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Clinical implications of *PON1* (rs662) and *TNF-α* (rs1799964) genes polymorphism in patients with coronary artery disease

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Abstract

Background: Coronary artery disease (CAD) is the most common kind of heart problem, currently became one of the leading causes of death worldwide and is predicted to persist so for the next 20 years. The global risk factors to CAD include atherosclerosis, genetic predisposition, environment and the lifestyle. This study is aimed to find out the genotypic association of *PON1* (rs662) and *TNF-α* (rs1799964) genes with CAD among North Indian populations. A total of 330 subjects including 175 CAD cases and 155 healthy controls were enrolled in this study. Single nucleotide polymorphisms were analyzed by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) method. χ2 and Student's t-tests were applied for the comparison of alleles and genotype frequencies in cases and controls. Logistic regression analysis was applied to calculate the 95% confidence intervals and odds ratios (OR) for assessing the association of genotype with disease.

Results: The *PON1* gene QQ, QR, RR genotypes frequencies were 36.57%, 50.29%, 13.14% in CAD cases and 60%, 38.71%, 1.29% in controls, respectively. OR for the genotype QQ, QR, RR was 0.38, 1.6, 11.57 ($P < 0.001$, $P = 0.035$, $P < 0.001$). The *TNF-α* gene CC, CT, TT genotypes frequencies in cases were 4.57%, 50.29%, 45.14% and controls 3.23%, 46.45%, 50.32%, respectively. OR for CC, CT, TT genotype was 1.437, 1.166, 0.812 ($P = 0.531$, $P = 0.487$, $P = 0.347$). We found significant difference in the genotype and allele frequencies of *PON1* gene between cases and control, while no significant difference was observed in *TNF-α* gene between cases and control.

Conclusions: The *PON1* (rs662) gene polymorphisms were significantly associated with an elevated risk of CAD, while no significant association was observed with *TNF-α* (rs1799964) gene polymorphism and the risk of CAD.

Keywords: Coronary artery disease, *PON1*, *TNF-α*, Genetic polymorphism

Background

Coronary artery disease (CAD) is the narrowing and blockage of the arteries, usually caused by the buildup of plaque inside the artery walls or atherosclerosis that results to a restriction of blood flow to the heart [1]. According to World Health Organization (WHO) 2015 estimation, 17.7 million people died of CAD that is 31% of all global death [2]. CAD is commonly found in people of every age, but it is more common in progressively older ages [3]. In 2016, the estimated prevalence of CAD in India was estimated to be 54.5 million [4]. One in 4 deaths in India is now because of CAD with ischemic heart disease and stroke responsible for > 80% of this burden. [4]. More than two hundred genetic loci have been identified to be significantly associated with CAD by genome-wide association studies (GWAS) [5].

A calcium-dependent enzyme, human paraoxonase 1 (*PON1*) is produced by the liver and released into blood [6]. The antioxidant properties possessed by HDL associated *PON1* inhibit LDL lipid peroxidation and prevent the atherosclerosis development and

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CAD [7, 8]. *PON1* gene is 26,857-bp long and presents on chromosome 7q21.3 and consists of 8 introns and 9 exons [9]. Among various genetic aberrations, the *PON1 (*Q192R) gene polymorphism present in the coding region exchanging an arginine (R) for glutamine (Q) at position 192 affect the PON1 activity toward paraoxon. A significant decrease in PON1 activity has been consistently reported in atherosclerosis and related cardiovascular diseases [10, 11]. Recently, several meta-analyses of clinical studies suggested that one of the risk factors for CAD is lower plasma *PON1* activity [12, 13].

Tumor necrosis factor (TNF-α), a multi-functional cytokine, is mostly synthesized and secreted by inflammatory cells (monocytes and macrophages) [14]. The human *TNF-α* gene present on chromosome 6p21.3 within the highly polymorphic major histocompatibility complex (MHC) class III region [15]. Previous study showed that TNF-α is a major participant in the progression of various atherosclerosis complications [16]. *TNF-α* 308G > A (rs1800629), 857C > T (rs1799724), 238G > A (rs361525), 1031 T > C (rs1799964) and 863C > A (rs1800630) gene polymorphisms are reported so far [17]. Previous studies observed the role of *TNF-α* rs1799964 gene variant with increased *TNF-α* secretion and probability of cardiovascular disease [17, 18]. On the basis of these observations, this study was made to examine the role of *PON1* and *TNF-α* genes polymorphism with CAD.

Materials and methods
Subjects
The study was complied with the declaration of institutional ethical/review committee, and written informed consent was obtained from all participants before sample collection. Total 330 subjects including 175 CAD cases (who experienced coronary angiography) and 155 healthy controls were enrolled in the study from January 2017 to December 2019. Blood samples for all the subjects were collected from the Cardiology Unit (Department of Medicine), Era's Lucknow Medical College & Hospital and from other hospitals of Lucknow. Clinical parameters including age, sex, body mass index (BMI), height, weight, blood pressure, lipid profile, etc., were collected from all the subjects with a standard case report form. Patients with coronary artery diameter's reduction of > 50% are considered to have CAD. On the basis of number of significant stenotic vessels, all cases were grouped as: angiographically normal vessel (*n* = 17), 1-vessel (SVD) (*n* = 42), 2-vessel (DVD) (*n* = 55) and 3-vessel (TVD) (*n* = 61). Control subjects include

individuals with no previous history of cardiovascular disease or diabetes.

Biochemical estimations
BMI was calculated by Quetelet equation. Random blood sugar (RBS) levels were measured by (glucose oxidase–peroxidase method), serum triglyceride (TG) (glycerol phosphate oxidase–peroxidase amidopyrine method), serum cholesterol (cholesterol oxidase–peroxidase), high-density lipoprotein (HDL) and cholesterol (immunoinhibition) were assessed by XL-300 Transasia Fully Auto Analyzer Transasia, Mannheim, Germany. The measurement of total cholesterol levels and low-density lipoprotein (LDL) was taken by using Friedewald's formula. Standard manufacturer's protocols were followed for all the assays. In this study, each experiment was implemented according to the ethical standards and the declaration of Helsinki.

DNA extraction
Genomic DNA was extracted from blood specimens according to the manufacturers' protocol using DNA extraction kit (MACHEREY-NAGE, Germany). Extracted DNA was quantified by the Thermo Scientific NanoDrop 2000 Spectrophotometers and stored at − 20 °C until analysis.

Analysis of polymorphisms
PON1 Polymorphism
Genotyping of *PON1* gene was done by PCR–RFLP method using forward primer 5′ TATTGTTGCTGT GGGACCTGAG 3′ and reverse primer 5′ CACGCTAAA CCCAAATACATCTC 3′. Final volume of 20 µL PCR reaction mixture consists of: 10 pmol of each primer, 1 × PCR master mix (Thermo Fisher Scientific Cat no: RR310A), 3 Mm MgCl^2, and 2 U Taq polymerase (G Biosciences). PCR conditions were: initial denaturation at 95 °C for 5 min, followed by 35 cycles each consisting of denaturation annealing and extension at 95 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min, respectively, and final extension for 10 min at 72 °C. PCR products were digested with 5 U of restriction enzyme *AlwI* (New England Biolabs, USA Cat no: R0513S) at 37 °C for 3 h in a total volume of 25 µl, and checked by 3% agarose gel electrophoresis (Fig. 1).

TNF-α polymorphism
Genotyping of *TNF-α* gene was done by PCR–RFLP method using forward primer 5′-TATGTGATGGAC TCACCAGGT-3′ and reverse primer 5′-CCTCTA CATGGCCCTGTCTT-3′. Final volume of 20 µL PCR
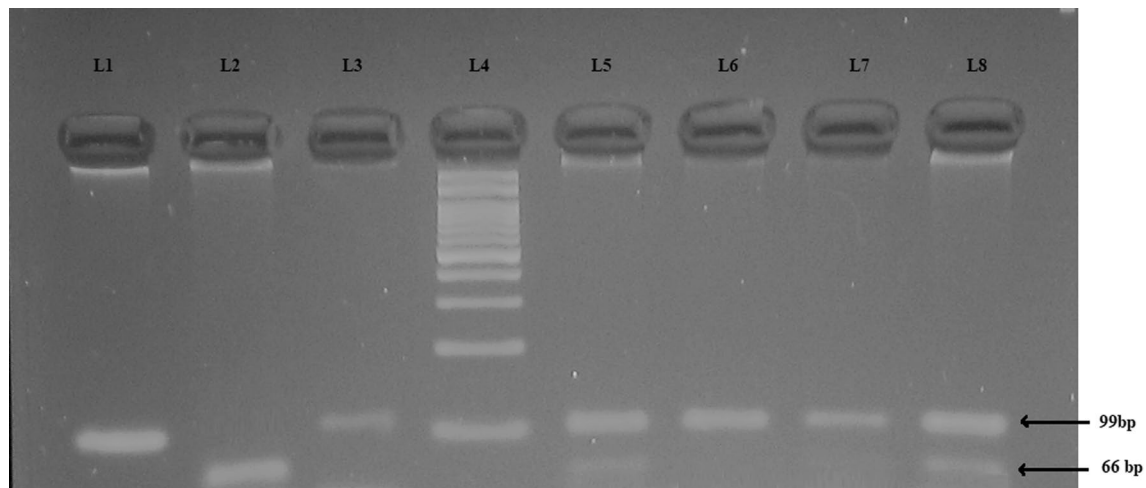
Fig. 1 The 3% agarose gel picture of *AlwI* digested products of *PON1* rs664 gene. Lane 1 shows undigested PCR product corresponding to a band of 99 bp, lanes 5 and 8 show QR genotype corresponding to three bands sizes of 99 bp, 66 bp and 33 bp, lanes 3, 6 and 7 show the QQ genotype corresponding to one bands of 99 bp and lane 2 shows RR genotype corresponding to two bands of 66 bp, 33 bp, whereas lane 4 shows 100 bp ladder

reaction mixture consists of: 10 pmol of each primer, $1 \times$ PCR master mix (EmeraldAmp GT PCR master mix), 3 Mm $MgCl^2$, and 2 U Taq polymerase (G Biosciences). PCR conditions were: initial denaturation at 94 ℃ for 12 min, followed by 35 cycles of annealing and extension at 94 ℃ for 30 s, 59 ℃ for 1 min, and 72 ℃ for 2 min and the final extension at 72 ℃ for 2 min. PCR products were digested with 3 U of restriction enzyme *BbsI* (New England Biolabs, USA Cat no: R0539S) at 37 ℃ for overnight in a total volume of 25 µl and checked by 4% agarose gel electrophoresis (Fig. 2).

Statistical analysis

Values for all the figures were expressed as means ± standard deviation for continuous variables and proportion/percentages for categorical variables. The genotyping data for cases and controls were compared by using Chi-square test and logistic regression analysis. $P < 0.05$ was represented as statistically significant. All of the statistical tests were performed with the statistical package for the social sciences (SPSS) version 17 software.
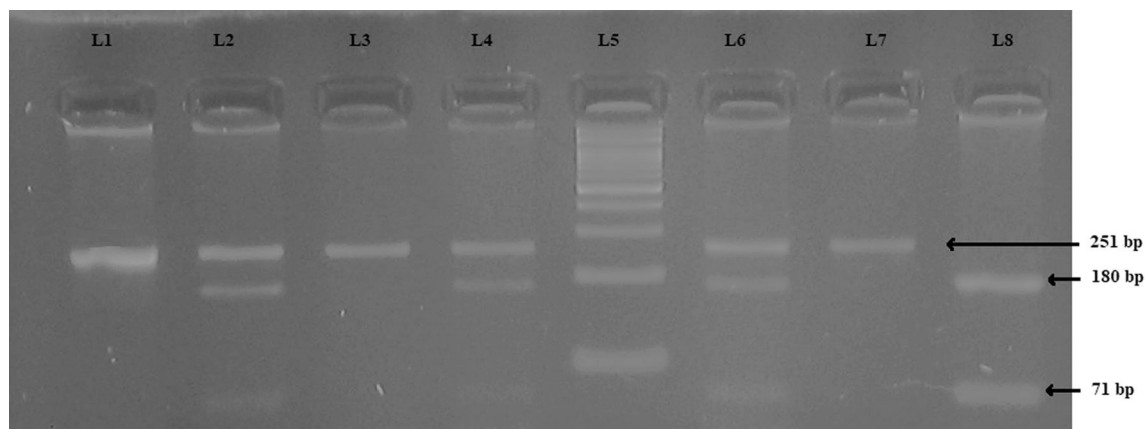

Fig. 2 The 4% agarose gel picture of *BbsI* digested products of *TNF-α* rs1799964gene. Lane 1 shows undigested PCR product corresponding to a band of 264 bp; lanes 2, 4 and 6 show CT genotype corresponding to three bands size of 251 bp, 180 bp and 71 bp; lanes 3 and 7 show the CC genotype corresponding to one bands of 251 bp, whereas lane 8 shows TT genotype corresponding to two band of 180 bp and 71 bp and lane 5 shows 100 bp ladder

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Results

Demographic and clinical characteristics

This case–control study comprised 330 subjects including 175 CAD cases and 155 ethnicity matched healthy controls. Clinical and biochemical parameters of CAD cases and controls are shown in Table 1. Demographic, clinical and biochemical characteristics of the studied population were recorded from both cases and controls. The mean ages of CAD cases and control were 54.89 ± 10.47 and 47.30 ± 9.53 years, respectively ($P = 0.065$). Clinical parameters such as systolic blood pressure (SBP), diastolic blood pressure (DBP), RBS, TG, HDL, LDL ($P > 0.05$) were significantly higher in cases as compared to controls, whereas no significant difference was observed in BMI (p = 0.797), serum cholesterol (p = 0.215) and VLDL (p = 0.722) in cases compared to the controls (Table 1).

Hardy–Weinberg equilibrium test

PON1 and *TNF-α* genotype distribution in this study was in line with Hardy–Weinberg equilibrium (all $P > 0.05$, data not shown).

Genetic polymorphism Analysis

PON1 polymorphism analysis (rs662)

The *PON1* gene QQ, QR, RR genotypes frequencies were 36.57%, 50.29%, 13.14% in CAD cases and 60%, 38.71%, 1.29% in healthy controls, respectively. Odds ratio (OR) for QQ, QR, RR genotype was 0.38, 1.6, 11.57 ($P < 0.001$, $P = 0.035$, $P < 0.001$). The frequencies of Q and R alleles were 61.71% and 38.29% in cases as compared to 79.35% and 20.65% in the controls. OR for Q was 0.419 ($P < 0.001$), and for R allele OR = 2.384 ($P < 0.001$) (Table 2). The frequency of QQ and RR genotype of *PON1* gene was found to be highly significant in cases with 0.3 and 11 fold higher risk of CAD ($P < 0.0001$) in comparison with control. Similarly, the frequency of Q and R allele of *PON1* gene was also found to be highly significant in cases with 0.4 and two-fold higher risk of CAD (p ≤ 0.0001).

Table 1 Comparison of biochemical parameters in cases and controls

Parameters	Control (155)	Cases (175)	p-value
Age	47.30 ± 9.53	54.89 ± 10.47	0.065
BMI	23.73 ± 2.58	23.65 ± 3.00	0.797
SBP(mm Hg)	120.70 ± 8.22	144.33 ± 18.50	**0.000**
DBP(mm Hg)	76.85 ± 13.01	83.09 ± 4.63	**0.000**
RBS (mmol/l)	117.68 ± 18.11	204.59 ± 58.97	**0.000**
Serum cholesterol (mmol/l)	166.72 ± 26.76	162.60 ± 31.68	0.215
Triglyceride(mmol/l)	142.9 ± 41.68	132.46 ± 35.26	**0.018**
HDL (mmol/l)	57.76 ± 10.49	29.02 ± 10.84	**0.000**
VLDL (mmol/l)	33.70 ± 10.73	33.25 ± 11.35	0.722
LDL (mmol/l)	92.69 ± 21.55	87.12 ± 16.85	**0.012**

*BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *RBS* random blood sugar, *HDL* high-density lipoprotein, *VLDL* very low-density lipoprotein, *LDL* low-density lipoprotein

Table 2 The genotype, allele frequencies of *PON1* (rs662) and *TNF α* (rs1799964) genes and their statistical analysis among CAD cases and controls

PON1 (rs662)		N	Control (155) Frequency (%)	N	CAD (175) Frequency (%)	OR	95% CI	x^2	p values	Bonferroni-corrected p values	Power
Genotype	QQ	93	60.00	64	36.57	0.384	(0.25—0.60)	18.09	**< 0.001**	< 0.001	0.977
	QR	60	38.71	88	50.29	1.601	0.40—0.97	4.45	**0.035**	0.105	0.927
	RR	2	1.29	23	13.14	11.576	2.68—49.96	16.49	**< 0.001**	< 0.001	0.989
Allele	Q	246	79.35	216	61.71	0.419	0.30—0.59	24.36	**< 0.001**	< 0.001	0.987
	R	64	20.65	134	38.29	2.384	1.68—3.38	24.36	**< 0.001**	< 0.001	0.987
TNF α (rs1799964)		**N**	**Control (155) Frequency (%)**	**N**	**CAD (175) Frequency (%)**	**OR**	**95% CI**	**x^2**	**p values**	**Bonferroni-corrected p values**	**Power**
Genotype	CC	5	3.23	8	4.57	1.437	0.46—4.49	0.39	0.531	1.000	0.859
	CT	72	46.45	88	50.29	1.166	0.76—1.80	0.48	0.487	1.000	0.856
	TT	78	50.32	79	45.14	0.812	0.53—1.25	0.88	0.347	1.000	0.895
Allele	C	82	26.45	104	29.71	1.175	0.84—1.65	0.86	0.352	0.705	0.842
	T	228	73.55	246	70.29	0.851	0.60—1.20	0.86	0.352	0.705	0.842

**N = number of subjects, OR = Odd ratio, CI = confidence interval, P = p-value, χ^2 = Chi-square test

TNF-α polymorphism analysis (rs1799964)

The *TNF-α* gene CC, CT, TT genotypes frequencies were 4.57%, 50.29%, 45.14% in CAD cases and 3.23%, 46.45%, 50.32% in healthy controls, respectively. OR for CC, CT, TT genotype was 1.437, 1.166, 0.812 ($P = 0.531$, 0.487, 0.347). The frequencies of C and T alleles were 29.71% and 70.29% in CAD cases as compared to 26.45% and 73.55% in the controls. OR for C was 1.175 ($P = 0.352$), and for T allele OR $= 0.851$ ($P = 0.352$) (Table 2).

Discussion

CAD has become the major cause of death worldwide, accounting for approximately 30% of deaths and the most common health problem in India [19]. As with most complex diseases, the risk of CAD development in an individual is affected by the genetic and lifestyle factors [20]. The associations of 163 genetic loci with CAD have been revealed so far by GWAS [21]. Our previous studies have also shown the association of gene polymorphism with CAD [22, 23]. Due to the presence in noncoding regions, most of the gene variants are associated with CAD resulting into the development of disease by regulating gene expression [24].

PON1 is a calcium-dependent antioxidant glycoprotein and is regarded as a cardiovascular protective factor. Clinical studies suggested that the lower plasma *PON1* activity is significantly associated with increased CAD risk [13, 25]. Approximately 160 polymorphisms of the *PON1* gene have been discovered so far, either present in exonic (coding sequences) or intronic (noncoding sequences) region and the regulatory parts of genes [26]. Majority of them include single-nucleotide polymorphisms; however, those in the coding regions were extensively studied, i.e., Q192R (rs662) and L55M (rs854560) [27, 28]. The antioxidative potential of Arg192 may disturb by the exchange of glutamine (Q) to arginine (R)Q in 192R polymorphism and result into the development of CAD [29]. There are different reports suggesting the distribution of Q and R allele in different ethnic populations. Among cases, we found Q and R to be major and minor alleles at codon 192 in the *PON1* gene, respectively. The distribution of Q and R allele of the *PON1* gene in the present study tended to be closer to that observed in Egyptian [32, 34], Chinese [35] and Asian Indians [36], but differed from South Indian [33] populations where the R allele was predominant (Table 3). The present study showed the significant increase in the QR and RR genotypes frequency of *PON1* Q192R polymorphism in cases compared to the controls, hence, indicating that it plays a crucial role in the pathogenesis of CAD. Studies examined the role of the Q192R polymorphism with CAD risk observed inconsistent results. Several studies have shown the positive correlation of Q192R polymorphism with CAD risk [31–36], whereas others have not [30]. The *PON1* gene Q192R polymorphism contains the ability to inhibit LDL oxidation; hence compared with 192Q allele, the 192R allele is less effective for the prevention LDL oxidation [37]. Thus, R allele's carriers are more susceptible to develop cardiovascular diseases than Q allele's carriers. In the present

Table 3 Allele frequencies of *PON1* Q192R (rs662) and *TNF-α* (rs1799964) genes polymorphism in CAD cases among different populations

Ethnicity	N	PON1 (rs662)		Significant	Ref
		Q allele (%)	R allele (%)		
American	356	70	30	No	[30]
Chinese	475	35	65	Yes	[31]
Egyptian	50	63	37	Yes	[32]
South Indian	200	54	46	Yes	[33]
Egyptian	134	67	33	Yes	[34]
Chinese	165	67	33	Yes	[35]
Asian Indians	412	60	40	Yes	[36]
North Indian	175	62	38	Yes	PS
Ethnicity	**N**	**TNF-α (rs1799964)**		**Significant**	**Ref**
		T allele (%)	C allele (%)		
Pakistani	310	75	25	No	[39]
African	418	81	19	Yes	[40]
China	961	82	18	No	[41]
North Indian	175	70	30	No	PS

*N = number of subjects, PS = present study

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study, significant association was observed between Q and R allele of *PON1* gene with CAD ($P < 0.001$), where Q allele confers the increase risk while R allele with decrease risk of CAD. We have observed that the *PON1* QR genotype frequency was 50.29% in CAD cases which is similar to Asian Indian 44.9%, South Indian 51.5% CAD cases [33, 36]. *PON1* QQ genotype frequency was 36.57% in our study which is significantly higher compare to South Indian 28% [33]. Further studies including larger sample size from different ethnic groups required to examine the association of *PON1* gene polymorphisms in the development of CAD.

One of the most typical pro-inflammatory cytokines *TNF-α* was suggested by many studies as a major contributor in the atherosclerosis development, complications and progression [38]. Relatively limited studies have been done so far based on the relation of *TNF-α*-1031T > C (rs1799964) gene polymorphisms and CAD. Hence, this study was carried out to examine the role of *TNF-α* gene polymorphisms with CAD among North Indian population. Our findings suggest no significant association of *TNF-α* gene-1031T > C polymorphism with CAD risk in the study population. The *TNF-α*-1031C allele frequency was 26% in control versus 30% in cases ($P = 0.46$), indicating that the − 1031C allele is not a risk factor of CAD in our population. This is in accordance with the results of Asifa et al. and Ghazouani et al., demonstrating no significant association between the *TNF-α* gene polymorphism at − 1031 T > C promoter sequence and CAD among Pakistani and Chinese, respectively [39, 41]. The present study observed the distribution of − 1031 T and − 1031C allele of the *TNF-α* gene relatively similar to Pakistani population [39] but slightly differed from that of African [40] and Chinese [41] [Table 3]. The frequency of *TNF-α* TT genotype was 45% in CAD cases which is lower compared to Pakistani population 57% [39].

To the best of our knowledge, this is the first study to look at the relationship between *PON1* (rs662) and *TNF-α* (rs1799964) genes polymorphism in patients with CAD. Further study on a larger cohort of cases with CAD is needed to validate this study.

Conclusions

Our results suggest that the *PON1* (rs662) gene polymorphisms were associated with an increased risk of CAD, whereas *TNF-α* (rs1799964) gene polymorphism was not conferring any risk of CAD. Thus, *PON1* (rs662) gene polymorphism may be a useful tool for diagnosis, prognosis, and prediction of the disease and may have an influence on more effective and specific treatment against the CAD.

Declarations

Ethical approval and consent to participate
Protocol and procedures in the study complied with the declaration of Institutional Ethical/ Review Committee (Ref no. ELMC &H/R-Cell-/2019/24) of Era's Lucknow Medical College and Hospital, Lucknow.

Consent for publication
Written informed consent for publication was obtained from all the participants before sample collection.

Competing interests
None.

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