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Screening of LDLR, APOB and TNNI3 Genes in Cardiovascular Patients in the Population of Indian Origin

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Abstract---

Background: CVD accounts for 31% of mortality, the majority of this in the form of CHD and cerebrovascular accident, the majority of this in the form of CHD and cerebrovascular accident.

Methods: Institutional Human Ethics committee approval was taken for the current study from Bharti hospital and Savitribai Phule Pune University. DNA was extracted from whole blood and PCR conditions were optimized for all exons, SSCP was done for screening of variations in all the exons of APOB, LDLR and TNNI3 gene. Samples showing abnormal band shifts with respect to control were Sanger sequenced for confirmation of variation. Biochemical estimations were done by commercially available kits.

Results: SSCP analysis reveals a band shift in exon 05 of APOB gene with respect to control samples on 8% gel concentration in proband 16 while other exons of APOB gene did not show any band shift with respect to control samples. T>G transversion at Chr. Location 55663368 of Intronic variant was located in exon 08 of proband 13by screening of all exons of TNNI3 gene. Sequences revealed. Patient showing G/A variation shows LVEF of 47% while CVD patients without variation shows 43%. The level of NO in control was lowest than MI level and IHD level but MI patients shows highest level of NO.

Conclusion: A reported silent mutation was found in intronic region without impact on splicing. This variation did not show a profound impact on physiological complication as compared to mutation negative probands.

Key words--- *Cardiac disorders, splicing, APOB, LDLR*

Acronyms---

CVD: Cardiovascular diseases

LDL: Low density lipoprotein

LDLR: Low density lipoprotein receptor

SDS: Sodium dodecyl sulphate

PCR: Polymerase chain reaction

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I. INTRODUCTION

Cardiovascular disease (CVDs) is a term used to describe a wide range of diseases that affects cardiovascular system i.e. heart and its blood vessels. CVDs are one of the leading health causes of disability and mortality worldwide (Mendis *et al.*, 2011). According to global burden disease report (GBD, 2011) in 1990 out of total 9.4 million deaths in India 2.3 million deaths i.e. 25% were due to cardiovascular diseases. According to World Health Organization in 2008, 17.3 million CVD deaths occurred globally (Mendis *et al.*, 2011). The Indian subcontinent region has the highest burden of CVD in the world. Cardiac disease prevalence is increasing at alarming rate in Indian subcontinent (Goyal *et al*., 2006). Various diseases are included under the umbrella of cardiovascular disease such as heart attack, stroke, heart failure, myocardial infarction, acute coronary disease, ischemic heart diseases, coronary artery disease, etc. INTERHEART and INTERSTROKE studies suggested that obesity, smoking, hypertension, lifestyle, psychological factors, metabolic disorders, poor diet, physical activities, alcohol consumption, diabetes, dyslipidaemia etc. are the most common risk factors for cardiovascular diseases worldwide (O'Donnell *et al*., 2010; Yusuf *et al*., 2004). Cardiovascular diseases affect Indian population a decade earlier, mainly in their most productive midlife years as compared to European population (Joshi *et al*., 2007; Xavier *et al*., 2008). A single 16 kbp long APOB gene transcript is present from 29 exons. This transcript have two isoforms of APOB protein: APOB-48 and APOB-100. Various health conditions are related to mutation in APOB gene such as Familial Hypobetalipoproteinemia (Kane, 1995; Schonfeld, 2003; Aggerbeck *et al*., 1992; Whitfield *et al*., 2003).Low density lipoprotein receptor (LDLR) is a membrane bound receptor maintaining cholesterolhomeostasis along with Apolipoprotein B (APOB), andother genes of lipid metabolism. LDL receptor is cysteine-rich protein in which disulphide bonds between two cysteine's are required for correct folding of 10 major modules necessary for protein functioning (Russell *et al*. 1989, Kurniawan *et al*. 2001). Endocytosis of cholesterol-rich LDL is mediates by LDL receptor and thus maintains the plasma level of LDL (Leren, 2014). Liver is the main site which removes ~70% of LDL from the circulation. LDLR has been identified as the primary mode of entry for the Vesicular stomatitis virus in mice and humans (Finkelshtein *et al*., 2013).

This study focuses on the distribution of gene variations in APOB, LDLR and TNNI3 genes in cardiac patients. The current study emphasizes estimation of marker enzymes and their correlation with pathophysiology of heart in Pune population.

II. MATERIAL AND METHODS

A) Approval from Ethics Committee and sample collection:

Institutional Human Ethics committee approval was granted for the present study from Bharti hospital, Katraj, Pune (DCG1 Reg. no. ECR518) and Savitribai Phule Pune University.

B) Inclusion criteria:

All CVD patients were examined by trained cardiologist for 2-D echo-cardiography. HCM patients showing interventricular septum of 12mm and more were taken as positive for HCM for the current study. Healthy donors were considered after normal ECG and without family history of CVDs.

C) Exclusion criteria:

Patients suffering from renal dysfunction, cancer, diabetes and hepatic dysfunction were excluded from current study.

DNA Extraction from Blood samples:

DNA was extracted from 300µl of blood sample by the method of phenol: chloroform: isoamyl alcohol method. Briefly 800µl of 1X SSC (saline-sodium citrate) buffer was mixed with 300 µl of blood. Samples were centrifuged at 10,000 rpm for 2 minutes at room temperature. 20µl of 10% SDS and 10ul proteinase K was also added to the sample and pipetting was done back forth to mix the constituents. Samples were incubated at 55^{o}C for 1 hour and phenol chloroform isoamyl alcohol was added to the solution and vortexed for 30 sec. the constituents were mixed thoroughly until DNA strands are visible.

D) Primer designing and PCR amplification:

Primer were designed for exon 2–20 in APOB gene and 1-18 exons for LDLR gene and all exons of TNNI3 gene. Primer sequences and PCR conditions are available on request.

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E) Single Strand Conformational Polymorphism (SSCP) and PAGE:

Single Strand Conformational Polymorphism analysis was done for PCR amplified product on 8% and 10% polyacrylamide gels. Each exon was optimized on different conditions for better results.

F) Silver staining of polyacrylamide gels:

Silver staining was done as per the method of Bassam and Gresshof (2007). All the reagents were of Merck Millipore U.S.A make and were of higher purity grade.

G) PCR purification and Sanger sequencing:

Samples showing abnormal bandshift with respect to control samples were forwarded for PCR purification by Sure Clean Plus purification kit (Bioline, India). Samples were then visualized on 2% agarose gels andwere sequenced on ABI 3730XL capillary DNA sequencer.

H) Statistical analysis:

Statistical analyses were performed by PAST3 and graphical representation in Graph Pad Prism 7. Data was expressed as mean ±SD. One-way ANOVA was used for statistical significance. $P< 0.05$ was considered significant for current study.

III. RESULTS

Hypertension (25.6%) was the most frequent comorbidities associated with CVD patients in this population. Other comordities include sinus tachycardia (13.6%), mitral regurgitation (11.36%) and dyspnea (13.6%) (Table 01). SSCP analysis reveals a band shift in exon 05 of APOB gene with respect to control samples on 8% gel concentration in proband 16 while as other exons of APOB gene did not show any band shift with respect to control samples (Fig.03). LDLR exons did not show any variation in any exons in this population. Screening of APOB gene for first 20 exons revealed a transition in exon 5 (G/A) at g.10952. This variation is already reported in status as per dbSNP (rs758633082) and is present in intronic region. Screening of TNNI3 gene in HCM patients shows an aberrant band in exon 08 of proband 13. Samples were sequenced bi-directionally on ABI 3730XL capillary DNA sequencer. Sequences revealed T>G transversion at Chr. Location 55663368 of Intronic variant (Fig. 04).

Table 01: Prevalence percent of most frequent comorbidities in CVD patients.

Complications	**Prevalence percent**
Sinus tachycardia	13.63
Mitral Regurgitation	11.36
Dyspnea	13.63
Hypertension	25.64

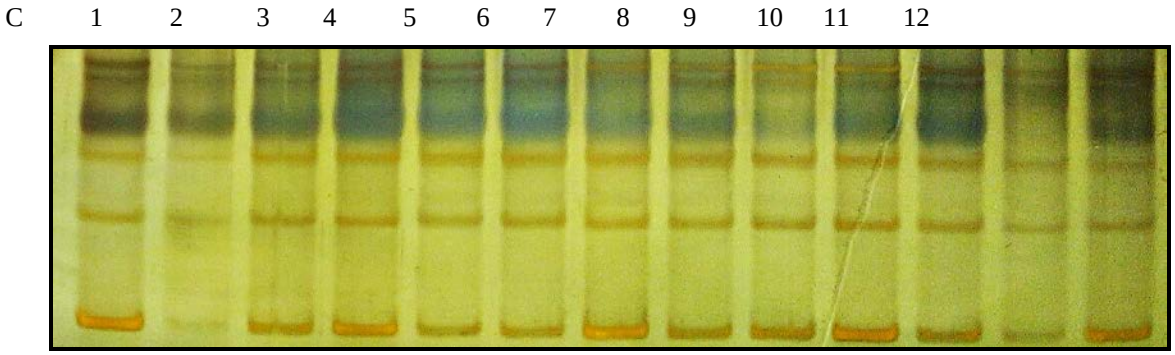

Figure 01: SSCP analysis in exon 8 of APOB gene at 8% PAGE showing no band shift as compared to control samples.

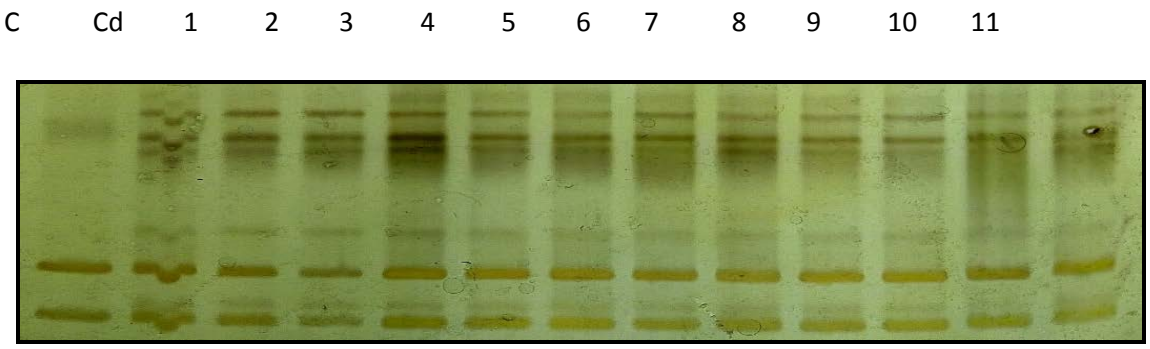

Figure 02: SSCP analysis of exon 13 of APOB gene at 10% PAGE shows no band shift as compared to control samples.

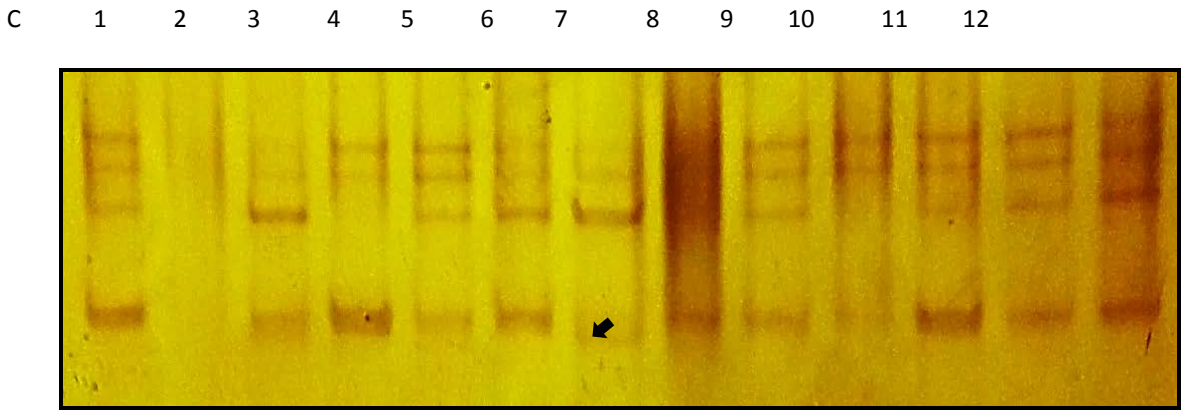

Figure 03: SSCP of exon 05 of APOB gene at 8% gel concentration showing band shift in proband 06 as compared to control samples.

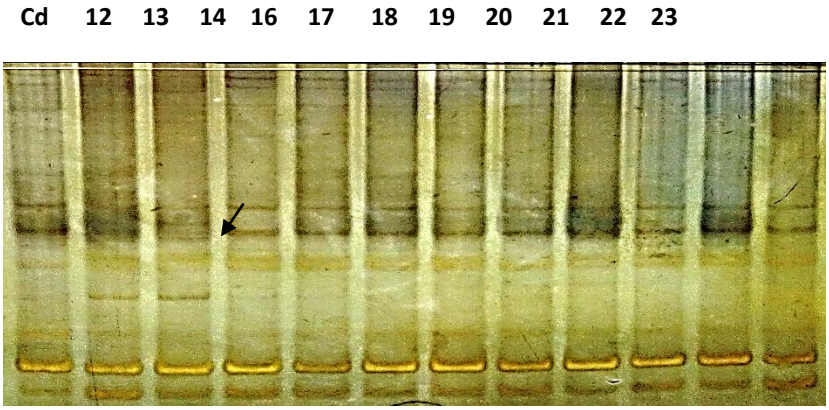

Figure 04: SSCP gel image of exon 08 proband 13. Arrow shows aberrant band shift as compared to control

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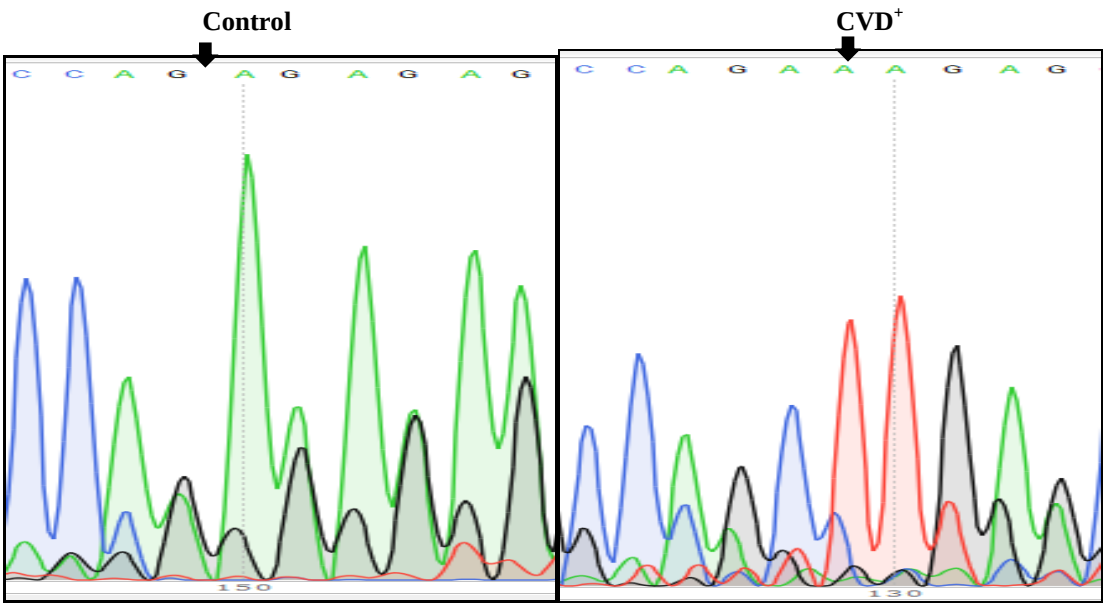

Figure 05: Electropherogram shows variation in APOB gene (G/A) at g.10952.

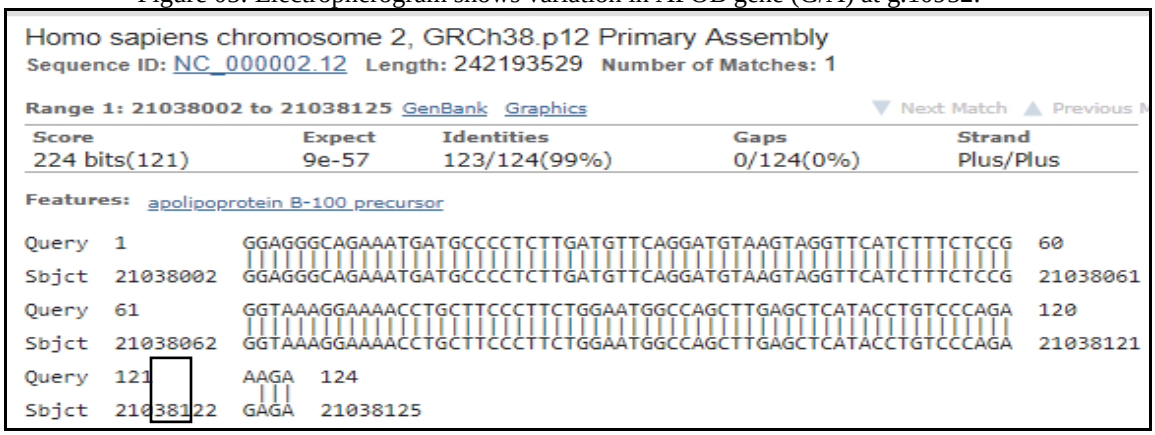

Figure 06: NCBI Blast analysis of exon 5 of APOB gene.

Human splicing finder (HSF V 3.1) did not reveal any splicing changes in exon splicing enhancers and exon splicing silencers by this variation.

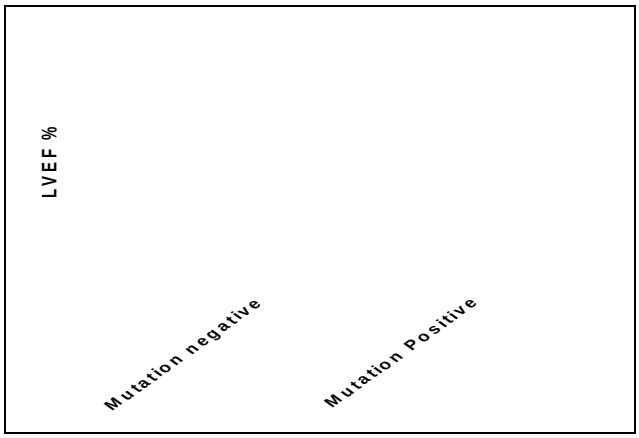

Figure 07: Impact of gene variation on ejection fraction of heart.

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Patient showing the G/A variation shows LVEF of 47% while CVD patients without variation shows 43%. This variation have small impact of functioning of ventricles may be due to presence in intronic region (Fig. 06). Screening of all the exons of LDLR in this population on 8% and 10% gel concentration did not reveal any variation in cardiac patients which may be due to the highly conserved nature of gene (Fig. 07).

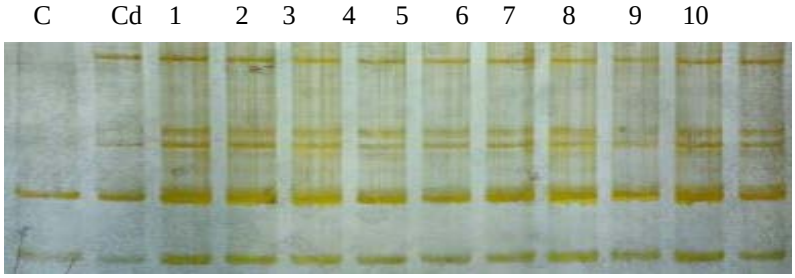

Figure 08: SSCP analysis of exon 9 in LDLR gene at 10% PAGE in 10CVD probands.

NO (nM/L) estimation was performed for control samples and disease patients. The level of NO in control was lowest than MI level and IHD level but MI patients shows highest level of NO (nM/L). MI stage is severe type of condition in which coronary arteries are deprived of oxygen. Higher levels of nitric oxide synthase have been reported in patients with chronic heart failure (Fig. 08).

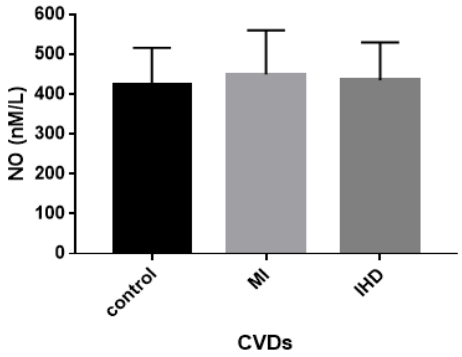

Figure 09: Estimation nitric oxide in plasma samples of CVD patients and control samples.

MI patients significantly show higher levels of CPK as compared to IHD patients and control samples (*p<0.05) (Fig. 09). CPK are the marker enzymes of heart pathophysiology. Higher levels of CPK represents greater amount of injury thus MI patients are at higher risk as compared to IHD patients.

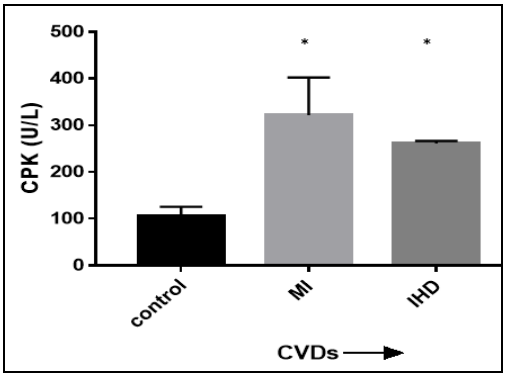

Figure 10: Estimation of CPK levels in plasma samples in control and CVD patients.*p<0.05.

IV. DISCUSSION

In our present study, screening of APOB gene revealed intronic variation in exon 5. G>A transition in Exon 5 resulted in a previously reported silent mutation at chromosome position 21260994. After the 30 to 39-years of age

in Indian population, age-specific trends in cardiovascular risk factors among the adolescent and youngreveals as cardiovascular risk factors increase exponentially (Gupta *et al.*, 2009). An additional cause of concern inIndians and South Asians is that they tend to have more severe manifestations of CVD andhigher fatality rates (Yusuf *et al*., 2014). It has been reported that apolipoprotein B could be a better risk predictor of cardiovascular diseases than concentrations of cholesterol in the LDL fraction (Pierre *et al*., 2006; Talmud *et al*., 2002; Ingelsson *et al*., 2007; McQueen *et al*., 2008). The probable explanation for this is that each atherogenic particle (chylomicrons, VLDL, IDL, LDL, etc.) contain only single molecule of APOB, therefore it is the measurement of total atherogenic particles in the body (Elovson *et al*., 1988).Left Ventricle Ejection Fraction is the measurement of percentage of blood being pumped from left ventricle during each heartbeat. The normal value of LVEF range from 55% to 65% (Poppe*et al*., 2013). The individuals suffering from hypertension and having LVEF value less than 50% are at 10 fold higher risk of heart failure than the individual with LVEF value greater than 50% (Verdecchia *et al*., 2005). In our study it was found that mutation positive patient have higher LVEF value than mutation negative. The mutation positive patient was suffering from long term hypertension and also with myocardial infarction and this was consistent with the previous studies (Verdecchia *et al*., 2005). In the current study the most admitters of CVDs were MI as seen more in males and less in females and also IHD percentage was more in males than females. Significant rises in total serum CPK activity after cardiac catherization have been reported frequently (Chahine *et al*., 1974; Harrison *et al*., 1972). Nitric oxide (NO) is a key signaling messenger in the cardiovascular system. (Bredt *et al*., 1994). NO is produced in cardiac smooth muscle, where it regulates cardiac contractility. Adequate levels of endothelial NO are important to preserve normal vascular physiology—in the face of diminished NO bioavailability, there is endothelial dysfunction, leading to increased susceptibility to atherosclerotic disease. (Grange *et al*., 2001).

V. CONCLUSION:

Hypertension is the most frequent secondary complication associated with CVD in this population. Sinus Tachycardia and Dyspnea were equally distributed in cardiac disorder patients. A reported silent mutation was found in intronic region without impact on splicing. This variation did not show a profound impact on physiological complication as compared to mutation negative probands. Thus APOB and LDLR gene mutations do not show any significant contribution in development of CVD in the selected population. However, further investigation with large sample size is suggested.

Conflict of Interest: None declared

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