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COMPARATIVE STUDY OF BRAF MUTATION IN SEROUS TUMORS OF OVARY BY MEANS OF IMMUNOHISTOCHEMISTRY AND REAL TIME POLYMERASE CHAIN REACTION

Pathology

Sharma Garima MBBS, Junior Resident, Dept of Lab Sciences & Mol Med, Army Hospital (Research &Referral) Delhi, India

Ahuja Ankur MD, DM, Assistant Professor, Dept of Lab & Mol Med, Army Hospital (Research &Referral) Delhi, India

Deb Prabal MD, Director Operations, Lab Director & Chief Histopathologist, Clinical Reference Lab, SRL Limited, Goregaon West, Mumbai

BS Sunita MD, DNB, Professor, Dept of Pathology, Armed Forces Medical College, Pune, Maharastra India

Trehan Arti MD, Associate Professor, Dept of Lab & Mol Med, Army Hospital (Research &Referral) Delhi, India

Gahlot Gaurav PS* MD, Associate Professor, Dept of Lab Sciences & Mol Med, Army Hospital (Research &Referral) Delhi, India *Corresponding Author

Joshi Vidushi MD, Graded Specialist Military Hospital Devlali, Maharastra, India]

Singh Beenu DLO, Medical Officer, Base Hospital Delhi Cantt, India

ABSTRACT

Background: The purpose of this study was to correlate the evaluation of BRAF V600E by IHC and RT-PCR to correctly categorize, prognosticate and offer the best possible treatment for the serous ovarian tumors thus contributing an attempt for better patient survival and quality of life.

Aims: To study the expression of BRAF mutation by Immunohistochemistry (IHC) and Real Time Polymerase Chain Reaction (RT-PCR) in various histological grades of serous ovarian tumors

Study design: This cross section observational study was performed in thirty cases of serous ovarian tumors to access expression of BRAF mutation

Subjects & methods: To correlate histomorphological grades of serous ovarian tumors with immunohistochemical and RT-PCR results of BRAF mutation in 30 cases.

Statistical Analysis Used: The correlation between BRAF scoring by IHC & RT-PCR, WHO histological grade and subtype of tumor was assessed using Pearson Chi Square Test with statistically significant p-value < 0.05

Results: This study confirmed the higher frequency of BRAF V600E mutations in benign serous tumors, than in high grade tumors as assessed by both IHC and RT-PCR, however no statistically significant correlation could not established between them. It is therefore suggested that the study to be continued with larger number of cases and as a multicentric project with clinical follow-up to comprehend the different facets of BRAF pathway in ovarian neoplasms.

KEYWORDS

Ovarian serous tumors, Low grade serous carcinoma (LGSC), High grade serous carcinoma (HGSC), BRAF V600E, Immunohistochemistry (IHC), Real Time Polymerase Chain Reaction (RT PCR),

INTRODUCTION:

Ovarian cancer is worldwide second most common gynaecological malignancy in women and in India is the third leading cancer trailing behind cervix and breast cancer with its incidence rates of 5.4-8.0/100,000 populations. [1] 95% of the primary ovarian neoplasm are epithelial in nature with serous carcinomas (30-70%) constituting the most common histopathological subtype followed by endometrioid (10-20%), mucinous (5-20%) and clear cell (3-10%). [2] Positive risk factors for ovarian carcinogenesis include old age, white ethnicity, red meat, excess fat consumption, obesity, perineal talc users, BRCA1, BRCA2 gene mutations and HNPCC/Lynch syndrome whereas negative risk factors are maternal age at last birth, hysterectomy, green leafy vegetables, whole grain bread, pasta, NSAIDs, oral contraceptive users and more parity.

Benign, borderline and malignant serous tumors comprise of 25%, 10-15% and 33% of all serous ovarian tumors presenting at 4th-5th decade, 5th decade and sixth decade respectively. Histomorphologically serous tumors resemble cells of internal lining of the fallopian tube with benign ones (>1cm size with single layer of flattened-to-cuboidal cells with uniform basal nuclei), borderline ones (micropapillae and small nests of cells infiltrating the stroma haphazardly) and malignant ones. Based on several clinicopathological, histomorphological, immunohistochemical and molecular studies, a dualistic, easy reproducible model of ovarian serous carcinogenesis i.e. low grade serous carcinoma LGSC (Type I) and high grade serous carcinoma HGSC (Type II) was suggested. [3-7] Majority of LGSC show micropapillary pattern of tumor cells with scant to moderate amount of eosinophilic cytoplasm, uniform, small, round to oval nuclei, high nuclear: cytoplasmic ratio, small nucleoli and mean mitotic index of 4 mitotic figures/10 high-power fields [6]. HGSC exhibit a papillary or solid growth pattern with tumor cells contain markedly atypical, large pleomorphic nuclei (≥3:1 variation in nuclear size and shape) and frequent mitotic figures. [6,7] HGSC can mimic endometrioid carcinoma. Tumors with irregular luminal contours, large complex papillae lined by stratified epithelium with irregular slit-like patterns, hobnail cells, bizarre tumor giant cells, and psammoma bodies, a diagnosis of HGSC is favored while tumors with solid islands, nests, squamous metaplasia, or a background of atypical proliferative (borderline) endometrioid tumor or endometriosis favors high-grade endometrioid carcinoma. Figure 1]

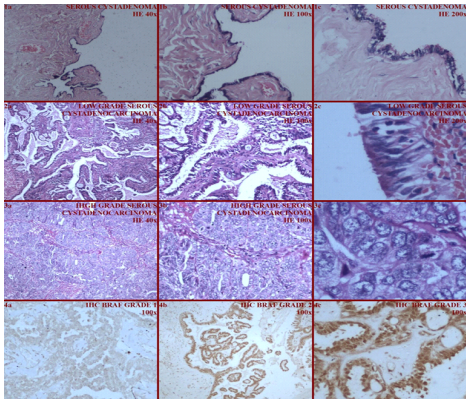

Figure 1a-1c: Serous cystadenoma: H&E stained sections show a cyst lined by flattened epithelium (1a; 40x), cyst lined by low cuboidal cells (1b; 100x), epithelial cells with round to oval nucleus and fine cilia (1c; 200x). Figure 2a-2c: Low Grade Serous Tumor: shows neoplastic cells arranged in papillary pattern with papillae anastomosing with each other (2a; 40x), papillae containing broad fibrous core lined by tumor cells (b; 100x), tumor cells having scanty cytoplasm, small round uniform, round to oval nucleus (2c; 200x). Figure 3a-3c: High Grade Serous Carcinoma: shows complex architecture with tumor cells arranged in sheets and papillae (3a; 40x), tumor cells arranged around thin fibrous core (3b; 100x), low cuboidal cells with high N:Cratio, marked nuclear pleomorphism and frequent mitotic figures including atypical mitosis (3c; 200x). Figure 4a-4c: Grades of immunohi stochemistry staining of BRAF Grade 1- shows some of the tumor cells stained weakly (4a; 100x), Grade 2- most of the tumor cells positive (4b; 100x) and Grade 3- intense cytoplasmic staining in all the tumor cells (4c;100x)

Type I tumors develop as "adenoma-borderline tumor- carcinoma" progression through a dysregulation of RAS–RAF signaling pathway. [8,9]. BRAF (v- Raf murine sarcoma virus oncogene homologue B1) serves as effector of MAPK (mitogen activated protein kinase) signalling cascade, a signal transduction pathway that modulates cellular proliferation. [10-12] In 95% cases T1796A point mutation of BRAF results in substitution of valine to glutamic acid at position 600. [13] Type II tumors develop *de novo* from the surface epithelium and grow in a rapid manner without morphologically identifiable precursor lesions. [14] Mutations of KRAS, BRAF, or ERBB2 are very infrequent in HGSC unlike LGSC. [10,11] In contrast, 80% HGSC show TP53 mutations with up/down regulation of other genes. [7,14] The other genes associated with ovarian carcinogenesis are BRCA1 (70% lifetime risk) and BRCA2 (10% lifetime risk). Somatic loss of BRCA1 and BRCA2 genes were found in 50%–70% and 30-50% of sporadic ovarian carcinomas respectively. In addition EGFR with consecutive activation of AKT by ligands like EGF, TGF are also responsible for ovarian carcinogenesis through its role in tumor invasion, metastasis and angiogenesis. Preusser et al, Grisham et al observed better outcome in LGSC positively stained for BRAF V600E [10,11,15-17]

Immunohistochemical staining for the BRAF V600E mutation-specific monoclonal antibody VE1 can be used to detect BRAF mutation in routinely processed, formalin-fixed and paraffin-embedded (FFPE) tissues. [15,18,19] Its advantages include requirement of minimal tissue, wide availability, economic and easiness of procedure, however in view of 30% false positivity, there is initial need of validation with sequencing. [17] On the other hand, more sensitive DNA RT-PCR based technique is expensive, less available, require sophisticated equipments and more expert manpower. Hence, the current study was undertaken to compare IHC method using VE-1 antibody with RT-PCR method, so that it can be used as specific, sensitive tool and useful screening test for detection of BRAFV600E mutation. [12]

SBTs and LGSC are resistant to chemotherapy therefore further clinical trials to access the effect of inhibitors targeting the MAPK pathway are under investigation. [20] In HGSC, studies (preclinical) demonstrating effect of MAPK pathway inhibition with the compound CI-1040 on tumor cells having BRAF or KRAS mutations showed some clinical potential. [10,11,21,22] The MEK inhibitor selumetinib has been used in a phase II treatment trial for HGSC. [23]

MATERIALS & METHODS:

30 cases of serous tumors of ovary of various histological grades operated at a Tertiary Care Hospital of North India from Jan 2015 to Dec 2017 were included in the study. In the initial screening part H&E (haematoxylin and eosin) stained slides of all the cases were reviewed and the original histopathological diagnoses, viz. tumor type and grade reconfirmed. Evaluation of BRAF was performed by IHC using antibody to BRAF V600E mutation and by RT-PCR.

Immunohistochemistry involves the binding of primary antibody to specific tissue antigens followed by interaction with biotinylated secondary antibody. The streptavidin/horse radish peroxide (HRP) complex is then applied. Streptavidin then attaches to the biotin on the secondary antibody and HRP acts as the indicator enzyme. On addition of a DAB chromogen, a coloured precipitate developed at the tissue antigen sites. The existing standard protocol of IHC procedure of our Department was followed using BRAF V600E mutated colon carcinoma as positive control with each batch. Mouse monoclonal (Spring Bioscience, USA) having immunogen of Synthetic peptide representing the BRAF V600E mutated amino acid sequence from amino acid 596 to 606 (GLATEKSRWSG), clone: VE1, Isotype: MouseIgG2a at dilution of 1:100 was used. BRAF positivity was assessed by visualization of cytoplasmic staining by the distinct brown colour of oxidized DAB. The intensity was graded on the basis of proportion of tumor cells positivity as 0 or negative (10%), 1+ or weak (11-25%), 2+ or moderate (26-50%), 3+ or strong (%) at high power magnification (400x) followed by its analysis with the tumor grade.

Detection of BRAF mutation by RT-PCR was performed by extracting DNA from formalin fixed paraffin embedded tissue using "QIAamp® DNA FFPE Tissue Extraction Kit" following the established standard protocols. RT-PCR was done by "PNAClamp™ BRAF" and "Qiagen BRAF" Mutation Detection Kits and their results/efficacy were compared. PNAClamp detected only most frequent BRAF V600E mutation while Qiagen kit in addition detected other BRAF V600D, V600K and V600R mutations. All experimental runs contain positive and negative controls. Assessment of data was based on "Ct value" i.e. the cycle number at which a signal is detected above background fluorescence. The Ct values of the Clamping control tube Non PNA mix#1 (C1=<30) and for BRAF PNA mix#2 (C2 >33) were accepted. DNA samples with Ct values of 22~35 were considered valid. The validity of the test can be decided by the Ct value of Non PNA mix (S1). [Table 1]

Calculation of ΔCt-1 values was done by subtracting the sample Ct values from the Standard Ct values (35). If the Ct of DNA samples is displayed as NA (not applicable), then the Ct value was set as 38 for further calculation.

*ΔCt-1 = [Standard Ct] – [Sample Ct (S2)] where Standard Ct = 35
**ΔCt-2 = [Sample Ct (S2)] - [Non PNA mix Ct (S1)]

Assessment of result along with the values of ΔCt-1 and ΔCt-2 was done as follows:

Assessment of the result of RT-PCR by PNA Clamp kit

ΔCt-1	ΔCt-2	Assessment
2≤ ΔCt-1	All value	BRAF V600 mutant
0< ΔCt-1 <2	ΔCt-2 ≤3	BRAF V600 mutant
	3< ΔCt-2	Wild
ΔCt-1≤ 0	All value	Wild

Qiagen BRAF mutation detection is based on "Allele - or Mutation Specific Amplification" (ARMS) and "Scorpions techniques" following the established standard protocols. [24]

Assessment of sample

- **Negative control:** Amplification in no template control was in the range of 32.53-38.16 in the Yellow (HEX) channel. Samples were rerun in case the values were beyond the specified standards.
- **Positive control:** The BRAF Positive Control (PC) gave a control assay C_T (FAM channel) of 27.82–33.85 (specified standard). Samples with values beyond this limit were considered as run failure. DNA re-extraction of respective samples done.

Procedure for BRAF mutation detection Negative control: Standard for no template control was taken in the amplification range of 32.53–38.16 in the Yellow channel. Samples were made to run again when values fell beyond the standards provided.

- **Positive control:** BRAF positive controls were considered valid when the values fell within the acceptable Ct ranges provided as follows.
- **Sample analysis- sample control FAM Ct values:** According to the specified standards each sample control C_T value must be within the range of 20.95–33.00 in the Green channel.
- **Sample analysis- sample mutation assays FAM Ct values:** The FAM values for all 4 reaction mixes were checked against the values listed below.

Acceptable C_T range for reaction controls sample mutation reaction values (FAM)*

Rreaction mix	Sample	Channel	CT range
CControl	PC	FAM	27.82–33.85
VV600E/Ec	PC	FAM	27.49–33.51
VV600D	PC	FAM	27.45–33.47
VV600K	PC	FAM	27.28–33.28
VV600R	PC	FAM	27.28–33.28

AAssay	Acceptable CT range	CT range
VV600E/Ec	15.00–40.00	≤7.0
VV600D	15.00–40.00	≤6.9
VV600K	15.00–40.00	≤6.0
VV600R	15.00–40.00	≤7.0

- If the FAM C_T falls within the specified range- FAM amplification positive.
- If the FAM C_T is above the specified range or there is no amplification- FAM amplification negative.

C_T value for each mutation tube that is FAM amplification positive was calculated as follows.

C_T= mutation C_T – control C_T

The correlation between BRAF scoring by IHC & RT-PCR, WHO grade and histologic subtype of tumor was assessed using Pearson Chi Square Test. P-value < 0.05 was considered significant.

RESULTS:

Thirty cases of serous tumors of ovary of various histological grades operated at a Tertiary Care Hospital in North India were included in the index study. Maximum cases including both benign and malignant, were within the age group of 41-60 years (13; 43.3%) followed by 61-80 years (8; 26.7%), <40 years (6; 20%) and of >80 years (3; 10%) with mean age of 61.2 years. The median age of presentation for benign tumors, LGSC, HGSC were 55.4, 43 and 55.5 years respectively. According to histological profile 17 cases (56.67%) were HGSC (grade 2), 1 case (3%) was LGSC (grade 1) and 12 cases (40%) were benign (grade 0) in nature. No statistically significant correlation between the grades of tumor and different age groups studied (p-value > 0.05) was observed. [Table 1]

Table 1: The acceptability of samples of RT-PCR by PNA Clamp kit

Acceptability	Ct value of Non PNA mix (S1)	Descriptions and recommendations
Optimal	22< Ct <30	The amplification and the amount of DNA sample are optimal
Acceptable	30≤ Ct <35	The target gene was amplified with low efficiency. For more reliable result, it is suggested that repeat PCR reaction with a higher amount of DNA
Invalid	Ct ≤22	Possibility of false positive is high. Repeat the PCR reaction with a lower amount of DNA
	35≤ Ct	The amplification was failed. Check DNA amount and purity. New DNA prep might be required

On immunohistochemistry for BRAF V600E by IHC, 21 (70%) positive and 9 (30%) negative cases were found irrespective of tumor grade. BRAF V600E mutation by RT-PCR using PNAClamp kit was positive in 23 cases (76.7%) and negative in 7 (23.3%) with similar results for V600D and V600K mutations using Qiagen kit. In addition Qiagen kit showed positivity in 24 cases (80%) and negativity in 6 cases (20%) for BRAF V600E and V600R mutation. The correlating IHC results, BRAF mutations by RT-PCR using PNAClamp kit and Qiagen kit with grades of tumor were also not statistically significant as p-value is >0.05. [Table 2]

Table 2: Distribution of age group with tumor grade, correlation between benign (grade 0) and low to high grade serous tumors (grade 2) with BRAF mutation by IHC, RT-PCR by Qiagen Kit and RT-PCR by PNAClamp Kit

Parameters		Grade			Total	Pearson Chi-Square	p-value
			Benign (Grade 0)	Low to high (Grade 2)			
Age Group		<=40 Yrs	3	3	6	1.62	0.655
		41-60 Yrs	4	9	13		
		61-80 Yrs	3	5	8		
		> 80 Yrs	2	1	3		
BRAF mutation by IHC		Negative	3	6	9	1.4	0.71
		Weak	1	3	4		
		Positive	4	3	7		
		Strong Positive	4	6	10		
BRAF mutations by RT-PCR (Qiagen kit)	V 600 E	Positive	8	16	24	2.22	0.136
		Negative	4	2	6		
	V 600 D	Positive	8	15	23	1.12	0.29
		Negative	4	3	7		
	V600 K	Positive	8	15	23	1.12	0.29
		Negative	4	3	7		
	V 600 R	Positive	9	15	24	0.312	0.576
		Negative	3	3	6		
BRAF mutations by RT-PCR (PNAClamp Kit)	V 600 E	Positive	9	16	25	1	0.317
		Negative	3	2	5		

Statistically insignificant results were obtained on correlating BRAF mutation by RT-PCR using PNAClamp and Qiagen kit with the p-value of 0.738. One (3%) high grade case out of 30 cases was positive by IHC but negative by RT-PCR using PNAClamp kit (V600E negative). [Table 3]

Table 3: Comparison of BRAF V600E mutation by RT-PCR using PNAClamp vs Qiagen kit

Cases	BRAF V600E Mutation		Pearson Chi-Square test	p-value
	PNAClamp	Qiagen		
Positive	25	24	0.1113	0.738
Negative	5	6		

DISCUSSION:

Existence of molecular diversity within specific histological subtypes of epithelial ovarian tumors, within individual tumor as well as between different tumors of an individual patient, has been clarified by various studies. The application of ancillary techniques IHC and advancement in methods to detect molecular behavior of serous tumors of ovary has led to tremendous increase in the existing knowledge. [4,5] In this study, we assessed the expression of BRAF V600E mutation by IHC and RT-PCR (using two different kits) in thirty cases of serous tumors of ovary and evaluated the clinicopathological prognostic features among various grades. LGSC showed an altered MAPK pathway with mutated BRAF and are resistant to conventional chemotherapy but overall favorable survival. [13,20] On the other hand BRAF mutation is very rare in HGSC which show early genetic instability with TP53 mutations, high mitotic rate and response to chemotherapy. [14,21,22] Hence, detection of mutation status of BRAF is necessary, which is emerging as important prognostic and therapeutic biomarker. Over 95% of BRAF mutations are of the V600E type and specific, small molecular inhibitors are currently under pre-clinical or clinical investigation to serve as an alternative regimen.

It has been proven that mutated BRAF is a useful predictive and prognostic marker of serous ovarian tumors. 17 cases (56.60%) showed positivity for BRAF V600E immunohistochemical staining whereas 13 cases (43.34%) were negative. Of all the negative cases; nine were completely negative (grade 0) however four showed very faint staining (grade 1). Out of twelve benign tumors, eight (60%) were positive for BRAF V600E expression by IHC, six (50%) cases were

positive for BRAF V600E mutation both by IHC and RT-PCR and the results were in near concordance with study by Bosmuller et al, where 71% cases of borderline/benign were positive for BRAF mutation by IHC. [15,18,19] However our results were not in corroboration with the study by Hayashi et al, where three out of 11 cases (27.2%) of serous borderline tumors were positive by IHC. One out of 30 (3%) cases of LGSC showed strong positivity for BRAF mutation by both IHC and RT-PCR. Among the high grade tumors, eight out of 17 cases (47%) were positive by IHC and this result was in contrary to Bosmuller et al who observed 100% of high grade tumors were negative for BRAF mutation by IHC. [19] In our present study, no significant correlation was observed between BRAF mutation by IHC and tumor grade with p-value of 0.626. This is not consistent with the study by Nakayama et al where significant correlation was noted between BRAF mutation and tumor grade with the p-value of 0.004. Four (13.34%) cases (two each of high grade and low grade) were negative for BRAF V600E mutation both by IHC and RT-PCR.

V600E is the most common (97%) mutation observed in BRAF till date in ovarian cancers. [11] RT-PCR was done using two different kits revealing presence BRAF V600D, V600K and V600R apart from V600E mutation. Our study had 15 (88%) BRAF mutation positive high grade cases (17) by RT-PCR using PNAClamp kit which is in contrast to Nakayama et al, Sadlecki et al and Bosmuller et al who observed only 4%, <1% and 0% positivity respectively among their high grade serous tumors for BRAF mutation. [12,19] However the 22 positive RT-PCR results out of 25 (88%) cases, pertaining to co-existence detection of mutation clones of V600D and V600E using Qiagen kit in our study were in concordance with Bosmuller et al. Eight (60%) out of 12 benign cases were detected to have BRAF V600E mutation by RT-PCR. [19] Seven out of 12 (58%) benign cases were positive for BRAF mutation both by IHC and RT-PCR (PNAClamp). In addition to these, it was also observed that nine (30%) out 30 cases which were negative by IHC were found to have mutated BRAF by RT-PCR (PNAClamp kit). The point to be noted here is that seven of these 09 (77.7%) cases were of high grade in which BRAF mutation is very uncommon. This indicated that IHC was more specific in identifying true negative cases and RT-PCR was found to be more sensitive. These results are partially corroborated with the study by Bosmuller et al, where a complete concordance was noted between VE1 IHC and mutational analysis by allele specific-PCR. [19] However, there was no statistical significance of the data observed in our study as the p-value came out to be 0.222.

Statistically insignificant correlation was noted on correlating BRAF V600E mutation status by PNAClamp and by Qiagen kit (p-value=0.738). Frequency of cases having other mutations i.e. V600D, V600K and V600R was similar to that of V600E (24) showing 23 (76.6%), 23 (76.6%) and 24 (80%) respectively.

IHC is a routinely performed, cost-effective method available; has limitation to recognize variant in point mutations and hence they are unreliable for other codon mutations in BRAF. PCR can, however, detect large proportions of cases as compared to IHC. It requires high quality of infrastructure with well trained staff and also time consuming. Inadequate number of tumor cells and poor quality of DNA are sources of error.

Validation of BRAF mutations as a potential prognostic marker in LGSC offers protection against their progression to HGSC and predict recurrence risk in operated cases. In progressive disease cases positive for BRAF V600E mutation; specific inhibitors therapy can be advised as an alternative regimen. Therefore further study of BRAF mutation will definitely have a significant impact on the management of ovarian tumors. [13]

CONCLUSION:

In today's era of personalized medicine and with the advancement in molecular techniques in the diagnostic field, understanding molecular profile of serous ovarian cancers holds immense importance with new dualistic grading system of low grade and high grade types based upon distinct tumor development pathways. This study confirmed that higher frequency of BRAF mutations in benign serous tumors, however significant number of high grade tumors were also positive for the mutation assessed by both IHC and RT-PCR. There was no significant correlation observed between BRAF V600E mutation by both the methods used as well as with the histological tumor grades studied. Also only one case of low grade serous tumor in our study more studies may be done on this subtype. Hence, IHC can be used as screening tool following confirmation by PCR based methods.

Recent advances in the molecular characterization of epithelial ovarian tumors provided the basis for a simplified classification scheme in which these cancers are classified as either type I or type II tumors having divergent disease pathogenesis and prognosis. Thus BRAF mutation detection, refined the molecular classification of serous ovarian tumors, providing a prognostic tool for clinicians and identifying unique therapeutic targets based on the individual patient profile. Therefore, detection of KRAS and BRAF mutations in ovarian cancers may identify patients who will benefit from MEK inhibitors like CI-1040 therapy. However, it is suggested that the study be continued with larger number of cases and as a multicentric study with clinical follow-up to comprehend the different facets of BRAF pathway in ovarian neoplasms.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Ethical clearance: The present study is in compliance with Ethical Standards.

Funding resources: No funding was obtained from any external source.

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