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RESEARCH

# Systematic detection of mosaicism by using digital NGS reveals three new *MEN1* mosaicisms

**Arnaud Lagarde[1], Grégory Mougel[1], Lucie Coppin[2], Magalie Haissaguerre[3], Lauriane Le Collen[4,5], Amira Mohamed[6], Marc Klein[7], Marie-Françoise Odou[8,9], Antoine Tabarin[3], Hedia Brixi[10], Thomas Cuny[11], Brigitte Delemer[4], Anne Barlier[1]** and **Pauline Romanet[1]**

[1]Aix Marseille Univ, APHM, INSERM, MMG, Laboratory of Molecular Biology Hospital La Conception, Marseille, France
[2]Univ. Lille, CNRS, Inserm, CHU Lille, UMR9020-U1277 – CANTHER – Cancer – Heterogeneity Plasticity and Resistance to Therapies, Lille, France
[3]Service d'Endocrinologie, Centre Hospitalier Universitaire, Hôpital du Haut Levêque, Pessac, France
[4]Endocrinology, Diabetology and Nutrition Unit, University Hospital of Reims, Reims, France
[5]Inserm/CNRS UMR 1283/8199, Pasteur Institute of Lille, EGID, Lille, France
[6]Laboratory of Molecular Biology, Hospital La Conception, APHM, Marseille, France
[7]Service Endocrinologie, CHU de Nancy, Hôpital de Brabois, Vandoeuvre-lès-Nancy, France
[8]CHU Lille, Service de Biochimie et Biologie Moléculaire 'Hormonologie, Métabolisme-Nutrition, Oncologie', Lille, France
[9]Univ. Lille, Inserm, CHU Lille, U1286 – Infinite – Institute for Translational Research in Inflammation, Lille, France
[10]Department of Gastroenterology and Digestive Oncology, Reims University Hospital, Reims, France
[11]Aix Marseille Univ, APHM, INSERM, MMG, Department of Endocrinology, Hospital La Conception, Marseille, France

**Correspondence should be addressed to P Romanet: pauline.romanet@univ-amu.fr**

## Abstract

*Purpose:* Mosaicism is a feature of several inherited tumor syndromes. Only a few cases of mosaicism have been described in multiple endocrine neoplasia type 1 (MEN1). Next-generation sequencing (NGS) offers new possibilities for detecting mosaicism. Here, we report the first study to systematically look for *MEN1* mosaicism, using blood DNA, in MEN1-suspected patients but without *MEN1* pathogenic variants (PV) in a heterozygous state.

*Methods:* Digital targeted NGS, including unique molecular identifiers (UMIs), was performed in routine practice, and the analytic performance of this method was verified.

*Results:* Among a cohort of 119 patients harboring from 2 to 5 MEN1 lesions, we identified 3 patients with *MEN1* mosaic PVs. The allele frequencies ranged from 2.3 to 9.5%. The detection rate of MEN1 mosaicism in patients bearing at least 3 MEN1 lesions was 17% (3/18). No cases were detected in patients with two lesions.

*Conclusion:* We report here three new cases with *MEN1* mosaicism. This study examined the performance of UMI in the diagnosis of *MEN1* mosaicism in routine practice, and our results underline that the frequency of mosaicism is probably underestimated in patients with suspected MEN1.

**Key Words**

- mosaicism
- MEN1
- NGS
- unique molecular identifier
- mosaic
- deep-sequencing
- de novo
- digital NGS
- thymic tumor
- pituitary adenoma
- hyperparathyroidism
- pancreatic neuroendocrine tumor

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## Introduction

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is an autosomal dominant disease due to mutation in the *MEN1* gene, characterized by a broad spectrum of clinical manifestations (1). The classic clinical triad includes primary hyperparathyroidism (PHPT), pituitary neuroendocrine tumors (PitNET), and duodeno-pancreatic neuroendocrine tumors (DPNET). Other endocrine tumors including adrenal cortical tumors and neuroendocrine thymic or bronchopulmonary tumors may also be present. Several non-endocrine manifestations have also been associated with MEN1: facial angiofibromas, facial collagenomas, lipomas, and

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meningiomas. Of the patients with MEN1, 28–70% die as a consequence of the disease, particularly due to the pancreatic and carcinoid lesions (2, 3). Classical genetic testing on blood DNA is positive in 90–95% of familial cases, and in 30–45% of sporadic cases who present with the classical triad (4, 5, 6). Mosaicism can explain some of these unresolved cases. Mosaicism has been described in several inherited tumor syndromes and corresponds to the spontaneous acquisition of a genetic variant during cell division during post-zygotic embryonic development (7, 8, 9, 10, 11). Mosaicism thus results in a fetus composed of a variable proportion of mutated cells, depending on how early and in which cell lines the variant occurs. Mosaic variants may be undetectable in blood samples using classical sequencing methods. Only a few cases of *MEN1* mosaicism have been reported to date (12, 13, 14, 15, 16). Indeed, identification of *MEN1* mosaicism remains challenging in the routine practice of diagnosis laboratories and consequently is not systematically performed. Next-generation sequencing (NGS) offers new possibilities for detecting mosaic variants (7, 8, 14, 16, 17, 18). Here, we set up targeted NGS using unique molecular identifiers (UMI, the UMI principle described in the Supplementary materials, see section on supplementary materials given at the end of this article) to systematically search for *MEN1* mosaicism using blood DNA in unresolved MEN1 index cases showing at least 2 lesions and determined the performance of such analysis in a cohort of 119 patients.

## Materials and methods

### Next-generation sequencing workflow

Genomic DNA was extracted from peripheral blood samples using QIAsymphony DSP DNA Midi Kit (Qiagen) according to the manufacturer's protocols.

Between 20 and 40 ng of genomic DNA was used to produce the library and perform target enrichment using the QIAseq Targeted DNA Custom Panel kit (Qiagen). The custom library included 62 kb of coding exons and 20 bp flanking regions of *MEN1* (NM_130799) and 27 other genes involved in endocrine diseases (see Supplementary materials). This library used UMIs. UMIs are unique oligonucleotide sequences which are added to DNA prior to any amplification and differentially label each molecule in the native DNA fragment. UMIs are usually used for improving the molecular detection of rare events in somatic DNA (19). Indeed, UMIs allow for a computational correction of amplification bias and sequencing errors by identifying PCR duplicates (see Supplementary Figure 1).

Quantitation and qualification of libraries was done using the Qubit Fluorometer (Thermo Fisher Scientific) and the TapeStation instrument (D100 ScreenTape, Qiagen) to enable equimolar pooling of barcoded samples. Twenty-four samples were sequenced during the same paired-end run (V2 2 × 150 bp) on a MiSeqDx (Illumina).

Alignment and variant calling were performed on the CLC Genomics WorkBench 20.0.4 (Qiagen) standalone analysis system against the Human genome reference GRCH37. The elements of optimization and validation of the variant calling for detection of mosaicism are described in the Supplementary materials. All variants that had an allele frequency (AF) greater than 0.5% were considered.

### Validation and analytical performance of mosaicism detection using library preparation with UMIs

First, we analyzed the DNA from a patient who presented with a known mosaic *MEN1* pathogenic variant, quantified at an AF of 5.1% using ultra-deep NGS (patient A, Supplementary Table 1) (14).

Secondly, we created artificial mosaic variants (AMVs) to simulate mosaic *MEN1* variants at different frequencies. For this, DNA from 2 patients (B and C) carrying *MEN1* pathogenic variants in a heterozygous state were mixed at 20, 10, 4, and 2% with DNA from a wild-type sample with a known genotype (patient D). All these patients had been referred to the molecular laboratory at the La Conception hospital for genetic testing. Patient B carried a heterozygous *MEN1* pathogenic variant c.1546dupC, p.(Arg516Profs*15) which is located in a repeat region (homopolymer track with $n=7$), and patient C carried a heterozygous *MEN1* pathogenic variant: c.1252G>A, p.(Asp418Asn) (4). The expected mutated AFs were respectively 10, 5, 2, and 1% in diluted samples. Variant calling using UMI groups was compared to variant calling using the same workflow but discarding the UMI group creating tool. The error rate of the method was taken as the number of false positives detected in a patient divided by the number of sequenced nucleotides and expressed as number of false positives/kb.

### Cohort of unresolved MEN1 cases

This study was performed on patients referred between March 2018 and March 2021 for *MEN1* genetic testing to the molecular laboratory of Marseille La Conception Hospital. Patients presented with at least two MEN1-related tumors

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but without *MEN1* pathogenic variants in the heterozygous state. Written informed consent for genetic analysis was obtained from all patients during one-on-one genetic counseling. NGS data produced during routine practice processes were retrospectively realigned and reanalyzed using the pipeline optimized for mosaic detection. All variants with an AF superior to 0.5% were analyzed. The study was approved by the ethics committee of Aix-Marseille University (approval number: 2018-13-12-004).

### Confirmation of mosaicism

Putative mosaic variants responsive for MEN1 were confirmed by searching for the variant either in the MEN1 lesions when these were available or in a second blood sample from the patient. NGS or Sanger sequencing was performed on DNA from formalin-fixed paraffin-embedded (FFPE) MEN1 lesions. FFPE-tissue DNA was extracted from samples using a QIAamp DNA FFPE tissue kit (Qiagen). For Sanger sequencing, DNA was amplified using PCR targeting the identified *MEN1* variation (primers available upon request) using the AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific). After ExoSap-IT purification (Thermo Fisher Scientific), PCR products were sequenced using the Sanger method on an AB3500XLDX genetic analyzer (Thermo Fisher Scientific). NGS sequencing used the same method as described earlier.

### Statistical methods

Data were compiled using R (https://www.r-project.org/). Statistical analyses were performed using Prism software v9.0 (GraphPad Software). All results are expressed as median (range min–max values). The correspondence between the theoretical and observed values was evaluated by calculating the $R^2$ of the Pearson correlation coefficient.

## Results

### Validation and analytical performance of *MEN1* mosaicism detection using library preparation with UMIs

First, we confirmed the presence of the known mosaic *MEN1* variant in patient A, with an AF of 5.9% (vs 5.1% by ultra-deep NGS (7, 14) Supplementary Table 1).

Then, a total of 10 DNA samples were sequenced: 2 undiluted samples from patients B and C, plus 4 dilutions for each sample (Supplementary Table 2). The sensitivity of the mosaic detection process was 100% with all *MEN1* AMVs being detected. The observed AF of the *MEN1* AMVs correlated well with the expected AFs (correlation coefficient $R^2$ = 0.95 y=0.8255*x+0.3102, Fig. 1A). Using the UMI-specific bioinformatic process, false positive *MEN1* variants were detected in only 3 out of the 10 samples (Fig. 1B) and only at low AF. One was a single nucleotide variation at an AF of 0.7% (*MEN1*: c.787C>G, p.(Leu263Val)), and 2 were the same deletion of 1 nucleotide in a homopolymer region at an AF of 1% (*MEN1*: c.1546delC, p.(Arg516Glyfs*43)), supported in both cases by only 1 UMI group.

Finally, at AF values greater than 0.5%, the error rate for *MEN1* was only 0.27 per kb (Fig. 1C), and at AF values greater than 1%, the error rate was zero, that is, no false positive *MEN1* variants were found (Fig. 1C). In contrast, without considering the UMIs in the bioinformatic process, the median number of false positives in *MEN1* was 37.5 per

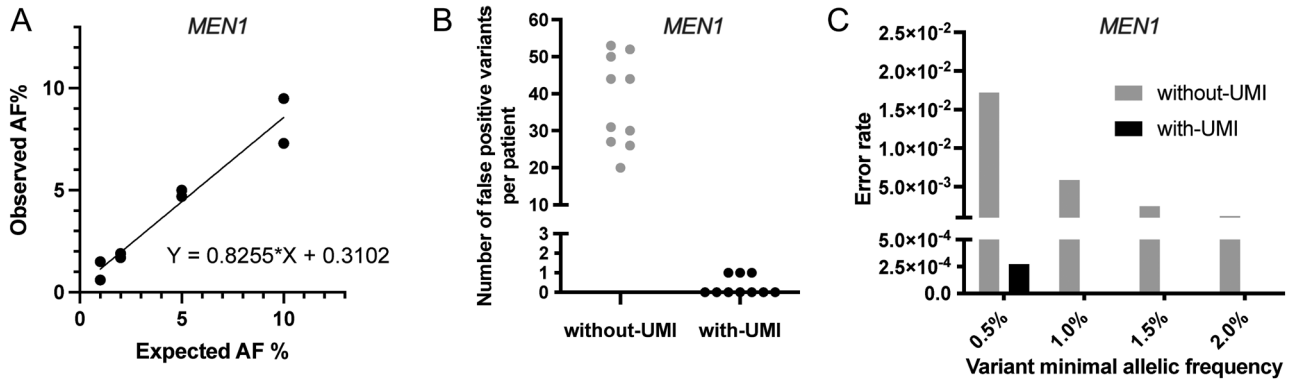



**Figure 1**
Analytic performance of the method. (A) Linear regression between expected and observed AF of the artificial mosaic variants in *MEN1*. (B) Comparison of the false positive variant count in *MEN1* with and without taking into account UMIs. (C) Error rate for variant calling in *MEN1*. AF, allele frequency; UMI, unique molecular identifier.

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sample (min 20–max 53) (Fig. 1B). At an AF superior to 0.5%, the error rate for *MEN1* was 17 per kb (Fig. 1C). These data validated the mosaic detection process using UMI for *MEN1* (see also Supplementary materials for the rest of the 62 kb panel).

## Cohort of unresolved MEN1 cases

One hundred nineteen patients (40 males and 79 females) were included in the study. Their average age at the time of genetic testing was 54 years (range 17–86 years). Patients presented with at least two MEN1-associated lesions (Table 1). PHPT and PitNET were the most frequent lesions (80 and 67% of patients, respectively). Sixty-five patients (54%) presented with both PHPT and PitNET.

The median coverage of depth for *MEN1* sequencing was 584X (48–2753X). In view of the results of the validation study, we restricted the analysis to variants supported by two UMI groups to avoid false positives. Among the 119 patients, 14 presented with variants at an AF greater than 0.5%. Eight patients harbored the *MEN1* variant c.787C>G, previously identified as a false positive in our validation study, at an AF between 0.6 and 1.22% (mean 0.9%). Two patients harbored the c.655-6dupC, p.(?) *MEN1* variant (AF 0.8 and 0.62%), and one patient harbored the c.655-5delC, p.(?) variant at an AF of 1.1%. These two variants were localized in a homopolymer region and were each supported by only one UMI group in two other patients. These variants were present in the general population in a heterozygous state (gnomAD v2.1 last access 07/05/2022) and using SpliceAI were not predicted to alter splicing, they were thus not selected as putative mosaic variants. The last three variants were not found in other patients even supported by only one UMI group (Table 2). These three variants have been reported in the literature, considered as pathogenic *MEN1* variants and consequently were selected as putative mosaic variants (Table 2 patients #1, #2, and #3).

The AF values of the three putative mosaic variants ranged from 2.3 to 9.7%, with coverage of depth between 312 and 921X. The number of UMI groups supporting mosaicism ranged between 3 and 23. The three mosaic *MEN1* variants were confirmed by a second method; the patient #1 variant was detected using NGS in somatic DNA from a parathyroid adenoma (AF: 78%) and from a thymic tumor (AF: 46%); the patient #2 variant was detected in somatic DNA from a thymic tumor using NGS (AF: 46%) and from a duodenal neuroendocrine tumor by Sanger sequencing; and the patient #3 variant was confirmed by Sanger sequencing using peripheral blood DNA and by NGS on a second blood sample.

**Table 1** Clinical characteristics of the cohort of 119 unresolved MEN1 cases.

| | PHPT | PitNET | DPNET | ADRE | THYM | LUNG | ≥4 Lesions | 3 Lesions | 2 Lesions | PHPT + PitNET | PHPT + DPNET | PitNET + DPNET |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of patients | 95 (80%) | 80 (67%) | 29 (24%) | 28 (24%) | 10 (8%) | 4 (3%) | 2 (2%) | 16 (13%) | 101 (85%) | 54 (54%[a]) | 17 (17%[a]) | 3 (3%[a]) |
| Mean age at diagnosis (years; min–max) | 57 (30–86) | 53 (18–80) | 55 (34–86) | 58 (19–86) | 52 (35–72) | 56 (43–84) | - | - | - | - | - | - |

ADRE, adrenal tumor; DPNET, duodeno-pancreatic neuroendocrine tumor; LUNG, bronchial neuroendocrine tumors; PHPT, primary hyperparathyroidism; PitNET, pituitary neuroendocrine tumor; THYM, thymic neuroendocrine tumor.
[a]Percentage among patients with two lesions.

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**Table 2** Clinical and genetic characteristics of patients with *MEN1* mosaicism in the cohort of unresolved MEN1 cases (patients #1, #2, and #3).

| | Patient #1 | Patient #2 | Patient #3 |
|---|---|---|---|
| Gender | Male | Male | Male |
| Age at molecular diagnosis (years) | 54 | 60 | 31 |
| Lesions (years) | PHPT (43)<br>THYM (43)<br>DPNET (53) | PHPT (56)<br>DPNET (56)<br>PitNET (56)<br>THYM (57) | PHPT (24)<br>DPNET (24)<br>PitNET (27) |
| *MEN1* mosaic pathogenic variant | Exon 3<br>c.496=/C>T<br>p.(Gln166=/*) | Intron 4<br>c.784-9=/G>A<br>p.(?) | Exon 2<br>c.252=/dup<br>p.(Ile85=/Tyrfs*32) |
| Allelic frequency | 9.7% | 2.3% | 9.3% |
| Number of reads supporting the mutation (singleton/UMI group) | 80 (57/23) | 21 (18/3) | 29 (23/6) |
| Proportion (singleton/UMIs) | 0.71 | 0.86 | 0.79 |
| QUAL | 200 | 200 | 200 |
| Coverage of depth of the mutation | 828X | 921X | 312X |

DPNET, duodeno-pancreatic neuroendocrine tumor; PHPT, primary hyperparathyroidism; PitNET, pituitary neuroendocrine tumor; QUAL, quality (0 to 200); THYM, neuroendocrine thymic tumor.

Overall, no mosaic variant was detected in the 101 patients with 2 MEN1 lesions (0/101), whereas 2 mosaic variants were detected in 2 out of the 16 patients with 3 lesions (2/16, 12.5%; patient #1 and #3), and in 1 of the 2 patients having at least 4 lesions (1/2; patient #2).

## Discussion

The diagnosis of mosaicism remains an unmet medical need in the routine practice of genetic laboratories (7, 8, 9, 10, 11). NGS offers the possibility of detecting mosaic variants using DNA from blood (7, 8, 14, 16, 17). The challenge is to lower the threshold of variant detection without including sequencing artifacts and also to distinguish variants from artifacts (17, 20, 21). UMIs are unique oligonucleotide sequences which are added to DNA prior to any amplification and these differentially label each molecule in the native DNA fragment. UMIs were initially developed to eliminate PCR duplicates in DNA or RNA seq in order to count the absolute number of molecules (22). In somatic or germline DNA context, UMIs can also be used to improve variant calling, by eliminating some artifacts coming from the amplification steps or from sequencing, thus improving the selection of true variants (23). This method is particularly appropriate for detecting variants at low AF. In specific regions, such as homopolymer tracks, UMI increases the sensibility and specificity of variant detection. Thus, in our validation study, we were able to detect a pathogenic mosaic variant in a homopolymer region at an AF as low as 1% (Supplementary Table 2 and Supplementary materials) but found two false positive variants in the same region at an AF of 1%, supported by only 1 UMI group. In our retrospective study, in order to increase the specificity of analysis, we considered variants supported by at least two UMI groups. When compared to bioinformatics methods without using UMIs, the number of false positives was reduced by 98.4% for *MEN1* and by 97.6% on the whole 62 kb panel, at an AF greater than 0.5% (Supplementary materials). Our method, using UMIs, enables a high level of specificity and consequently rapid decisions made regarding further molecular explorations to confirm the *MEN1* mosaicism, thus improving management for both the patient and their family. In our study, we detected *MEN1* mosaicism at a very low AF (2.3%), a threshold that has not been reported previously for this pathology.

Due to the technical difficulties involved in setting up systematic mosaicism detection in the routine practice of laboratories, the frequency of mosaicism is probably underestimated in most genetic diseases. We report here the first blood-based systematic analysis of *MEN1* mosaicism in a cohort of clinically suspected MEN1 patients without heterozygous pathogenic variant. In our cohort of 119 patients with at least two MEN1-related lesions, we found mosaicisms in 2.5% of patients, and notably only in those with 3 or more lesions (3/18, 17%), showing that searching for *MEN1* mosaicism is required in such a context.

In neurofibromatosis type 2 (NF2), in which mosaicism is more frequent (24, 25), it has been proposed that two independent tumor samples are analyzed in each patient with clinically diagnosed NF2, based on the Manchester criteria (26). MEN1 is a lifelong disease in which the lesions may be widely spaced and earlier biopsy specimens


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are not always available. Moreover, some lesions may have benefited from first-line medical treatment or are not always systematically surgically removed. In our cohort, the rate of mosaicism was significantly lower than that reported in NF2, although 91 patients presented with clinically diagnosed MEN1 (27), based on the presence of at least 2 MEN1 primary lesions. No *MEN1* mosaicism was identified in patients bearing only two MEN1-related lesions. This is in agreement with the low rate of heterozygous *MEN1* mutations in such a population, ranging from 2 to 19% depending on the type of MEN1 lesions involved (5, 6). In the Dutch cohort, three-quarters of *MEN1* genetically negative patients who had two MEN1-related lesions had a PTHP and a PitNET and they had developed their lesions at a later age than *MEN1* genetically positive patients (28). In our series of patients with 2 MEN1-related lesions, 54% had a PHPT and PitNET and the other patients had a mild phenotype, with one of the tumors occurring at over 50 years of age. In these conditions, a complementary strategy involving systematic analysis of multiple tissues seems to be difficult to justify, both medically and economically, in all patients with two MEN1-related lesions. Nevertheless, somatic analysis of MEN1-related lesions should not be forgotten in case of strong clinical suspicion and negative blood-based genetic testing, in particular in patients with multiple lesions including DPNET and thymic neuroendocrine tumor. Recently, a patient with a macroprolactinoma at 24 years of age, harboring a *MEN1* mosaic variant at an AF of 11%, has been described (29). This case shows that we should not limit the detection of mosaicism to unresolved cases with very specific phenotypes. Our work highlights the need to optimize the sequencing process in diagnostic laboratories to allow the detection of mosaicism on blood samples in routine practice.

If a mosaic variant is also present in germinal tissue, the variant can be transmitted to the offspring. Thus, undiagnosed mosaicism in someone planning children leads to the lack of adjusted genetic counseling and to a possible loss of a chance for having children, particularly in the case of inherited cancer syndromes. Conversely, molecular diagnosis of mosaicism could have a strong benefit for patient care and also for genetic counseling. Family genetic investigation revealed no mutation in the three children of patient #2. The children of patient #1 refused genetic testing, and patient #3 has no children to date.

Of the two patients who had at least four MEN1 lesions, we failed to identify a *MEN1* mosaic pathogenic variant in blood of one patient. *CDKN1B* analysis for this patient was also negative. Further analysis, such as whole genome sequencing, is required to identify variants in other regions, possibly as a deep intronic variant or a variant located in the promoter.

Regarding our entire panel of 62 kb, during our validation study, the median false positive per sample was 25.5 vs 1073 per sample when UMIs were not taken into account, showing the interest of using UMIs for the genetic diagnosis of other syndromes, such as neurofibromatosis type 1, von Hippel Lindau syndrome or tuberous sclerosis (see Supplementary materials).

## Conclusions

We present here the first study to systematically search for *MEN1* mosaicism using blood DNA in unresolved MEN1 cases in which we identified mosaicism in 17% of patients bearing 3 or more MEN1-related lesions. This study suggests that the frequency of *MEN1* mosaicism may be underestimated and underlines the need to develop tools to detect it in routine practice, with UMI being potentially an accurate and powerful tool.

**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/EC-22-0093.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Ethics declaration**

Informed consent was obtained from all subjects involved in the study. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the or Ethics Committee of Aix Marseille Univ (ref 2018-13-12-004, date of approval: XII/14/2018).

**Author contribution statement**

Contributions: Conceptualization, methodology A L, P R and A B; software, A L, A M; validation, A L, L C, and M-F O; formal analysis, A L; resources, M H, A T, L L, B D, H B and M K; writing – original draft preparation, P R, A L, and A B; writing – review and editing, G M, T C, L C, M H, L L, A M, M K, H B, M-F O, A T, and B D; supervision, A B and P R; funding acquisition, A B. All authors


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have read and agreed to the published version of the manuscript. A B and P R: contributed equally to this work.

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