# Association of ABCA1 R219K and C69T single nucleotide polymorphisms with type 2 diabetes mellitus: A case control study

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**Abstract.** Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder associated with alterations in the metabolism of carbohydrates, proteins and lipids. ATP binding cassette transporter 1 (ABCA1) encoding the ABCA1 protein plays a critical role in the reverse cholesterol transport of high-density lipoprotein-cholesterol (HDL-c) metabolism. ABCA1 gene single nucleotide polymorphisms (SNPs) have been found to be associated with abnormalities in the serum levels of HDL-c, thereby disrupting the cholesterol removing ability of cells. The aim of the present study was to determine the association between the R219K and C69T SNPs of ABCA1 gene variants and T2DM. For this purpose, a case control study was conducted among 50 patients with T2DM who were selected as cases, and 50 age- and sex-matched patients without comorbidities were selected as the controls. Venous blood samples were collected from all participants following overnight fasting. Plasma glucose, glycated hemoglobin (HbA1c) and lipid profile estimations were performed using an autoanalyzer with standard methods. ABCA1 gene polymorphisms were determined using the polymerase chain reaction-restriction fragment length polymorphism method. The body mass index for both the case and control groups was found to be above the revised cut-off value for obesity. HDL-c levels were below the optimum level in the case and control group. The distribution of genotype and dominant alleles of the C69T and R219K SNPs of the ABCA1 gene did not differ significantly between the case and control groups. The present study suggests the existence of a defective ABCA1 genotype in the studied population, predisposing these individuals to a decrease in serum HDL-c levels. This interferes with cholesterol sequestration from foam cells and creates an inflammatory state, which is aggravated by obesity. Thus, HDL-c becomes dysfunctional from inflammation-mediated structural modifications and paves the way for atherogenic dyslipidemia associated with T2DM.

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*Key words:* diabetes mellitus, single nucleotide polymorphism, ATP binding cassette transporter 1, R219K, C69T

## Introduction

Diabetes mellitus (DM), the foremost non-communicable disease is a complex metabolic disorder related to alterations in the metabolism of carbohydrates, proteins and lipids (1). Type 2 DM (T2DM) is evolving as the major cause for morbidity and mortality in India and is considered as the equivalent of cardiovascular disease due to its principal complication, atherosclerosis, ensuing from dyslipidemia. As regards the Indian population, epidemiological studies on dyslipidemia are inadequate (2). The South Asian population which encompasses Indians, has been found to have atherogenic dyslipidemia, that is characterized by decreased levels of high-density lipoprotein cholesterol (HDL-c) and increased triglycerides (TGL) which are associated with T2DM (3).

In India, with the rapid increase in the population, urbanization and lifestyle changes, including an unhealthy diet and sedentary habits are paving way for dyslipidemia. According to the ICMR INDIAB phase I study, 79% of the Indian population have fasting dyslipidemia with low HDL-c levels, accounting for 72.3% of the population (4). HDL-c maintains the intracellular cholesterol homeostasis through a process known as reverse cholesterol transport, through which excess cholesterol is removed from peripheral tissues. The important steps involved in the reverse cholesterol pathway are the following: i) The HDL-c-mediated uptake of cellular cholesterol; ii) the esterification of cholesterol present in HDL-c-mediated by lecithin cholesterol acyl transferase; and iii) the confiscation of cholesterol esters from HDL-c by the liver for excretion through bile (5). It has been discovered that a protein, namely ATP-binding cassette transporter A1 (ABCA1), plays a crucial role in the first step of reverse cholesterol transport.

ABCA1 belongs to a family of proteins that pair the hydrolysis of ATP to the binding of a substrate, thereby enabling its transport through the plasma membrane. The ABCA1 protein is coded by a gene mapping to chromosome 9 in humans (6). It is made up of two transmembrane domains, two nucleotide binding domains, two regulatory domains and two extracellular domains (ECDs). The ECD has a hydrophobic hollow interior, which serves as a potential passage for the transportation of lipids to apolipoprotein A-I (ApoA-I) from the interior of the membrane. Lipid flopping mechanism causes conformational

change, to facilitate the delivery of the lipids to the ECD from the transmembrane cavity. It is hypothesized that ABCA1 and the lipid bilayer bind cooperatively with ApoA-I and deliver the substrate for nascent HDL-c formation (7).

According to a meta-analysis of proteomic and genomic studies, it was found that an impairment in the ABCA1 pathway attributed to the quantitative decline in HDL-c levels, causing the intracellular accumulation of lipids (8). Therefore, the aim of the present study was to determine the association between single nucleotide polymorphisms (SNPs) of the ABCA1 gene and T2DM.

## Patients and methods

A case control study was conducted among 50 patients with T2DM selected as the cases and 50 age- and sex-matched patients without comorbidities were selected as the controls. An ethical clearance was obtained from the Institutional Human Ethics Committee of the PSG Institute of Medical Sciences and Research, Coimbatore, India (Ref. no. 15/376). The diagnosis of T2DM was based on the American Diabetic Association (ADA) criteria (1). Patients satisfying the diagnostic criteria were provided with an explanation of the study. A written informed consent was acquired from the patients for the use of their samples for scientific research, prior to the collection of blood samples. The samples were collected between June, 2016 to June, 2017. Both males and females aged >30 years, diagnosed with T2DM with a maximum duration of 2 years on treatment (oral drugs/insulin) were included as the cases. Subjects with other malignancies, type 1 DM, T2DM with microvascular and macrovascular complications, acute illnesses, genetic malformations and pregnancy were excluded from the study.

Venous blood samples were collected after following overnight fasting and were processed for the estimation of routine parameters, including fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), total cholesterol, high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c) and triglycerides (TGL). The samples for DNA extraction were collected in BD vacutainer® EDTA tubes (BD Biosciences) and transferred to a labeled, sterile cryovials and stored at -80˚C until analysis. HbA1c levels were measured using turbidimetric inhibition immunoassay. Plasma glucose and lipid profile estimations were performed on a Cobas Integra 400 analyzer (Roche Diagnostics) using standard methods.

*SNP genotyping*. The SNPs in the ABCA1 gene, namely R219K (rs2230806) and C69T (rs1800977) were selected for investigation in the present study based on population genetics. The mutation in the R219K polymorphism was (G→A), whereas for C69T it was (C→T). DNA extraction was performed using a kit from Bio Basic, Inc. (EZ-10 Spin Column Genomic DNA Minipreps kit). The quality of the DNA was assessed using 0.8% agarose gel electrophoresis and viewed using a Gel Doc system (Syngene). DNA was quantified using a NanoDrop UV absorption spectrophotometer (Thermo Fisher Scientific, Inc.) at A260.

The nucleotide sequences of the two SNPs were determined, and forward and reverse primers for the flanking sequence of these nucleotide sequences were designed and were as follows: R219K forward, 5'-CCTCTTGTGCTTGTC TCTCTTTGCATG-3' and reverse, 5'-TTGGCTTCAGGATGT CCATGTTGG-3'; C69T forward, 5'-CAGCGCTTCCCGCGC GTCTTA-3' and reverse, 5'-CCACTCACTCTCGTCCGC AATTAC-3'. DNA amplification by polymerase chain reaction (PCR) was carried out in a DNA Thermal Cycler (Eppendorf). Reactions were performed with 1 $\mu$mol of each of the primers of R219K and with 10 $\mu$mol of each of the primers of C69T. The total reaction volume was 20 $\mu$l as follows: Forward primer, 1 $\mu$l; reverse primer, 1 $\mu$l; Taq DNA Polymerase master mix RED (Ampliqon A/S), 10 $\mu$l; DNA, 0.8 $\mu$l; and milli Q water (Merck KGaA), 7.2 $\mu$l. The annealing temperature was standardized at 60˚C for the primers of R219K and at 63.5˚C for the primers of C69T. The PCR program was performed as follows: Initial denaturation at 94˚C for 2 min, cycle denaturation at 94˚C for 30 sec, annealing for 45 sec, extension at 72˚C for 45 sec, and a final extension at 72˚C for 2 min. A total of 35 cycles were run, and the PCR product was held at 4˚C till it was removed. PCR amplification products were confirmed using 2% agarose gel electrophoresis. The PCR product size was determined [211 base pairs (bp) for SNP R219K and 345 bp for SNP C69T] using the Gel Doc system (Syngene) by comparing with a 100-bp DNA ladder (Invitrogen; Thermo Fisher Scientific, Inc.).

For restriction fragment length polymorphism, 1 $\mu$l of PCR product was mixed with 0.8 $\mu$l of restriction enzyme, 5 $\mu$l of 10X NE buffer and the reaction volume was made to 50 $\mu$l with milli Q water. The PCR product of SNP R219K was digested with *Eco*NI (*Xag*I) and incubated at 37˚C for 1 h and was inactivated at 65˚C for 20 min. The PCR product of SNP C69T was digested with *Bsm*AI and incubated at 55˚C for 1 h with no inactivation. Samples along with the 100 bp ladder and undigested PCR product were run on a 15% polyacrylamide gel for the analysis of restriction fragments. The gel was stained using ethidium bromide and the fragments were visualized using a Chemiluminescence Gel Doc System (Figs. 1 and 2).

Restriction did not occur when the recessive G allele was present, yielding a 211-bp fragment (GG-homozygous wild-type), whereas restriction occurred in the presence of the dominant A allele, yielding 149- and 61-bp fragments (AA-homozygous mutant) and in the presence of both the A and G allele, yielding fragments of 211 + 149 + 61 bp (AG-heterozygous wild-type).

Restriction did not occur when the recessive C allele was present, yielding a 345-bp fragment (CC-homozygous wild-type), whereas restriction occurred in the presence of the dominant T allele, yielding a 310-bp fragment (TT-homozygous mutant), and in the presence of both the C and T allele, yielding fragments of 310 + 35 bp (CT-heterozygous wild-type).

*Statistical analysis*. Statistical analysis was performed using R i386 3.6.3 software for Windows. Continuous variables are presented as the mean ± standard deviation. Categorical variables are presented as frequency tables. Continuous data were compared using an independent t-test/Welch's t-test or the Mann-Whitney U-test. Categorical data were compared using Chi squared test and the data that did not follow the assumptions of Chi squared test were analyzed using Fisher's exact test. Odds ratios (ORs) with 95% confidence intervals

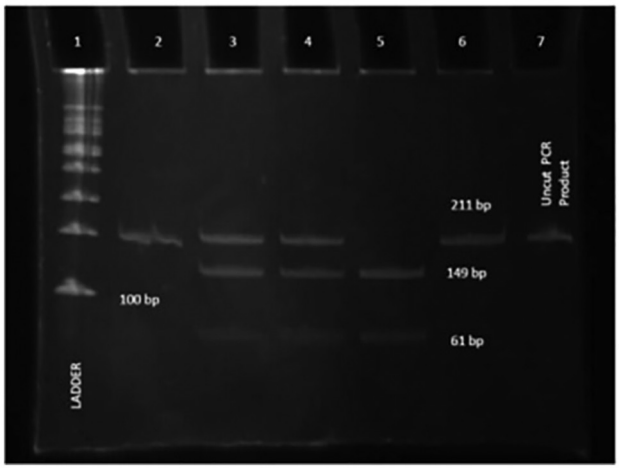


Figure 1. Gel image depicting the restriction fragments of the R219K single nucleotide polymorphism. Lane 1, DNA ladder; lanes 2 and 6, GG; lanes 3 and 4, AG; lane 5, AA; lane 7, uncut PCR product.

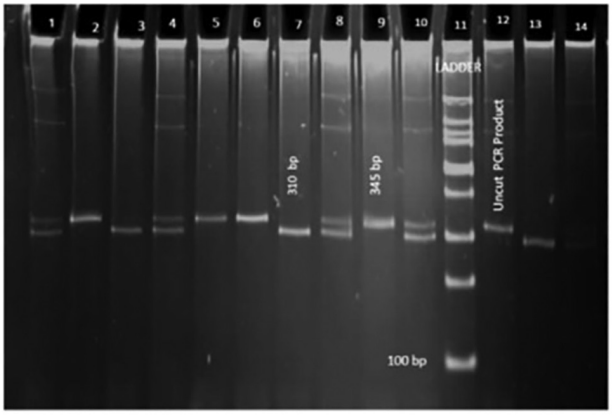


Figure 2. Gel image depicting the restriction fragments of the C69T single nucleotide polymorphism. Lanes 1, 4, 8, 10 and 14, CT; lanes 2, 5, 6 and 9, CC; lanes 3, 7 and 13, TT; lane 11, DNA ladder; lane 12, uncut PCR product.

(95% CIs) were used to assess the strength of the association between the R219K and C69T ABCA1 SNPs, and T2DM.A value of P<0.05 was considered to indicate a statistically significant difference.

## Results

A total of 100 subjects consisting of 50 patients in each group were used in the present study. The demographic, anthropometric and laboratory data of the cases and controls are presented in Table I. The mean age of the subjects in the case and control group was 54.08 and 53.9 years, respectively. The mean body mass index (BMI) of the subjects in the case and control group was 26.51 and 27.48 kg/m$^2$, respectively. The mean FPG levels were significantly higher in the cases than in the controls and the distribution of HbA1c differed significantly between the case and control group. In addition, the mean LDL-c levels were significantly higher in the cases compared with the controls.

The present study found that the distribution of the CC, CT and TT variants of the C69T genotype was 30, 52 and 18% in the case group, and 32, 58 and 10% in the control group, respectively. In addition, the distribution of the AA, AG and GG variants of the R219K genotype was 14, 44 and 42% in the case group, and 8, 60 and 32% in the control group, respectively. Using the Chi-squared test, it was noted that the distribution of the C69T and R219K SNPs did not differ significantly between the two groups (Fig. 3 and Table II).

Using Fisher's exact test, it was also that the dominant T allele of the C69T genotype and the dominant A allele of the R219K genotype were not significantly associated with the case group in the different additive models (Table III).

In addition, it was observed that the mean levels of total cholesterol, HDL-c, TGL and LDL-c did not differ significantly between the mutant and non-mutant variants of both the C69T and R219K genotypes in the case and control groups (Table IV).

Table I. Summary of the demographic and lipid profile data of the study participants.

| Parameter | Cases (n=50) | Controls (n=50) | P-value |
|---|---|---|---|
| Age (years) | 54.08±10.82 | 53.9±10.99 | 0.9344$^{T}$ |
| Sex | | | |
| Male | 28 (56%) | 29 (58%) | 0.8399$^{C}$ |
| Female | 22 (44%) | 21 (42%) | |
| BMI (kg/m$^2$) | 26.51±3.48 | 27.48±2.63 | 0.1209$^{T}$ |
| WHR | 0.91±0.09 | 0.92±0.08 | 0.4502$^{T}$ |
| FPG (mg/dl) | 137.36±32.68 | 100.78±8.8 | <0.0001$^{M}$ |
| HbA1c (%) | 7.64±1.5 | 5.77±0.38 | <0.0001$^{M}$ |
| Total cholesterol (mg/dl) | 186.36±40.15 | 186.8±28.01 | 0.9494$^{T}$ |
| HDL-c (mg/dl) | 41.44±8.67 | 42.28±10.12 | 0.6567$^{T}$ |
| TGL (mg/dl) | 134.62±48.16 | 130.3±48.59 | 0.6562$^{T}$ |
| LDL-c (mg/dl) | 127.46±32.96 | 116.26±21.3 | 0.0234$^{WTO}$ |

The superscript letters next to the P-values indicate the following: T, t-test; WT, Welch's t-test; M, Mann-Whitney U-test; C, Chi-squared test; O, one-tailed test. BMI, body mass index; WHR, waist-to-hip ratio; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; HDL-c, high-density lipoprotein cholesterol; TGL, triglycerides; LDL-c, low-density lipoprotein cholesterol.

Table II. Comparison of genotypes of R219K and C69T polymorphisms among the study groups.

| Genotype | Sub-category | Cases, n (% ) | Controls, n (%) | P-value |
|---|---|---|---|---|
| C69T | CC | 15 (30%) | 16 (32%) | 0.512 |
| | CT | 26 (52%) | 29 (58%) | |
| | TT | 9 (18%) | 5 (10%) | |
| R219K | AA | 7 (14%) | 4 (8%) | 0.2561 |
| | AG | 22 (44%) | 30 (60%) | |
| | GG | 21 (42%) | 16 (32%) | |

Table III. Effects of dominant allele of genotypes of SNP R219K and SNP C69T among the study groups.

| Allele and model | Cases, n (%) | Controls, n (%) | OR (95% CI) | P-value |
|---|---|---|---|---|
| C69T additive model 1 | | | | |
| TT | 9 (18%) | 5 (10%) | 1.98 (0.61-6.38) | 0.3881 |
| CT + CC | 41 (82%) | 45 (90%) | | |
| C69T additive model 2 | | | | |
| CT + TT | 35 (70%) | 34 (68%) | 0.91 (0.39-2.13) | >0.99 |
| CC | 15 (30%) | 16 (32%) | | |
| R219K additive model 1 | | | | |
| AA | 7 (14%) | 4 (8%) | 1.87 (0.51-6.85) | 0.5246 |
| AG + GG | 43 (86%) | 46 (92%) | | |
| R219K additive model 2 | | | | |
| AA + AG | 29 (58%) | 34 (68%) | 1.54 (0.68-3.49) | 0.4076 |
| GG | 21 (42%) | 16 (32%) | | |

CT + CC and CT+ TT, and AG + GG and AA + GG were considered as references and the control was considered as a reference while calculating the OR values. Data were analyzed using Fisher's exact test. OR, odds ratio; 95% CI, 95% confidence interval.

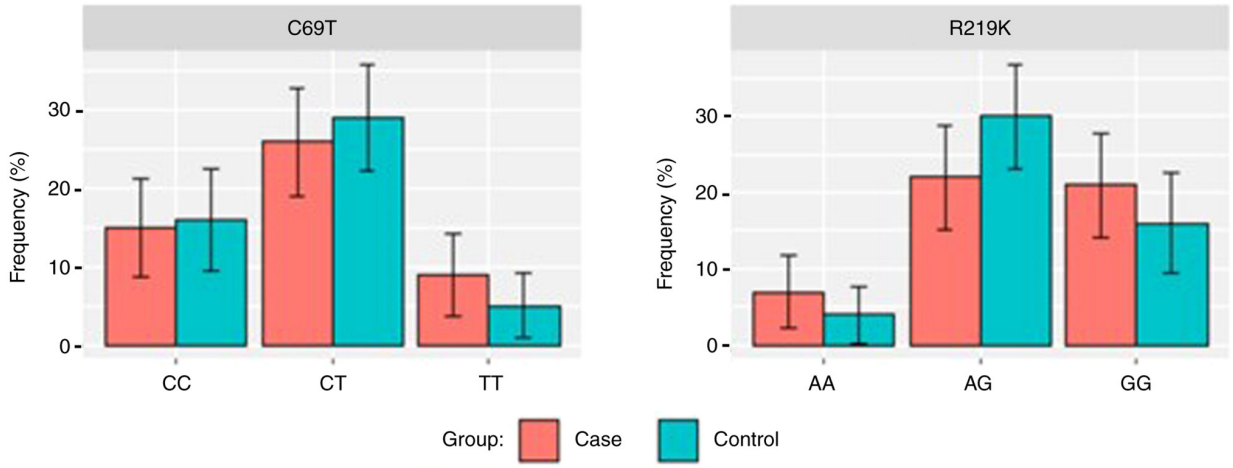


Figure 3. Distribution of genotypes of R219K and C69T polymorphisms among the study groups.

## Discussion

The global prevalence of T2DM has markedly increased; thus, it is critical to carry out research to assess the risk factors and multigenetic factors associated with this condition. The most severe complication of T2DM is atherogenesis ensuing from dyslipidemia (9). One of the aberrations in lipid metabolism commonly encountered in T2DM is a decrease in HDL-c levels. The association between the genetic polymorphism in the ABCA1 gene and the decrease in HDL-c levels has been demonstrated in recent years (10). A systematic review and meta-analysis on studies that determined the association between the ABCA1 gene polymorphism and T2DM, revealed inconsistent results (11). The divergence may be due to the influence of ethnicity, genetic susceptibility, and environmental factors.

One of the contributing factors for dyslipidemia is obesity and the association between obesity and dyslipidemia is illustrious. Recently, there has been a revision in the cut-off value for obesity for Asian Indians, and as per this revision, a BMI ≥25.0 kg/m$^2$ is defined as obese (12). According to this criterion, both the patients with T2DM and the controls in the present study fell under the obese category, as reflected by their mean BMI values (Table I). In addition to this, the mean waist-to-hip ratio of both the groups was above the cut-off value for obesity as per the WHO (13) (Table I). This reveals the high prevalence of obesity among the Indian population, which is in agreement with the study conducted by Ahirwar and Mondal (14).

In the present study upon assessing the lipid profile parameters between the cases and controls, it was found that the mean LDL-c values were significantly higher in the cases than in the controls (Table I). This may be explained by the decrease in the insulin-mediated expression of LDL-c cell surface receptors in T2DM, which causes a decrease in the clearance of LDL-c, thereby contributing to these increased levels (15).

Table IV. Comparison of lipid profile parameters of the R219K and C69Tgenotypes among the cases and controls.

C69T

| | Cases | | | Controls | | |
|---|---|---|---|---|---|---|
| Lipid profile parameters | Mutant | Non-mutant | P-value | Mutant | Non-mutant | P-value |
| Total cholesterol (mg/dl) | 179.78±33.07 | 187.80±41.76 | 0.5922[T] | 177.00±25.14 | 187.89±28.35 | 0.4151[T] |
| HDL-c (mg/dl) | 44.11±9.87 | 40.85±8.40 | 0.3121[T] | 41.80±8.17 | 42.33±10.39 | 0.9123[T] |
| TGL (mg/dl) | 133.00±53.48 | 134.98±47.63 | 0.9126[T] | 104.60±7.99 | 133.16±50.39 | 0.2071[M] |
| LDL-c (mg/dl) | 121.89±19.83 | 128.68±35.27 | 0.5808[T] | 121.4±15.21 | 115.69±21.93 | 0.3003[M] |

R219K

| | Cases | | | Controls | | |
|---|---|---|---|---|---|---|
| Lipid profile parameters | Mutant | Non-mutant | P-value | Mutant | Non-mutant | P-value |
| Total cholesterol (mg/dl) | 177.86±49.27 | 187.74±38.98 | 0.5511[T] | 192.75±31.57 | 186.28±28.00 | 0.6624[T] |
| HDL-c (mg/dl) | 38±.05 | 42±8.85 | 0.2617[T] | 47±6.22 | 41.87±10.33 | 0.3359[T] |
| TGL (mg/dl) | 122.14±39.52 | 136.65±49.53 | 0.4655[T] | 135.50±59.12 | 129.85±48.32 | 0.7206[M] |
| LDL-c (mg/dl) | 122.29±32.63 | 128.30±33.32 | 0.6589[T] | 100.5±5.07 | 117.63±21.64 | 0.124[T] |

The superscript letters next to the P-values indicate the following: T, t-test; M, Mann-Whitney U-test. R219K mutant, AA; R219K non-mutant, AG + GG; C69T mutant, TT; C69T non-mutant, CT + CC; HDL-c, high-density lipoprotein cholesterol; TGL, triglycerides; LDL-c, low-density lipoprotein cholesterol.

According to the National Cholesterol Education Program (NCEP) ATP III guidelines, the optimal HDL-c level is 50 mg/dl (16). When the levels of HDL-c decrease, it is considered as a risk factor for the development of cardiovascular disease (17). In the present study, the HDL-c levels were low in both the cases and the controls (Table I). It is often considered that this decrease in the levels of HDL-c is due to the action of cholesterol ester transfer protein (CETP), which enhances the formation of TGL-rich HDL-c, thereby priming for HDL-c catabolism (18). However, CETP is only partially responsible for the decrease in HDL-c levels, as it has been previously reported that genetic variations in the ABCA1 gene are associated with decreased serum HDL-c levels (19).

In the present study, the genotypic distribution of the ABCA1 gene for the R219K and C69T SNPs did not reveal any statistically significant differences in the distribution of homozygous mutants (AA and TT) among the cases when compared to the controls (Table II). Additionally, additive models for ORs were used to determine whether the presence of the dominant allele alone (the A allele in R219K and the T allele in C69T) is sufficient to cause pathological changes in HDL-c metabolism in T2DM (Table III). No statistically significant differences were observed between the case and control groups, depicting that there is a possibility of an inherent disruption in HDL-c metabolism among the Indian population. A previous meta-analysis conducted by Shim *et al* (11), reported on the inconsistencies in lipid levels and ABCA1 variants. The association between the two SNPs and lipid profiles was assessed in the present study, and no substantial association (P>0.05) was found for these parameters and the genotype distribution (Table IV). This was in accordance with the studies of Alharbi *et al* (20) and Ergen *et al* (21).

The lack of significant difference in HDL-c levels, the genotype and allele distribution of the ABCA1 gene among the cases and control subjects in the present study may be due to a defective ABCA1 genotype in the Indian population, thereby predisposing these individuals to a decrease in HDL-c levels. According to the study conducted by Salinas *et al* (22), it was found that apart from HDL-c metabolism, ABCA1 plays a role in cholesterol homeostasis in β-cells of pancreas and adipocytes. Hence, a defective ABCA1 genotype causes obesity due to enlarged adipocytes and T2DM due to decreased insulin secretion (22). This predisposes the population to a greater risk of developing dyslipidemia. Similar genetic variations in ABCA1 genotypes leading to dyslipidemia and subsequent cardiovascular diseases have been reported in studies conducted across South Asia and Saudi Arabia (17,19,20,23).

Dyslipidemia due to a decrease in the levels of HDL-c is merely one end of the spectrum. On the other end, the functional capacity of HDL-c is equally attributed to dyslipidemia in recent years. In addition to reverse cholesterol transport, HDL-c also has anti-inflammatory, antioxidant, antithrombotic and endothelial cell maintenance functions. A decrease in HDL-c levels causes a decrease in cholesterol sequestration from foam cells. This results in an inflammatory state, which causes a modification in the HDL-c structure, resulting in a detrimental effect on its function and thereby rendering it

dysfunctional (24). This produces a vicious cycle of decreased HDL-c levels, an inflammatory state and dysfunctional HDL-c. In the Indian population, this inflammatory state is aggravated by obesity due to the release of inflammatory mediators, paving the way for a pro-inflammatory state and oxidative stress (25). The increase in the prevalence of T2DM in India may be ascribed to dysfunctional HDL-c and obesity. Overall, there is an increased risk of developing atherosclerosis associated with T2DM at an early age. In the present study, the levels of HDL-c were below the optimum in both the cases and the controls, most likely due to the defective ABCA1 genotype. The present study, however, did not evaluate the functional capacity of HDL-c due to a lack of availability of diagnostic methods. The identification of ABCA1 gene polymorphisms leading to a disruption in HDL-c metabolism may aid in the early assessment of the risk of developing T2DM and its associated complications (26).

A limitation of the present study was the small sample size. Hence, it is recommended that further proteomics and genomics studies are conducted in the future to evaluate the association of ABCA1 variants with T2DM in larger sample sizes, in order to assess the contribution of dysfunctional HDL-c on overall cardiovascular disease risk in T2DM in the Indian population. However, the findings of the present study, may shed light into the association between these polymorphisms and T2DM and may aid future studies on this topic.

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Not applicable.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

DB was involved in the conception and design of the study, in the screening of the patients, in the selection and recruitment of the study participants, the provision of informed consent, laboratory investigations, laboratory report interpretations, data collection and the monitoring of data, interpretation of the data, statistical analysis and interpretation, maintaining a master file of project and in drafting the final manuscript. GB was involved in the conception and design of the study, in the drafting of the final manuscript, laboratory report interpretations, data collection and in the monitoring of data and interpretation of data. Both authors have read and approved the final manuscript. DB and GB confirm the authenticity of all the raw data.

**Ethics approval and consent to participate**

The present study was approved by Institutional Human Ethics Committee of the PSG Institute of Medical Sciences and Research, Coimbatore, India (Ref. no. 15/376). Consent was obtained from all the participants after explaining the objectives of the study.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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