Rare P376L variant in the SR-BI gene associates with HDL dysfunction and risk of cardiovascular disease

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ABSTRACT

Background: Scavenger receptor class B type 1 (SR-BI) encoded by SCARB1 gene serves as a multifunctional HDL receptor, facilitating the uptake of cholesteryl esters from HDL to the liver. Recent studies have identified the association between the P376L missense mutation of the SCARB1 gene with increased serum HDL-Cholesterol level. However, the contribution of this variant to the development of cardiovascular disease (CVD) remains unclear.

Objective: We have investigated the association between the P376L polymorphism with the properties of HDL and CVD outcomes in a population sample recruited as part of the Mashhad-Stroke and Heart-Atherosclerotic-Disorders (MASHAD) cohort.

Methods: Six hundred and fifteen individuals who had a median follow-up period of 7 years were recruited as part of the MASHAD cohort. Anthropometric, biochemical parameters and HDL lipid peroxidation (HDLox) were assessed. Genotyping was performed using TaqMan-real-time-PCR based method. The association of P376L-rs74830766 with cardiovascular-risk-factors and CVD events were evaluated.

Results: Carriers of the P376L variant were significantly more likely than non-carriers to develop CVD using multivariate analyses adjusted for traditional CVD risk factors defined as: age, sex, BMI, presence of diabetes, or hypertension, positive smoking habit, and total cholesterol (OR: 3.75, 95%CI: 1.76–7.98, p = 0.001). In an adjusted model, there was a two fold increase in cardiovascular endpoints among individuals who were heterozygous for the P376L variant (hazard ratio, 2.08; 95% CI, 1.12-to 3.84, p = 0.02). Although there was no association between the presence of the P376L variant and HDL-C level, serum HDLox, measured as dysfunctional HDL, was 13% higher among carriers of the P376L variant than non-carriers.

Conclusion: We have found that carriers of the P376L variant possessed higher HDLox and were at increased risk of CVD in a representative population-based cohort, as compared to non-carriers.

1. Introduction

Cardiovascular disease (CVD) is one of the leading causes of mortality and disability globally [1]. Despite the progress made in prevention and treatment of CVD, it remains the most common cause of death in Iran [1,2]. High-density lipoprotein (HDL) is considered to be an anti-atherogenic lipoprotein because of its role in reverse cholesterol transport, from the peripheral tissues to the liver [3,4]. Epidemiological investigations confirm a negative association between the level of circulating HDL-C and risk of CVD [1,5–8]. Recent genome-wide association studies (GWAS) have been used to identify disease susceptibility loci, suggesting novel polymorphisms at new loci related to CVD and

serum HDL-C concentrations [9]. The results of genomic and sequencing studies have reported that high HDL-C levels may not always be protective against CVD [6] and so raising HDL-C may not be an appropriate treatment target [10]. Furthermore, clinical trials using drugs such as the cholesteryl ester transfer protein (CETP) inhibitors aiming at increase serum HDL-C levels revealed no association between high HDL-C level and clinical CVD outcomes [11]. The scavenger receptor class B Type 1 (SR-B1) is generally known as the primary receptor for HDL-C [12]. This integral membrane protein is encoded by a gene on chromosome 12 and is expressed in numerous tissues, but particularly by the liver and endocrine organs [13,14]. Recent experimental studies have shown that SCARB1 has an important role in reverse cholesterol transport and secretion of cholesterol into the bile and elevate the hepatic selective uptake of cholesterol from HDL [6]. Studies on mouse models have shown that genetic deletions and deficiency of SCARB1 [15] are associated with decreased biliary cholesterol and increased HDL-C levels. Many studies have shown that SCARB1 gene polymorphisms are associated with variations in lipid levels [16,17]. Three rare missense mutations of SCARB1, V111M, G319V, and V32M are associated with raised serum HDL-C levels. Recently, four other missense variants, P376L (rs74830766), S112F, P297S, and T175A have been shown to be associated with increased HDL-C levels and an increased risk of CVD [18,19]. It is conceivable that the rare mutation encoding P376L at the SCARB1 locus, is associated with increased serum HDL-C level and in turn the risk of CVD by inhibiting reverse cholesterol transport [6]. Recent studies have suggested that increased HDL-C as a result of SCARB1 loss of function, variant P376L, is not an indicator for CVD risk [20] and this has remained a controversial issue. The aim of the current study was to evaluate the association between P376L polymorphism with HDL-C level, an indicator of HDL dysfunction (serum HDLox concentrations) and clinical CVD outcomes in a population recruited from the Mashhad-Stroke and Heart-Atherosclerotic-Disorders (MASHAD) cohort.

2. Material and methods

2.1. Patient samples

One hundred and sixty one subjects with CVD and 454 healthy sex- and age-matched randomized individuals without clinical coronary artery disease (CAD), stroke and peripheral arterial disease were recruited from cohort the MASHAD cohort study. Eligibility was assessed based on physical examination, medical interviews and review by cardiologists. Assessments comprised evaluation of demographic, anthropometric and CVD risk factors (smoking status, fasting blood glucose, lipid profile, blood pressure measurements, history of diabetes and hypertension). lipid profile (total cholesterol, TG, LDL and HDL-C) and fasting blood glucose were measured using a Cobas Modular P800 analyzer (Roche Diagnostic, USA). CVD patients were confirmed by an electrophysiologist and two interventional cardiologists by using several medical investigations that included: angiography, CT angiography and ETT. The primary end-point was cardiovascular event including: stable and unstable angina, myocardial infarction, and also coronary revascularization (percutaneous coronary intervention or coronary-artery bypass grafting). Informed consent was completed by all participants utilizing protocols conform to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Committee of the Mashhad University of Medical Sciences.

2.2. HDL lipid peroxidation assay

ApoB depleted serum was prepared at baseline samples using PEG precipitation. HDL lipid peroxidation was determined by previously cell-free fluorometric assay [21]. Concisely, ApoB depleted sera which prepared from 20% PEG 6000 with the proportion of 1:2.5,were added to the well plates in duplicate. $1 \times$ reaction buffer was considered as a negative control and 20 mM hydrogen peroxide (H2O2) working solution was the positive control. The fluorescence readout of Amplex Red conversion to resorufin was assessed in each well plate at wavelengths of 530/590 nm by a plate reader (Biotek, Vermont, USA). In order to minimize variations, ApoB depleted of serum control samples were pooled as an experimental control in each plate. Were normalized using the Mean fluorescent of the pooled control and HDL-C level were used for normalization of mean fluorescence of samples by the calculation of: "normalized" oxidized HDL (nHDLox) = [HDLox_sample × 40 (mg/dL)]/[HDLox_control × HDLC sample (mg/dL)], where 40 mg/dL represents HDL-C of the pooled serum control. In order to minimize buffer autoxidation, catalase was added to the solution. Therefore, the oxidation of the fluorochrome Amplex Red lead to quantify hydroperoxides in HDL. Variation of Inter-assay and intra-assay of experiments was < 15% and inter-assay and intra-assay CV was also reported as 3–10% and 1–7% respectively.

2.3. DNA extraction and genotyping

Genomic DNAs were extracted from peripheral blood leukocytes using QIAamp® DNA Mini-Kit (Qiagen, San Diego, CA, USA) according to the manufacturer's protocol. The concentration and purity of DNA samples were determined using the NanoDrop®-1000- Detector (NanoDrop-Technologies, Wilmington, DE, USA). Extracted DNA samples were stored at −20 °C. Genotype analysis of loss of function variant of P376L in SCARB1 gene was carried out using Taqman-probes-Based assay; Real-Time PCR System were performed in 12.5 μL total volume, using 20 ng of DNA in TaqMan® Universal Master Mix with specific primers and probes (Applied Biosystems, Foster City, CA, USA). The ABIPRISM-7500 instrument equipped with the SDS version-2.0 software was utilized to determine the allelic content of the samples.

2.4. Statistics

All statistical analyses were performed by SPSS software, version 22 (IBM Corp, 2013). Results for normal and non-normally distributed data were reported as mean ± SD, or median and interquartile range, respectively. Baseline characteristics of participants with and without CVD were compared by student's *t*-test for normally distributed parameters, chi-square for categorical ones. Demographic and biochemical variables were compared across mutations using Pearson's $\chi 2$ tests. The presence of normal distribution within the subgroups was assessed by Kolmogorov-Smirnov tests. The observed genotype frequencies of the variant of P376L in SCARB1 gene were assessed with $\chi 2$ tests. The Hardy-Weinberg equilibrium assumption was determined by comparing the genotype frequencies using the Pearson χ distribution. The association between P376L variant and risk of cardiovascular disease was assessed by logistic regression model after adjustment for potential confounders including age, sex, BMI, smoking status, total cholesterol, diabetes and hypertension. Cox regression was applied to investigate the effect of P376L variant upon the time CVD event (myocardial infarction, Coronary revascularization, stable angina and unstable angina) took to happen. p values < 0.05 were defined as statistically significant.

3. Results

3.1. Association of the genetic variant with clinical characteristics of population

To evaluate whether there was a relationship between the SCARB1 rs74830766 (A/G) polymorphism and CVD risk, genotyping was carried out utilizing extracted DNA. Genotyping was successfully accomplished in DNA samples with no discrepancies in the samples analyzed in duplicate. Genotype frequencies of P376L variant are shown in Table 1, and these were in Hardy-Weinberg equilibrium (HWE) (p > 0.05). The

Table 1

Distribution of genotype frequencies and their association with CVD.

Genetic models		Total	Control	CVD	OR (95% CI)	p value
Dominant	GG	583(94.8%)	439(75.3%)	144(24.7%)	Reference	
	AG + AA	32(5.2%)	15(46.9%)	17(53.1%)	3.45(1.7–7.09)	0.001

Abbreviation: CVD; Cardiovascular Disease, OR; Odds Ratio.

Table 2

Association of the genetic variant with clinical characteristics of population with and without CVD.

Variable		Without CVD			With CVD		
		Genotype					
		GG	AG + AA	p value	GG	AG + AA	p value
Anthropometrics							
	Age (year)	47.83 ± 8.86	47.93 ± 8.5	0.9	54.4 ± 6.5	51.7 ± 7.28	0.1
	Gender (female) n (%)	241(56%)	11(73.3%)	0.1	71(49.3%)	6(35.3%)	0.2
	Weight (kg)	71.42 ± 13.38	69.3 ± 13.16	0.5	73.68 ± 12.7	75.28 ± 11.07	0.6
	BMI (kg/m^2)	27.4 ± 4.83	27.62 ± 3.9	0.8	28.46 ± 4.4	28.6 ± 4.34	0.9
	WC (cm)	93.26 ± 11.33	89.68 ± 14.74	0.2	98.5 ± 11	97.65 ± 8.1	0.7
	Waist hip (cm)	0.9 ± 0.07	0.9 ± 0.1	0.6	0.95 ± 0.07	0.93 ± 0.06	0.4
	PAL	1.62 ± 0.3	1.65 ± 0.15	0.5	1.52 ± 0.28	1.47 ± 0.19	0.5
Smoking n (%)	Non smoker	298(69.5%)	10(66.7%)	0.8	90(62.9%)	9(52.9%)	0.6
	Ex-smoker	41(9.6%)	2(13.3%)		23(16.1%)	4(23.5%)	
	Current smoker	90(21%)	3(20%)		30(21%)	4(23.5%)	
Blood pressure							
	BSP (mmHg)	120.4 ± 19	118.3 ± 13.6	0.6	134.2 ± 21.47	124 ± 24.1	0.07
	BDP (mmHg)	78 ± 11.37	77.15 ± 8.2	0.7	83.42 ± 10.96	81.19 ± 11.3	0.4
Lipid profile							
	Cholesterol (mg/dl)	183 ± 38.5	194.8 ± 50.5	0.2	197.74 ± 42.35	205.71 ± 49.34	0.4
	TG (mg/dl)	139.5 ± 89.93	156 ± 109.2	0.4	169.94 ± 94.34	189.8 ± 103.6	0.4
	HDL-C (mg/dl)	42.2 ± 11.67	42.5 ± 6.6	0.8	39.5 ± 8.97	44.06 ± 12.06	0.06
	LDL (mg/dl)	104.13 ± 34.4	107.34 ± 43.5	0.7	121.34 ± 35.5	117.93 ± 44.65	0.7
Fasting blood glucose							
	FBG (mg/dl)	91.2 ± 33.6	85.4 ± 23.2	0.5	118 ± 59.9	131.24 ± 88.6	0.4
Inflammation							
	Hs-CRP (mg/dl)	4.6 ± 9.05	3.6 ± 4.5	0.6	4.94 ± 7.87	5.27 ± 8.5	0.8
HDL lipid peroxidation							
	HDLox	0.84 ± 0.18	0.88 ± 0.13	0.3	1.27 ± 0.28	1.53 ± 0.26	0.001

Abbreviations: BMI, Body mass index; WC, Waist circumference; PAL, physical activity level; BSP, Blood systolic pressure; BDP, Blood diastolic pressure; TG, Triglyceride; HDL-C, High density lipoprotein cholesterol; LDL, Low density lipoprotein; FBG, Fasting blood glucose; Hs-CRP, High sensitive C-reactive protein; HDLox, HDL lipid peroxidation.

frequency of GG genotype was 94.8% and also AA and AG genotypes frequency were 5.2% for rs74830766 in total population. Furthermore, the frequency of risk A allele was 2.8% in the population. Baseline clinical characteristics of individuals based on presence or absence of CVD across the genotypes are illustrated in different groups and genetic model (Table 2). Analysis indicated that participants without CVD who carried the A allele had higher serum lipid values including cholesterol, triglyceride, HDL-C and LDL than those with GG genotypes, although this did not attain significance. Furthermore, serum HDL-C concentration among CVD patients with carriers of A allele were higher than patients with GG genotype. In contrast to the previous studies indicating inverse association of HDL-C level and CVD outcomes in our study. Serum HDL-C is reported to be increased among subjects with CVD, as compared with control group. Interestingly, this elevated HDL-C level is limited to the CVD groups with AA, AG genotypes. In addition,

the A allele was associated with higher HDL lipid peroxidation in the CVD subjects (1.53 ± 0.26) with respect to the healthy controls (0.88 ± 0.13) (p = 0.001). However, we found no statistically significant differences between serum HDL-C level in two groups of studied population across genotypes (p = 0.06). In line with this result, carriers of A allele had a higher serum HDLox compared with those who were homozygous for the G allele (p = 0.001). Analysis showed significant increase in HDLox values from 0.88 ± 0.13 to 1.53 ± 0.26 in heterozygotes of CVD subjects.

3.2. Association of P376L variant with risk of incident CVD

We also explored the association between the presence of the P376L variant and CVD in patients undergoing coronary angiography with obstructive coronary artery disease (Table 3, Table 4). Indications for

Table 3

Genotype characteristics of studied population (n = 615).

Variant	Risk allele	Gene	Dominant model OR (95%CI)	Codominant model OR (95%CI)	Recessive model OR (95%CI)	Additive model OR (95%CI)
rs74830766	A	SCARB1	3.45(1.7–7.09) p value = .001	AG: 3.75(1.76–7.98) p value = .001 AA: 1.52(0.13–16.93) p value = .73	1.41(0.12–15.5) p value = .77	1.52(0.13–16.93) p value = .73

Table 4
The association of P376L with CVD risk.

	Univariate				Multivariate[a]		
Variable	B	OR(95% CI)	p value		B	OR(95% CI)	p value
rs74830677	1.32	3.75(1.76–7.98)	0.001		1.22	3.40(1.47–7.88)	0.004

[a] Adjusted for age, sex, BMI, diabetes, hypertension, smoking, and total serum cholesterol.

coronary angiography were stable or unstable angina, MI, and recurrence of symptoms after revascularization. According to our results a positive association was found among heterozygotes of P376L variant and likelihood of CAD risk. In both univariate and multivariate analyses adjusted for traditional CVD risk factors (age, sex, BMI, diabetes, hypertension, smoking, and total serum cholesterol), carriers of P376L variant had an increased risk of CAD, compared to non-carriers (OR: 3.75, 95%CI: 1.76–7.98, p = 0.001).

3.3. Association of genotypes with cardiovascular events

161 participants were confirmed to have a primary cardiovascular disease over the follow-up period of 7 years. Kaplan–Meier curve and hazard ratios are shown for the genetic model, derived from Cox proportional-hazards models. The adjusted model included as traditional risk factors for cardiovascular disease (Fig. 1). There was an increased risk of cardiovascular events in participants with AG or AA genotype as compared to those with genotype GG. Moreover, after adjustment for potential confounders including age, sex, BMI, smoking, total cholesterol level, presence or absence of diabetes, and hypertension, the associations also remained significant (hazard ratio, 2.08; 95% CI, 1.12-to 3.84).

### 3.4. Relationship of HDLox with CVD risk factors and CVD risk

Evaluation of association between HDLox and CVD risk markers were revealed that HDLox was reversely correlated with HDL-C level (p = 0.001). Furthermore, our analysis showed positive association of HDLox with triglyceride (p = 0.009), blood glucose (p = 0.001) and

also BMI (p = 0.001). Additionally, hsCRP as an inflammatory biomarker indicated a significant positive association with HDLox (p = 0.02). We also assessed the association of HDLox, a potential marker of CVD, with risk of cardiovascular disease. Multivariate analysis (adjusted for age, sex, BMI, diabetes, hypertension, smoking, and total serum cholesterol) showed a significant positive association of HDLox and CVD risk (OR: 1.7, 95%CI: 1.51–1.89, p = 0.001).

4. Disscussion

In the current study, we found an association between carriers of P376L and increased risk of CVD. Following adjustment for traditional CVD risk factors, the association between heterozygotes of the polymorphism and risk of CVD development remained significant. Heterozygotes at this locus, has a 3.5 fold higher CVD risk over the seven year period of the follow-up. Moreover, we found that patients with a verified diagnosis of clinical CVD had significantly higher HDL lipid peroxidation among those who were heterozygotes for the P376L variant, as compared to non-carriers. Although serum HDL-C concentration among CVD patients with carriers of A allele was higher than non-carriers, there was no significant association between serum HDL-C level and genotypes among CVD and healthy groups.

Furthermore, in contrast to the previous studies indicating an inverse association between serum HDL-C level and CVD outcomes [7], in our study circulating HDL-C were found to be higher among subjects with CVD, as compared with control group. Interestingly, this elevated HDL-C level is restricted to the CVD groups who were carriers of the A allele, although the increased HDL-C observed within CVD subjects was not significant between groups of carriers and non-carriers. In addition,
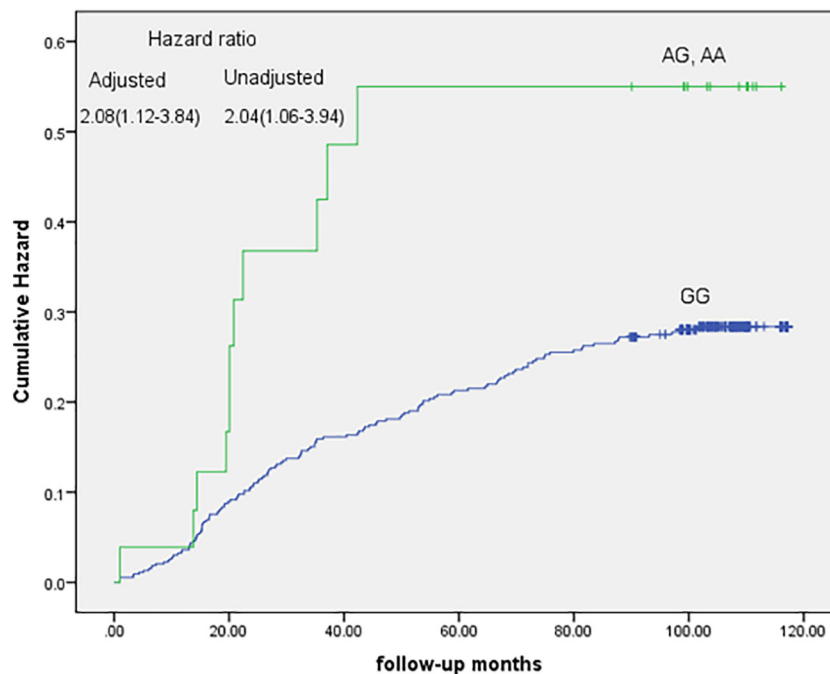
Fig. 1. Kaplan–Meier curve and hazard ratios, derived from Cox proportional-hazards models.
The hazard ratio was adjusted for age, sex, smoking status, body mass index, total cholesterol level, presence or absence of diabetes, and hypertension.

the A allele was associated with higher HDL lipid peroxidation among the CVD subjects with respect to the healthy controls. Lipid oxidation contributes to arterial wall inflammation. Clinical studies indicated that the rate of LDL oxidation and also lipid peroxide formation accelerates in a condition of systemic inflammation [22]. HDL undergoes several changes in response to inflammatory situations, such as altering its lipidome and proteome and increased high levels of lipid peroxides, resulting in dysfunctional HDL [21]. Enhanced oxidation of HDL is associated with a reduced antioxidant activity of HDL [23]. The association between HDLox and CVD has been demonstrated using atherosclerosis animal model and in vivo studies [21,24].

We also found a significant increase in HDLox values about 13% higher in heterozygotes of P376L than among non-carriers. Our findings show that HDL lipid peroxidation may be a useful measure of HDL dysfunction and an important predictor of clinical CVD risk rather than serum HDL-C level. To our knowledge, the association of HDLox and P376L variant has not been previously assessed in a cohort study with available clinical outcomes of CVD. Multiple studies, consistent with our data, indicated the positive relationship between HDLox and hsCRP as an inflammation factor that contributes to CVD development. [25,26]. Regarding to our findings which are concentrated on the association of the genetic variant with clinical features of population, no relationship was observed between hsCRP and studied genotypes.

Design and implementation of very large GWAS in patients has allowed novel opportunities to study the effect of rare genetic variants associated with HDL-C levels and the risk of CVD. The first human heterozygous mutations in SCARB1 were reported in 2011 [18,19]. The participants had high HDL-C levels, altered functions of platelets and reduced adrenal steroidogenesis. The inverse association between serum HDL-C and CVD has been questioned based on recent genetic studies. Mendelian randomization (MR) studies using different variants related to increase HDL-C concentrations has found no association between these variants and a reduced risk of atherosclerosis, suggesting despite of high HDL-C level, subjects with P376L are exposed to elevated risk of coronary artery disease [6]. Our analysis consistently showed that in both unadjusted and adjusted models comprising traditional CVD risk factors, there was twofold increased risk of CVD in heterozygotes for the P376L variant compared to the non-carriers. It can be concluded that positive associations existed among allele A carriers and CVD outcomes comprising myocardial infarction, stable angina, unstable angina or coronary revascularization (percutaneous coronary intervention or coronary-artery bypass grafting).

Daniel Rader and colleagues used next generation sequencing (NGS) of DNA from 328 patients with high HDL-C levels. Their results showed that it is proline 376 exchange with leucine causes a disruption in HDL uptake [6]. Carriers of this variant are associated with high risk for CVD than non-carriers. Additional experimental and in vitro studies confirmed that P376L may affect the function of the SCARBI protein, by impairing post-translational variation with potent impacts on homozygous SCARB1 than in heterozygous ones [27,28]. In addition, by delivering an adeno-related virus gene into the mice with SCARB1 deficiency, wild type of SCARB1 but not P376L, exposed to the loss of function of HDL. Therefore, because of the P376L cell surface reduction, abnormal high HDL-C levels in the homozygote can be explained by defective elimination of HDL-C from the body, while a partial dysfunction in SCARB1 elimination of HDL-C would clarify the moderately increased HDL-C in heterozygotes ones [29]. There are several studies indicate the association of P376L variant with HDL-C elevation [17,30,31]. Notably, this variant primarily appeared in Ashkenazi Jewish people (abundant about 1 in 20 Ashkenazi Jewish people compared with about 1 in 10,000 Europeans Except Ashkenazi Jews) [20,32]. To evaluate the potential relation of P376L with CVD risk, 16 meta-analysis were performed with > 300,000 individuals of case control studies. The results showed a reasonable relationship of the P376L variant in SCARB1 with increased risk of CVD (OR 1.79, P = 0.018). Also, multiple studies showed an apparently 79% raised

coronary risk in P376L carriers compared to non-carriers [5,6,22,33]. These findings support the notion that inactivation of SCARB1, although increasing HDL-C levels, promotes high risk of CVD [5]. Large population studies are required for evaluation of P376L as an important biomarker for CVD risk assessment.

5. Conclusions

This study showed an association between rare variant of P376L in the SCARB1 gene with HDL dysfunction. We have found that carriers of the P376L variant were exposed to higher risk of CVD, as compared to non-carriers in a representative population-based cohort.

Authors' contributions

All authors reviewed, considered and approved the manuscript.
Conflict of interest: The authors have no conflict of interest to disclose.

All authors have approved the final version of this article. SS, GAF, MGM and AA designed the experiments and revised the manuscript. ZF, MM, HFS performed the experiments. SS, ZSH wrote the manuscript. HE, AHM and MT carried out the data analysis.

Declarations of interest

None.

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