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RESEARCH

Dystrophinopathy patient data as a guide to interpretation of pregestational female population screening for DMD gene variants

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Abstract
Pregestational population screening of healthy females for copy number variants in DMD gene has raised numerous challenges regarding the interpretation and disclosure of these findings. Our objective was to analyze data from a local dystrophinopathy patient database, in comparison to population screening results. Utilizing the "Little steps" association registry for children with dystrophinopathy, we classified genetic findings (out-of-frame, in-frame, or difficult-to-predict) in 231 DMD and 90 BMD male patients. A comparison was made with a previously published cohort of 162 female carriers identified through population screening. Duplications classified as "difficult to predict" were absent in DMD/BMD patients, as opposed to 45.1% of women analyzed in the scope of population screening ($p < 0.0001$). While the distribution of deletions did not differ between the groups, significantly higher proportion of duplications initiated at the proximal hot spot in the DMD/BMD cohort (87.1%), vs. only 11.7% in women analyzed through population screening ($p = 0.0038$). Notably, duplications initiating in the dp427c promoter area were noted only in the latter cohort ($n = 62$). Local databases of dystrophinopathy patients can facilitate analysis and reporting of pregestational female population screening results. These conclusions facilitate future introductions of population screening genetic tests for diseases with variable presentation.

Keywords Dystrophinopathy · MLPA · Population screening

Introduction

Dystrophinopathies, encompassing Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), refer to a cluster of genetic disorders associated with genetic variants in the dystrophin encoding *DMD* gene [1]. DMD and BMD are mainly characterized by progressive symmetric muscle weakness with later cardiac and respiratory involvement, exerting a severe impact on overall health and leading to premature mortality. DMD is primarily caused by variants that disrupt the open reading frame, while the less severe BMD is caused by variants that maintain the open reading frame. Since DMD affects approximately 1 in every 3,500-5,000 male births, Multiplex ligation-dependent probe amplification (MLPA) genetic testing for DMD deletions and duplications was recently introduced into national population screening program for reproductive purposes [2]. However, notable challenges in the interpretation of screening results were promptly identified. Main reasons for these challenges were the rich diversity of genetic variants in the 79-exon *DMD* gene, the variable penetrance and expressivity, as well as false-positive MLPA results due to underlying sequence variants. While a framing rule may facilitate interpretation, it does not always capture the full spectrum of genetic alterations associated with dystrophinopathies [3]. According to a recently published manuscript, representing 85,737 MLPA testing results of first year implementation of pan-ethnic screening testing for dystrophinopathy, of the

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162 deletions and duplications in the dystrophin gene, 82 (one in 1,072 tests) were annotated as "difficult to predict" by the available reading frame checkers [4]. In addition, 373 cases (one in 230 tests) with single exon deletions were determined to be false positives resulting from underlying single nucleotide variants. The study discussed numerous challenges, primarily stemming from the limited available information regarding the precise risk of severe early onset disability and other health consequences in many cases with true-positive abnormal MLPA results.

The complexities of dystrophinopathy screening extend further to the realm of population-specific variations. Genetic diversity and the prevalence of specific mutations differ significantly among various demographic and ethnic groups [5–7]. These variations can significantly influence the effectiveness of screening and diagnostic strategies, highlighting the importance of considering population-specific factors in the context of dystrophinopathy screening.

In Israel, a country with a diverse and multi-ethnic population, comprehensive data on dystrophinopathy screening outcomes are notably scarce [4, 8]. Thus, the purpose of the current study was to examine a large local database of dystrophinopathy patients and compare these findings with the data of pregestational population screening.

Methods

Patient-specific data concerning individuals diagnosed with dystrophinopathies and women with familial history of dystrophinopathy was retrieved from a database maintained by a non-profit organization, namely the "Little Steps Association" registry https://www.littlesteps.org.il/eng/. This assoc iation plays a pivotal role in offering support and guidance to individuals and families grappling with the challenges of dystrophinopathy. DMD and BMD patients, as well as female carriers examined due to positive family history, voluntarily register with the organization, providing extensive personal and medical information.

Data acquired for this study encompassed various clinical characteristics, including age, prevalence of autism spectrum disorder, inability to walk, walking aid assistance, age of absolute walking aid dependance, cardiac involvement, need for respiratory support, scoliosis, and need for scoliosis surgery. In addition, the data included the results of genetic testing, also based on patients' reports.

The results of the genetic testing were categorized into three distinct groups: deletions, duplications, or point variants. The deletions and duplications were defined as out-of-frame/in-frame/difficult to predict according to Leiden Open Variation Database v.3.0 (LOVD, https://www.lovd.nl/). In case no reports were included in Leiden Muscular Dystrophy pages (https://www.dmd.nl/) involving difficul t-to-predict duplications, additional searches in the UMD-DMD France database (http://www.umd.be/DMD/W_DMD/index.html) as well as PubMed (https://pubmed.ncbi.nlm.nih.gov/) were performed.

Data of national pan-ethnic population screening testing for reproductive purposes was retrieved from a recently published manuscript, representing MLPA testing results of 85,737 healthy females [4]. The rate and location of deletions and duplications were compared between the group of patients with dystrophinopathy to that of healthy female carriers.

Categorical variables were expressed as counts (percentages) and analyzed using Fisher's exact test. Continuous variables were presented as means ± standard deviations and assessed through Student's t-test, while ordinal variables were represented as medians (interquartile range, 25th percentile to 75th percentile) and evaluated using the Mann-Whitney test. Statistical significance was defined as a p-value below 0.05.

Results

A total of 464 patients from Little Steps registry were included in the study – 231 male patients with DMD, 90 male patients with BMD, and 143 female carriers diagnosed due to family history of DMD/BMD. Clinical characteristics of the male patients are presented in Table 1. As anticipated, compared to BMD patients, DMD patients were younger, had a greater need for walking aid assistance, a younger age at which they became completely dependent on walking aids, and higher rates of requiring respiratory support, including full respiratory support. Among the 143 female carriers, no cardiac disease was reported, while 6 (4.2%) reported motor difficulties manifesting as pain and/or fatigue.

The analysis of cases with known genetic diagnoses is detailed in Table 2. Notably, genetic diagnoses were not noted in 9 cases of DMD, 15 cases of BMD, and 10 cases of female carriers. Within the cohort of DMD/ BMD cases with established genetic diagnoses, the majority (80.9%) exhibited exon deletions or duplications (78.4% for DMD and 88.8% for BMD). Point mutations accounted for 19.1% of the cases, with 21.6% in DMD and 12.0% in BMD. No statistically significant differences were observed in these parameters among the analyzed cohorts.

A comprehensive analysis of genetic variants within the examined cohort and a previously published cohort of women screened for DMD/BMD is depicted in Fig. 1. Notably, out-of-frame deletions were identified in 61.9% of DMD patients, whereas none were observed in BMD cases

Table 1 Clinical characteristics of patients with dystrophinopathies

	Overall ($n=321$)	DMD ($n=231$)	BMD ($n=90$)	
Age (years)	16.5 (11.1–23.5)	15.1 (10.3–21.2)	20.6 (14.4–27.5)	0.0114
Deceased	10 (3.1)	9 (3.9)	1 (1.1)	ns
Autism	19 (5.9)	15 (6.5)	4 (4.4)	ns
Inability to walk	46 (14.2)	36 (15.6)	10 (11.1)	ns
Walking aid assistance	181 (56.0)	148 (64.1)	31 (34.4)	<0.0001
Age of absolute walking aid dependance (years)	11 (9–13)	11 (9–12) $n=78$	14 (12-15.75) $n=10$	0.00386
Cardiac involvement	60 (18.6)	46 (19.9)	14 (15.6)	ns
Need for respiratory support	53 (16.4)	48 (20.8)	5 (5.6)	0.0007
Need for full respiratory support	22 (6.8)	21 (9.1)	1 (1.1)	0.0116
Scoliosis	41 (12.7)	32 (13.9)	9 (10.0)	ns
Need for kyphosis surgery	22 (6.8)	19 (8.2)	3 (3.3)	ns
Muscle biopsy	112 (34.7)	76 (32.9)	37 (41.1)	ns

ns – not significant

Categorical variables are expressed as counts (percentages), and ordinal variables are represented as medians (interquartile range, 25th percentile to 75th percentile)

Table 2 Genetic test results of the examined cohort

	Overall ($n=430$)	DMD ($n=222$)	BMD ($n=75$)	Female carriers ($n=133$)
Deletions	304 (70.7)	149 (67.1)	59 (78.7)	96 (72.2)
Duplications	44 (10.2)	25 (11.3)	7 (9.3)	12 (9.0)
Overall deletions + duplications	348 (80.9)	174 (78.4)	66 (88.8)	108 (81.2)
Point mutations	82 (19.1)	48 (21.6)	9 (12.0)	25 (18.8)

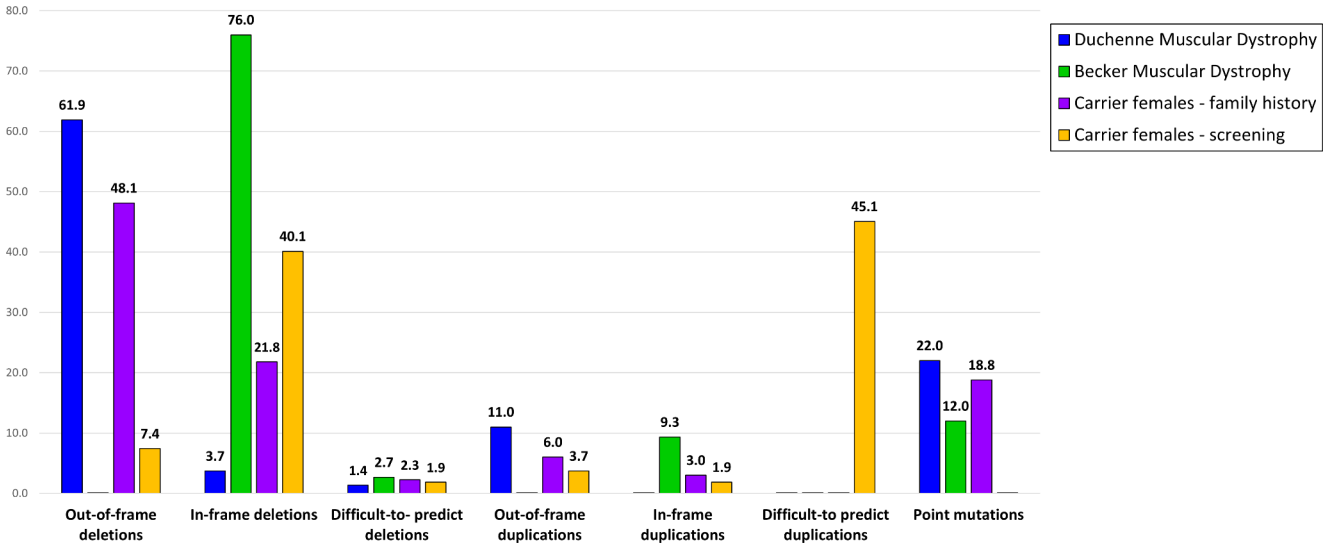

Fig. 1 The distribution of genetic testing results (in percentages), including the impact of deletions and duplications on the reading frame, in the cohort of individuals with personal/familial dystrophinopathy vs. those diagnosed through population screening

($p<0.0001$). Similarly, out-of-frame duplications were found in 11.0% of DMD patients, with none detected in BMD cases ($p=0.001$).

Conversely, in-frame deletions were prevalent in 76.0% of BMD patients, compared to 3.7% of DMD patients ($p<0.0001$). In-frame duplications were noted in 9.3% of BMD patients, but none were observed in the DMD cases ($p<0.0001$). Intriguingly, duplications categorized as "difficult to predict" were absent in both DMD/BMD patients and female carriers analyzed due to a family history of dystrophinopathy, as opposed to 45.1% of women analyzed through population screening ($p<0.01$).

Both deletions and duplications displayed a nonrandom distribution with two distinct hot spots (Fig. 2). Among 205

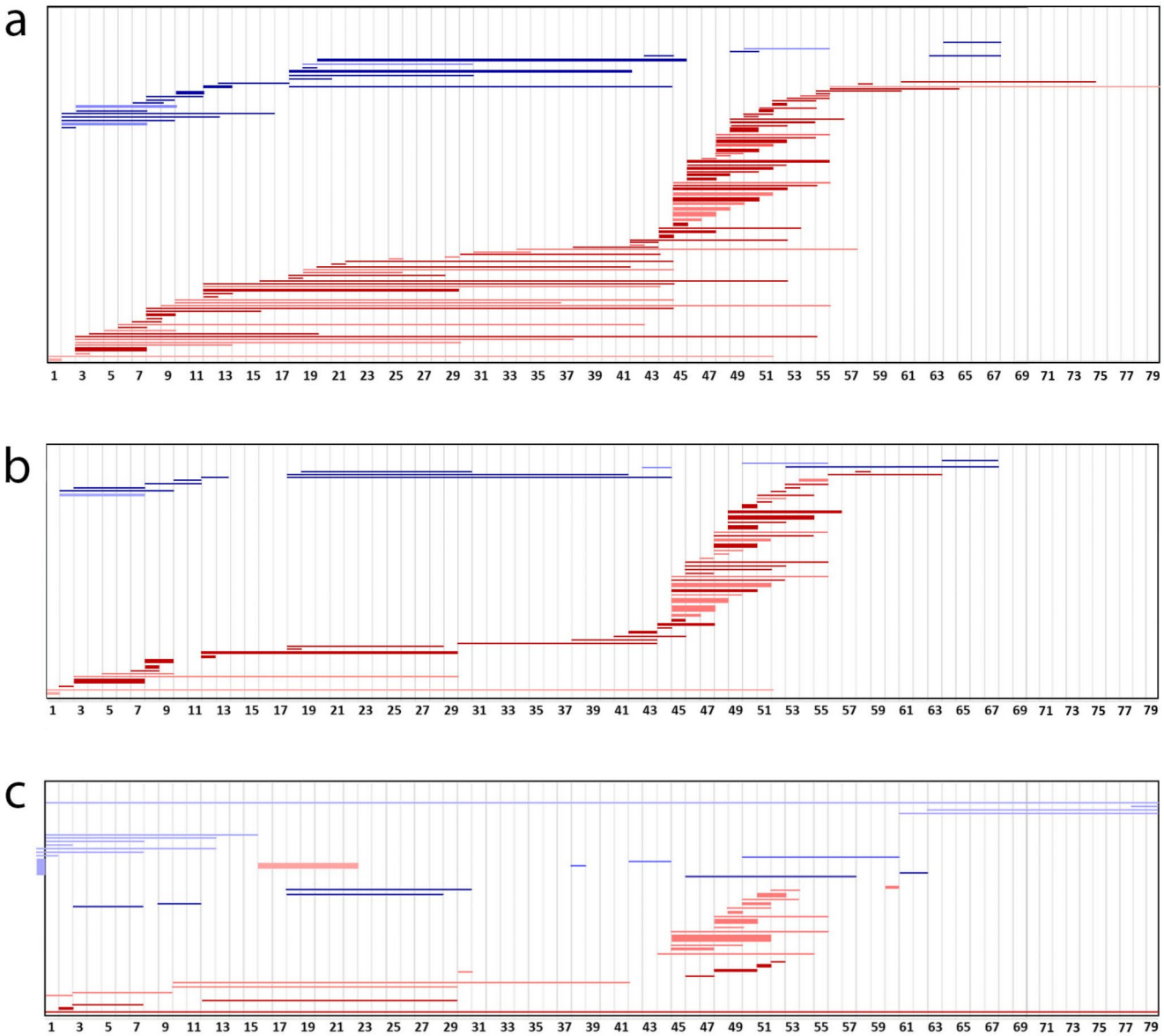

Fig. 2 The distribution of deletions and duplications including the reading frame effect in the cohort of personal (**a**) and familial (**b**) dystrophinopathy vs. diagnosis through population screening (**c**) Figure annotation: Red indicates deletions, while blue represents duplications. Dark colors signify out-of-frame mutations, medium colors indicate in-frame mutations, and light colors denote mutations that are challenging to predict. The variation in bar thickness reflects the number of similar cases

deletions with documented exon locations in DMD/BMD cohort, 135 (65.8%) commenced between introns 43–55, which corresponds to the well-known distal hot spot. Meanwhile, 53 deletions (25.8%) initiated between introns 1–20, representing the proximal hot spot. Similar proportions were observed for the 96 deletions in the cohort of women diagnosed due to a family history of dystrophinopathy, with 63 (66.3%) beginning in the distal hot spot and 26 (27.1%) in the proximal hot spot. The numbers did not differ for the 77 deletions in the cohort of women analyzed through population screening, as 59 (76.6%) started in the distal hot spot, and 15 (19.4%) in the proximal hot spot.

Contrasting patterns emerged for duplications: the majority of duplications in the DMD/BMD cohort and among women analyzed due to a family history of dystrophinopathy initiated in the proximal hot spot, constituting 27 out of 31 (87.1%) and 9 out of 12 (75.0%), respectively. This was significantly higher compared to the cohort of women analyzed through population screening, where only 10 out of the 85 duplications (11.7%) began in the proximal hot spot ($p = 0.0038$). Notably, duplications initiating in the dp427c promoter area were noted only in the latter cohort ($n = 62$). As the Dp427c is located at the promoter, while the commonly referred-to proximal hotspot typically encompasses

exons 1–20, Dp427c duplications not involving any DMD exons were considered as distinct genetic variants.

Discussion

In accordance with previous studies, deletions and duplications in the dystrophin gene were identified in approximately 80% of individuals with DMD/BMD, predominantly clustering in both the proximal and distal regions of the gene [5–8]. Nevertheless, the distribution and the predicted effect on the reading frame of these genetic changes detected among patients with dystrophinopathy compared to women analyzed through population screening offers valuable insights for the interpretation and reporting of DMD MLPA testing results in the context of national population screening programs.

First, as expected, out-of-frame variants were more strongly associated with clinical DMD than with BMD, while in-frame duplications and deletions in the dystrophin gene were predominantly linked to BMD. As BMD is linked to later-onset morbidity compared to DMD, these finding prompt an intriguing question regarding the necessity of reporting in frame variants in the context of pre-pregnancy population screening, which aims to detect severe disorders with significant childhood morbidity and/or mortality. However, our data revealed a substantial degree of functional impairment among BMD patients, one-quarter developing absolute walking aid dependance at a median age of 14 years (12-15.75 years). These findings highlight the considerable adverse impact of BMD on patients' daily functioning, emphasizing the need for ongoing monitoring and support for this population. In addition, clear distinction between DMD and BMD is not always possible, supporting the reporting of DMD duplications in the scope of pre-pregnancy population screening.

The pathogenic variants mostly followed the reading frame rule, with few exceptions. No out-of-frame variants were found in BMD patients. The in-frame deletions associated with DMD were deletions of exon 3–13, exon 3–29 (2), exon 3–37, exon 6–42, exon 9–55, exon 25 and exon 31–34 [3]. It is known that large in-frame deletions (deletion of exon 3–37, 6–42 and 9–55) can result in DMD, as all actin-binding domains are deleted and/or too much of the central rod domain is deleted for the dystrophin protein to be functional. As such one would expect DMD rather than BMD for these deletions. Furthermore, in-frame deletions in the beginning of the gene (deletion of exon 3–13 and 3–29) generally result in more severe BMD. Notably, these deletions are longer and remove not only the actin binding domain but also the first hinge and spectrin repeat domains, which may be more crucial for protein function and stability. Furthermore, it is known that in-frame deletions removing the N-terminal actin binding domains lead to more severe BMD and with improved care, the distinction between severe BMD and milder DMD is becoming blurred.

The deletions of exon 25 and exon 31–34 remove small parts of the central rod domain. These deletions would be expected to result in BMD. However, deletions are detected on DNA and it is possible that out-of-frame transcripts are produced by cryptic splicing events where part of an exon is not included into the transcript, e.g. because of the location of the intronic deletion breakpoints in close proximity to an exon. Without mRNA and protein analysis it is unknown whether these individuals make dystrophin or not. However, this analysis would involve a muscle biopsy, which is an invasive procedure that has an impact on patients and families [9]. It would provide knowledge about dystrophin presence or absence and satisfy scientific curiosity but would not influence the disease progression in any way.

An additional interesting finding emerged in our study regarding duplications classified as "difficult to predict" according to the LOVD database. These elusive genetic variations were notably absent in the cohort of individuals with personal or familial BMD/DMD. In stark contrast, a relatively high proportion of such genetic findings was observed in the cohort of healthy women diagnosed as carriers through population screening [4]. This observation raises a pertinent issue concerning the reporting of these "difficult to predict" duplications in the context of healthy female population screening for reproductive purposes. The results of our study support the notion of not reporting these particular genetic changes in the scope of population screening, as they are likely not pathogenic. Nevertheless, this conclusion should be applied with caution, since no functional tests were performed on the healthy women in the screening cohort, such as CK levels, echocardiography, MRI assessments, or mRNA analysis, highlighting the need for further studies.

Conversely, deletions annotated as "difficult to predict" according to the LOVD were detected in both the DMD and BMD populations. This underscores the importance of exercising caution by reporting and recommending familial segregation in cases involving such deletions when detected in the scope of population screening.

Subsequent to our study, the findings were presented and deliberated upon during a meeting of the Israeli Society of Medical Genetics. A collective decision was reached, indicating that duplications annotated as "difficult to predict" and located in the initial or terminal regions of the gene, especially those involving the promoter region, would not be reported to women tested through the national population screening.

It is essential to acknowledge certain limitations in our study. First, our data relies on patient self-reporting of genetic testing results, which might have introduced a level of subjectivity and potential recall bias. Furthermore, the specific point mutation data was mainly lacking; nonetheless, this data has limited relevance in the context of population screening, as the screening process for DMD/BMD in authors' country primarily relies on MLPA testing. Therefore, the availability of detailed point mutation information had a relatively lower impact on the specific focus of our study. In addition, that the prediction regarding DMD duplications was made under the assumption that these duplications were in tandem, without any confirmatory studies, such as mRNA sequencing.

Finally, our study mainly focused on male dystrophinopathy. It is known that females carrying a pathogenic variant on one allele can have symptoms as well (female dystrophinopathy) and have an increased risk for developing cardiomyopathy [10]. This aspect may be the topic of future efforts.

In summary, our study demonstrates the potential utility of local databases of dystrophinopathy patients in streamlining the analysis and reporting of pregestational female population screening results. Notably, the absence of "difficult-to-predict" duplications in our local DMD/BMD database suggests that these genetic changes should likely not be disclosed within the context of population screening. These findings hold significance in the ongoing effort to refine the reporting of genetic screening results.

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Author contributions Sagi-Dain Lena conceived and designed the work that led to the submission, drafted the manuscript, and approved the final version. Singer Amihood conceived and designed the work that led to the submission, acquired data, revised the manuscript, and approved the final version. Echar Moran and Grinshpun-Cohen Julia acquired data, revised the manuscript, and approved the final version. Aartsma-Rus Annemieke played an important role in interpreting the results, revised the manuscript, and approved the final version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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