Original research

High frequency of BRCA recurrent mutations in a consecutive series of unselected ovarian cancer patients

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Abstract

Hereditary predisposition to breast and ovarian cancer (HBOC) is diagnosed by molecular analysis of deleterious mutations in BRCA genes, allowing oncogenetic follow-up of patients and of their families. BRCA testing addresses only to HBOC families, using restrictive inclusion criteria based on familial history of cancer and age at diagnosis. Sporadic ovarian cancer has high incidence and mortality in Romania, with low median age of diagnosis and possibly a higher magnitude of hereditary contribution comparing to othe populations. However, sporadic ovarian cancers do not qualify for BRCA testing according to inclusion criteria, and a complete BRCA screening of all cancers is neither feasible nor recommended. Despite the large diversity of BRCA mutations worldwide, some recurrent mutations have higher frequencies in diverse populations. Precisely screening for recurrent mutations in a target population allows to rapidly identifying mutation carriers without sequencing the entire BRCA genes. In Romanian population and neighboring countries, several recurrent mutations have already been described. In a consecutive series of 50 sporadic ovarian cancer patients, not qualifying for BRCA complete testing, we screened for 9 most common BRCA mutations, by multiplex-PCR, RFLP and targeted Sanger sequencing. Our results revealed 6 different BRCA mutations in 8 unrelated patients, with a frequency of 16%, much higher than expected. We further recommend screening for the identified mutations in larger series of cancer patients. The results are highly beneficial to cancer patients, healthy relatives, and overall, considering prevention in cancer a priority, to public health system and future of oncogenetics in Romania

Keywords: *ovarian cancer, hereditary predisposition, BRCA genes, recurrent mutations*

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Introduction

Ovarian cancer belongs to top 10 cancer cases in women worldwide (1, 2) with increasing incidence and mortality in central-eastern Europe during the last decades (2, 3). In Romania, OC is following this ascending trend, with 13.9/100.000 incidence and 7.3/100.000 mortality (1). The lifetime risk of OC in general population varies between 1.3 and 1.8% (4-6), and the majority of risk factors have been described in the last decades (7).

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Family history of breast and/or ovarian cancer is the strongest risk factor for OC (8), especially when first degree relatives are affected (9) or when Hereditary Breast and ovarian cancer Syndrome (HBOC) or Lynch syndrome are present in the family (10, 11). While up to 80% of all OC cases are sporadic, some others involve hereditary predisposition, the main genes involved being the tumor suppressors *BRCA1* and *BRCA2* (12). Lifetime risk of OC is significantly higher in *BRCA* mutation carriers comparing to general population, the overall estimation being a 50-fold raise (13). Therefore, the hereditary factor is the only risk factor with positive predictive value justifying medical and oncogenetic follow-up. Lifetime risk of OC is estimated at 40% for *BRCA1* mutation carriers and less than 20% for *BRCA2* carriers (14).

It is known that taken together, BRCA deleterious mutations are responsible for about 1/3 of HBOC families in heterogeneous and up to 80% in isolated western populations (15). In less developed countries, where incidence of OC is rising but still lower, the proportion of cases due to hereditary predisposition could be even higher. Also, the hereditary factor could be much more involved in sporadic OC cases outside family syndromes such as HBOC or Lynch. Less than 0.2% of the general population is thought to carry a deleterious *BRCA* mutation, while the proportion of carriers is estimated up to 10% in sporadic breast or ovarian cancer cases (16).

BRCA testing is counseled today in HBOC cases according to a familial scoring by BRCAPRO, BOADICEA, Manchester or other risk evaluation algorithms (17-20). However, mutations are generally detected in about ½ of the tested families, while it is estimated that 30% of carriers are not included in testing because of not respecting the inclusion criteria (21-23). Even with emerging NGS technologies, a complete *BRCA* screening of the whole population or even in all cancer cases is not possible. Therefore, modern oncogenetic diagnostic focuses on targeting the mutations, by adapting the diagnostic algorithm to local cases and mutational profiles. There is little evidence of mutational hotspots in both *BRCA1* and *BRCA2*, the main regions involved being the Ovarian Cancer Cluster Regions (OCCR) in exon 11, between nucleotides 2401-4190 in *BRCA1* and 4075-7503 in *BRCA2*, respectively (24). However, in many populations, including Romania, most of the mutations are found outside OCCRs and are scattered over all exons or exonic regions in *BRCA* genes (15, 21). Therefore, the main pre-screening approaches are targeting common and recurrent mutations with higher probability to occur (22,25,26). These may be ethnicity specific founder mutations or population specific recurrent mutations, given each population has a proper mutational profile (27). Previous study demonstrated the higher occurrence of some mutations in the Romanian population (*BRCA1* 5382insC or *BRCA2* c.8680C>T), while some other mutations, very common in neighboring populations, proved to be almost absent in Romania (25, 26-29).

In order to better understand the local *BRCA* mutational profile, but also to extend *BRCA* testing to ovarian cancer patients not qualifying for oncogenetic diagnostic, we chose 9 most common *BRCA* mutations to be screened for in a consecutive series of 50 ovarian cancer cases not respecting oncogenetic inclusion criteria.

Patients and methods

Patients

Ovarian cancer patients were recruited at the Oncology Institute of Iași, Romania, patients agreeing to participate by written informed consent. This study was approved by the local Ethical Committee, UMF Iasi. No participant declared significant family cancer history to qualify for diagnostic through Eisinger (20) or Mancester (19) scores.

Molecular analysis

Genomic DNA was extracted from 3 ml peripheral EDTA-collected blood using Wizard™ Genomic DNA purification kit (Promega™ Inc, Madison, WI, USA). DNA was quantitatively and qualitatively evaluated by spectrophotometry.

BRCA1 5382insC was screened by allele-specific multiplex PCR, using a common reverse primer, one forward wild-type specific and one forward mutation specific primer, as previously reported (25). *BRCA2* 8908C>T was screened by restriction fragment length polymorphism (RFLP), with a differential digestion of wild-type and mutant amplicons by TaaI digestion enzyme, as previously reported (26). For all mutations, respective exons or exonic regions were sequenced by Sanger dideoxy sequencing. PCR was performed in 20 μl reaction, containing one unit ApliTaq® Polymerase with appropriate Buffer (Applied Biosystems™ Inc, Foster City, CA, USA), 0.4 mM each dNTP, 0.4 μM of each primer, 100 ng genomic DNA. Primers were designed using Primer-BLAST and Primer3 freeware, to flank exonic regions and exon/intron boundaries. Amplicons were evaluated through 1,5% agarose gel electrophoresis and purified by ExoSAP-IT™ (ThermoFisher), following provider's instructions. Sequencing reactions were performed on forward strand, using the BigDye® Terminator Cycle Sequencing Kit (ThermoFisher) and purified with BigDye XTerminator™ Purification Kit (ThermoFisher), then migrated by capillary electrophoresis on an Life Technologies 3500 Series Genetic Analyzer (ThermoFisher). For mutation verification, both forward and reverse strands were sequenced on a second independent sample. Sequence alignment and data analysis were performed using Seqman® (DNA Star™ Inc, Madison, WI, USA) and Variant Reporter™ Software v2.0 (ThermoFisher).

Data interpretation and analysis

All mutations and sequence variants are described according to the recommendations from the Human Genome Variation Society (HGVS), with first nucleotide of DNA numbering being the A from initiator translated ATG (HGVS). We used reference sequences NG_005905.2, NM_007294.3 and NP_009225.1 for *BRCA1* and NG_012772.3, NM_000059.3, NP_000050.2 for *BRCA2*. For known BRCA mutations, usual Breast Information Core (BIC) database nomenclature is also employed. For bioinformatics prediction of variants, Alamut® (Interactive Software™) was used.

Results

Our cases consisted in 50 ovarian cancer patients as a consecutive series, unselected for family history of cancer or age at diagnostic. Briefly, age at diagnosis ranged between 21 and 76 years, with a mean of 55 years, most of the patients being diagnosed between 40 and 60 years. Most of the patients (61%) were from urban areas and had an average level of education. Almost half of the patients were diagnosed with the serous histological type, but mucinous, mixed and endometrial histological types were represented as well. 16% of our patients declared a family history of cancer, of which 12% had at least one relative with breast and/or ovarian cancer, while 4% declared one relative with gastrointestinal cancer. None of the patients qualified for molecular BRCA testing through high Eisinger or Manchester scores.

Mutation analysis

A total of 9 mutations were screened for in our patients. A synthesis of our results can be found in Table 1. Overall, the mutation frequency was 16%, with a total of 6/9 different mutations identified in 8/50 patients. The clinical and histo-

Table 1. BRCA mutations searched for in this study and their respective occurrence

GENE	Exon	cdot (NM_007294.4)	pdot (NP_009225.1)	Complete pdot	dbSNP	BIC	gdot (NG_005905.2)	Occurrences
BRCA1	2	c.68_69delAG	p.Glu23fs	p.Glu23Valfs*17	rs80357914	185delAG	g.93953_93954del	1
BRCA1	5	c.181T>G	p.Cys61Gly	p.Cys61Gly	rs28897672	300T>G	g.111497T>G	1
BRCA1	11-3	c.1687C>T	p.Gln563Ter	p.Gln563Ter	rs80356898	1806C>T	g.124140C>T	1
BRCA1	11-9	c.4035delA	p.Glu1346fs	p.Glu1346Lysfs*20	rs80357711	4154delA	g.126488del	0
BRCA1	20	c.5266dupC	p.Gln1756fs	p.Gln1756Profs*74	rs80357906	5382insC	g.160921dup	2
GENE	Exon	cdot (NM_000059.3)	pdot (NP_000050.2)	Complete pdot	dbSNP	BIC	gdot (NG_012772.3)	Occurrences
BRCA2	11-13	c.5946delT	p.Ser1982fs	p.Ser1982Argfs*22	rs80359550	6174delT	g.29822del	2
BRCA2	21	c.8680C>T	p.Gln2894Ter	p.Gln2894Ter	rs397508002	8908C>T	g.66238C>T	0
BRCA2	23	c.9097dupA	p.Thr3033fs	p.Thr3033Asnfs*11	rs397507419	9326insA	g.69414dup	0
BRCA2	25	c.9371A>T	p.Asn3124Ile	p.Asn3124Ile	rs28897759	9599A>T	g.84324A>T	1

Table 2. Clinical and histopathological data of the ovarian cancer patients carrying causative BRCA variants

Patient code	Age at diagnostic	Ovarian neoplasm localization	Histopathological type	Variant
OVR020	62	Right	Signet ring cell carcinoma	BRCA2 6174delT
OVR054	67	Left	Invasive mucinous carcinoma	BRCA2 6174delT
OVR055	57	Left	High grade serous carcinoma	BRCA1 5382insC
OVR058	38	Bilateral	Mixed epithelial-stromal carcinoma	BRCA1 5382insC
OVR062	60	Left	Papillary serous adenocarcinoma	BRCA1 300T>G
OVR079	55	Bilateral	High grade serous carcinoma	BRCA2 9599A>T
OVR083	43	Bilateral	High grade serous carcinoma	BRCA1 185delAG
OVR094	47	Right	Papillary serous chistadenocarcinoma	BRCA1 1806C>T

pathological data of the ovarian cancer patients carrying causative BRCA variants is presented in Table 2.

BRCA1 5382insC, the most common mutation in our population and elsewhere, was firstly pre-screened by allele-specific multiplex PCR, and then positive samples were sequenced on *BRCA1* exon 20 as a 259 bp amplicon. As expected, this mutation was detected in 2 different unrelated patients. Both were diagnosed with high grade serous carcinoma, at 59 respectively 41 years old. Both reported poor family history including only lung cancer isolated cases, and both declared multiple births and lactation.

BRCA2 6174delT, the most common *BRCA2* mutation in neighboring populations, but previously not identified in Romania, was screened for by sequencing a 483-bp amplicon within *BRCA2* exon 11. Partly surprising, this mutation was identified, for the first time in our population, in two distinct unrelated patients, aged 67 and 69 respectively, both declaring total absence of family cancer history, multiple births and lactation, and both diagnosed with ovarian carcinoma with ring seal cells.

BRCA2 c.8680C>T, previously identified in multiple Romanian families, was firstly screened by the in-house developed PCR-RFLP (26), but it was not identified in the present study.

BRCA1 300T>G is another previously reported mutation in our population, located in *BRCA1* exon 5 and sequenced as a 278-bp amplicon. This mutation was also identified in the present study, in a 49 year-old patient with some family cancer history, no births but multiple abortions.

For *BRCA1* 185delAG identification, we sequenced exon 2 of *BRCA1* gene, as a 338 bp amplicon. *BRCA1* 185delAG was identified here for the first time in a Romanian population, in a 49 year-old patient diagnosed with bilateral serous ovarian cancer, declaring one first-degree relative with lung cancer and one third-degree possible relative with breast cancer. The patient had no births, abortions or miscarriages.

Two mutations were searched for in different regions of *BRCA1* exon 11. The Gln563Ter (c.1687C>T), within the third 510-bp amplicon of exon 11, was identified in a 53-year-old patient with third-degree possible relatives with cancer, few births, and abortions. The p.Glu1346fs (c.4035delA), in the eighth region of exon 11, was not identified in the present study.

A quite common *BRCA2* mutation, p.Thr3033fs, located in *BRCA2* exon 23 and originated from a c.9097dupA duplication, was searched for by sequencing a 380-bp amplicon, it but was not found in any of our patients.

The last mutation screened for was the previously identified *BRCA2* c.9371A>T (p.Asn3124Ile), within a 406-bp amplicon in *BRCA2* exon 25. Similarly with the first identification of this mutation, the patient was relatively young (age 55), and declared only colon cancer family history, no births, abortions or miscarriages.

In Figure 1 (A-F), the identified mutations appear as double peaks on the electropherogram, representing heterozygous sequence.

Discussion

The *BRCA* mutation frequency was much higher than expected for a consecutive series of cancer cases. While in familial selected HBOC cases an expected frequency is below 50% (21), less than 0,2% of the general population is thought to carry a deleterious *BRCA* mutation, while the proportion of carriers is estimated up to 10% in sporadic breast or ovarian cancer cases (16). A previous study on 1,342 unselected ovarian cancer patients in Ontario revealed a 13% mutation frequency which includes large genomic rearrangements (30). Therefore, the present study reveals a surprisingly high mutation frequency among Romanian ovarian cancer cases.

As expected, *BRCA1* 5382insC was again identified in Romanian population, and even twice. Although some previous results on 170 consec-
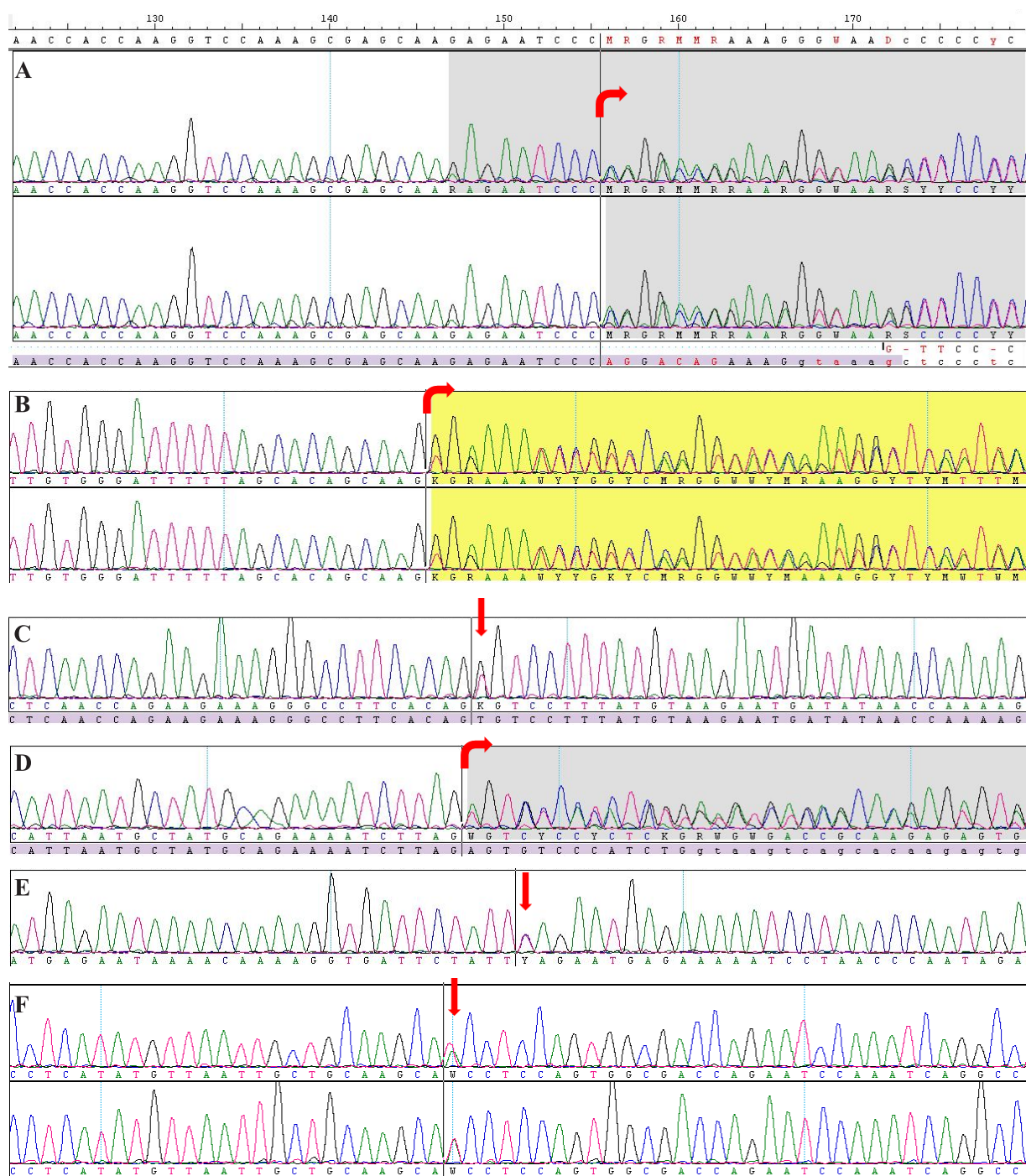
Fig. 1. The BRCA mutations identified in the present study (A-BRCA1 5382insC; B-BRCA2 6174delT; C-BRCA1 300T>G; D-BRCA1 185delAG; E-BRCA1 1806C>T; F-BRCA2 9599A>T). The arrows indicate the precise localization of each mutation.

utive breast cancer cases did not reveal the presence of 5382insC (22), this Ashkenazi founder mutation proved to have significant distribution in Eastern European populations, including our country (21, 25, 27-29, 31-36); our database is already counting today 6 unrelated 5382insC carriers. It could mean that 5382insC is very common in family and ovarian cancer sporadic cases, while not being frequent at all in sporadic breast cancer patients.

The other common founder eastern European mutations are known as *BRCA1* 185delAG and *BRCA2* 6174delT (27, 31-36). While none of these recurrent mutations have been previously identified in Romanian population, our study revealed for the first time the presence of 185delAG, and also twice the presence of *BRCA2* 6174delT. Taken together, such results bring Romanian population much more in the "normal" regional genetic context, comparing to what it was initially believed.

Other good candidates for recurrence were the previously identified *BRCA1* 300T>G and *BRCA2* c.8680C>T, the former being also recurrent in neighboring populations, while the latter seemed more like a local candidate. In this study, only *BRCA1* 300T>G was identified (for the third time in our population), but not *BRCA2* c.8680C>T. One interesting fact is that previously, *BRCA2* c.8680C>T had rather been identified in Romanian breast cancer cases (26), while it looks more rare among ovarian cancer cases.

Apart from the 5 recurrent mutations mentioned above, we chose 4 other possible candidates for appearing in our population. *BRCA1* c.1687C>T (p.Gln563Ter) represents a common mutation prevalent in other European countries, especially in Slovenia, Austria and Sweden, with a common founder ancestor of those 3 populations (27). We also found this mutation in our study. *BRCA1* c.4035delA is one of the most common *BRCA* mutations found in Poland and the Baltic countries, being detected in 16.3% of unrelated

Baltic ovarian cancer patients (37). Surprisingly, this mutation has neither been previously identified in Romania, nor in the present study. *BRCA2* c.9097dupA is the most common *BRCA2* mutation in the Turkish population, being very frequent in eastern populations (38). We did not find it in our population. We did find another *BRCA2* mutation, c.9371A>T (p.Asn3124Ile), previously identified in Romania but not very frequent in the populations around.

Overall, the observed mutational profile is much alike the expected one for our population, with a higher mutation frequency than expected. *BRCA1* 185delAG, 300T>G and 5382insC, as well as *BRCA2* 6174delT make a strong batch of essential-to-screen priority mutations for any further study. We would add, according to this study, *BRCA1* c.1687C>T and *BRCA2* c.9371A>T, as well as *BRCA2* c.8680C>T, although this last one is more likely to appear in breast cancer patients. Other mutations usually appearing in neighboring countries, such as *BRCA2* c.9097dupA for Eastern and *BRCA1* c.4035delA for Western neighbors, look more alike population-specific variants, and may not be included in routine *BRCA* screening.

BRCA testing is essential for saving lives, for longer and higher quality of life in breast and ovarian cancer patients. Obviously, extending the molecular testing would benefit the whole population and the health system as well (39-41). Our study proposed an evidenced shortcut for applying oncogenetic diagnostic to larger series of patients than those being currently included in HBOC testing. Better knowing the mutational profile in Romania helps improving the mutation detection strategy. But also, knowing that mutation frequency is higher than expected among the Romanian ovarian cancer cases brings again the question of *BRCA* testing all ovarian cancers. We highly recommend this to be implemented.

Although our study reveals a high frequency of recurrent *BRCA* mutations in unselected ovarian

cancer patients, therefore proving the necessity of testing all ovarian cancer cases, the image of local mutation landscape is still limited by a relative small number of cases. This might be the explanation for some discrepancies between this study and previous ones. As studies in Romanian population will continue with much larger groups of familial and non-familial patients, a clearer mutational profile will be designed, and the diagnostic algorithm will be consequently adapted.

Conclusions

An efficient *BRCA* mutation detection strategy in Romanian patients should include a pre-screening step for identifying a series of mutations with higher probability to appear. According to our study, *BRCA1* 185delAG, 300T>G, 5382insC and c.1687C>T, as well as *BRCA2* 6174delT, c.9371A>T, and c.8680C>T are the 7 mutations firstly to be included in a pre-screening test. We also highly recommend testing all ovarian cancer patients in Romania.

Abbreviations

BRCA – Breast cancer genes
HBOC – Hereditary breast and ovarian cancer
HGVS – Human Genome Variation Society
NGS – Next generation sequencing
OC – Ovarian cancer
OCCR – Ovarian Cancer Cluster Region
PCR – Polymerase chain reaction
RFLP – Restriction fragment length polymorphism
UMF – University of Medicine and Pharmacy

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Authors's contributions

AC (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Validation; Writing – original draft)
LN (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing – review & editing)
RB (Data curation; Investigation; Methodology; Writing – original draft)
AM (Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Writing – original draft)
AN (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Validation; Writing – review & editing)
AC (Investigation; Methodology; Writing – original draft)
CL (Conceptualization; Formal analysis; Project administration; Supervision; Validation; Visualization; Writing – review & editing)

Conflicts of interests

The authors declare no conflict of interests.

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