc.) must be adjusted according to the instrument manufacturer's recommendations. As it is a rather labor-intensive technique, factors like contamination, integrity and quantity of DNA, PCR inhibitors present in the samples, insufficient denaturation of DNA, DNA depurination, partial evaporation of samples, long pipetting time, improper use of capillary electrophoresis protocols (incorrect: time of injection, voltage, preparation of samples and size standards) may compromise the multiplex ligation-dependent probe amplification results by altering the enzymatic reactions and ultimately resulting in reduced or absent peak signals (14).

## General data interpretation

Data analysis can be performed using multiple software, such as MRC-Holland proprietary software Coffalyser.Net (4), or third-party software and the final decision has to take into account the specimen type and MLPA kit being used.

As a general rule, the final relative quantification should only be performed on samples and reference DNA specimens that were included in the same MLPA experiment (both PCR amplification and capillary fragment analysis) and were tested with the same probemix lot in order to exclude any possible inter-run variation.

For normal samples, the Dosage Quotient DQ (comparison of probe fluorescence within the sample, between samples and signal sloping corrections) is approximately 1, with accepted levels between DQ=0.8-1.2 (see Figure 1, a sample analyzed with a 3500 Genetic Analyzer

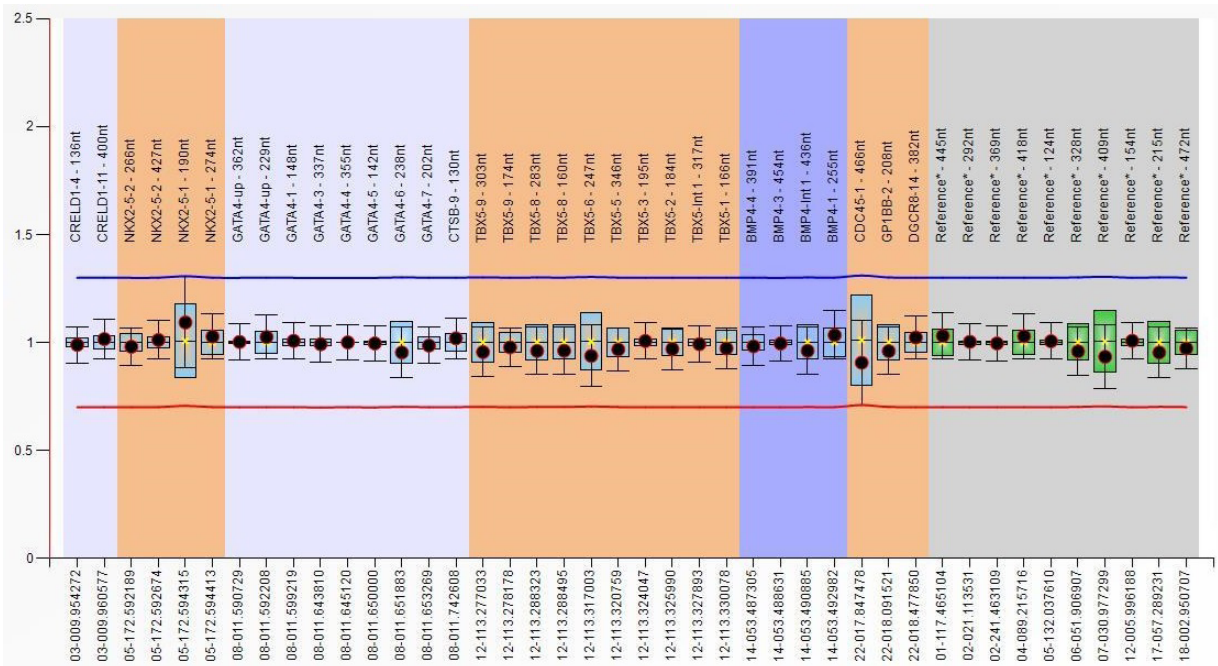


**Fig. 1. Normal sample analyzed with the SALSA MLPA P311 Congenital Heart Disease probemix on a 3500 Genetic Analyzer from ThermoScientific, USA: Dosage Quotient – DQ between 0.8-1.2 for all investigated fragments. Graph ordinate: DQ; Graph abscissa: the chromosomal position of investigated fragments**

from ThermoScientific, USA), and a standard deviation below 10%. When detecting a heterozygous deletion, the expected DQ is 0.40-0.65, while for heterozygous duplication the awaited DQ is 1.30-1.65, but MLPA can also detect homozygous deletion (DQ=0), or multiple copies (DQ between 1.75-2.15). All samples with copy number variations will naturally have a higher standard deviation, induced in the mathematical computation by the presence of the mutation itself.

MLPA probemixes also contain internal control fragments that have to be analyzed before the final result calculation. These include the Benchmark fragment, Q-fragments (DNA quantity and ligation indicators), D-fragments (DNA denaturation indicators), and gender indicator fragments (for chromosomes X and Y) (4) (see Figure 2, a sample analyzed with a 3500 Genetic Analyzer from ThermoScientific, USA).

Interpretation of MLPA results is delicate when there are deletions that involve only one exon, and the MLPA kit being used contains a single probe for each investigated exon. In these cases, the apparent deletion, which is observed in some cases, may be the result of a change in the exon sequence that inhibits the correct hybridization or ligation of the MLPA probes. Caused by pathogenic point mutations or by polymorphisms that do not affect normal gene function, these sequence variations causing apparent MLPA single exon deletions should be confirmed by other techniques, such as long-range PCR, qPCR, droplet digital PCR (5), or even gene sequencing. However, if the MLPA kit contains multiple probes for each investigated exon,

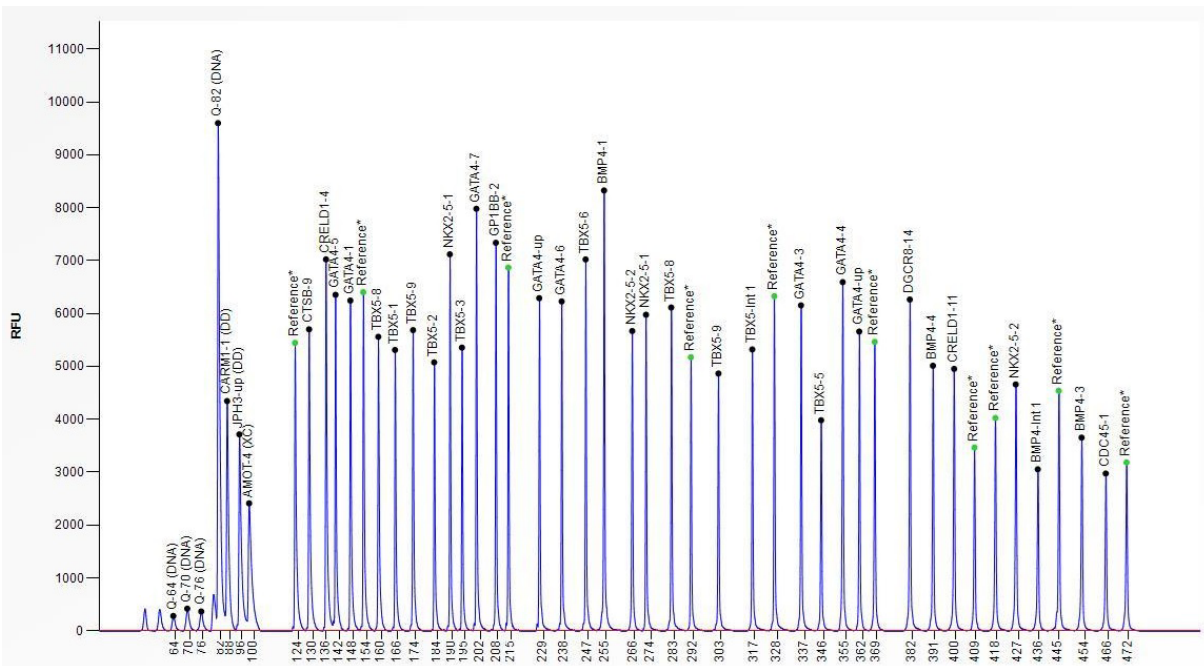


**Figure 2. Electropherogram of a normal sample analyzed on a 3500 Genetic Analyzer from ThermoScientific, USA, with the SALSA MLPA P311 Congenital Heart Disease probemix, containing control fragments and investigated fragments. Graph ordinate: RFU=relative fluorescent units; Graph abscissa: length of investigated fragments (in base pairs).**

a minimum of two probe DQs within the same exon have to be decreased in order to consider an exon deletion.

Nonetheless, there are MLPA probemixes able to discriminate known point mutations in the gene or within the genomic region of interest, and which contain probes designed to ligate directly at the point mutation site, for example, the SALSA MLPA probemix P080-C1 (17). Hence, by adding specific MLPA probes for common point mutations to regular MLPA gene dosage probemixes, both copy number variations and limited point mutation analysis can be performed altogether.

## MLPA limitations

When analyzing DNA samples extracted from cell mixtures, MLPA yields the medium copy number per cell. Therefore, in the case of tumor analysis, the detection of duplications or deletions is often difficult if the proportion of cancer cells is lower than 50% in the original sample according to some authors (5). Additionally, the existence of a mosaicism may remain undiscovered by MLPA as the presence of cells with anomalies (CNV) may be masked by the presence of normal cells.

MLPA is not useful in the diagnosis of balanced rearrangements (12), considering that this technique compares studied DNA samples against healthy controls, being well-known that balanced rearrangements produce a modification in DNA sequence order, and sometimes in their function, but not in the total DNA quantity.

Single cells cannot be investigated by current conventional MLPA probemixes (as opposed to FISH (2)), mainly because of the need for an initial 50ng of DNA (2) with good purity ratio. However, newer MLPA-based techniques, such as next-generation sequencing-based MLPA-digital MLPA (2) and Reverse Transcriptase MLPA (RT-MLPA) can be used in this respect, and, additionally, these techniques allow the simultaneous use of up to 1000 probes.

## MLPA variants

Methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA) is a variant of the original technique that allows the simultaneous investigation of CNVs and epigenetic modifications such as DNA methylation patterns within the CpG islands with the help of HhaI restriction enzyme which is methylation-sensitive, being very useful in the diagnosis of imprinting diseases, for example.

Reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) uses RNA as the initial sample, which has to be reverse transcribed to cDNA in order to be further handled using the standard MLPA protocol. This technique is especially useful for mRNA profiling, with various applications in apoptosis, inflammation, and malignancy investigations.

Next-generation sequencing-based MLPA, or digital MLPA, permits the simultaneous analysis of large numbers of samples [up to 1000 samples (2)], using the same MLPA principle, the difference being that oligonucleotide adapters are attached to the MLPA probes, thus allowing the amplicon quantification by next-generation sequencing analyzers [such as the Illumina next-generation sequencing systems (2)].

## Conclusions

Since its discovery, the MLPA assay has turned into one of the most extensively used investigation methods for the molecular analysis of CNVs, and together with its ability to detect methylation patterns and even point mutations, the technique may still provide adequate data for disease diagnosis or malignancy progression, thus reducing or even eliminating the need for further genetic testing for the patient.

## Abbreviations

Array CGH - Array comparative genomic hybridization
BARD1 - Breast cancer type-1 associated RING domain-1
BRCA1 - Breast cancer type-1
BRCA2 - Breast cancer type-2
CNV - Copy number variation
DAX-1 - Nuclear Receptor Subfamily-0 Group-B Member-1
DNA - Deoxyribonucleic acid
DQ - Dosage Quotient
EGFR - Epidermal growth factor receptor
FFPE - Formalin-fixed paraffin-embedded
FGFR3 - Fibroblast growth factor receptor-3
FISH - Fluorescence in situ hybridization
IVD - In Vitro Diagnostic
MLPA - Multiplex Ligation-dependent Probe Amplification
MS-MLPA - Methylation specific-multiplex ligation-dependent probe amplification
NGS - Next generation sequencing
PCR - Polymerase Chain Reaction
RT-MLPA - Reverse Transcriptase Multiplex Ligation-dependent Probe Amplification
RUO - Research use only
SF-1 - Splicing Factor-1
SNP - Single nucleotide polymorphism
SOX9 - Sex-determining region Y Box Transcription Factor-9
SRY - Sex-determining region Y
sSMC - Small supernumerary marker chromosomes
TE - Tris-Ethylenediaminetetraacetic acid
WNT4 - Wingless-Type MMTV Integration Site Family, Member-4

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## Authors' contributions

VM: conceptualization, methodology, investigation, writing - original draft preparation, writing – review and editing, visualization, supervision.
EM: conceptualization, resources, writing - original draft preparation, writing – review and editing, visualization, funding acquisition.

## Conflict of interest

The authors declare no conflict of interest.

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