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Original Article

Biochemistry Section

Distribution of Apolipoprotein A1 Polymorphism (G-75A and C+83T) in Patients with Diabetic Foot Ulcers- A Parallel Group Hospital Based Observational Study

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ABSTRACT

Introduction: Diabetic Foot Ulcer (DFU), a serious complications of diabetes mellitus is a result of persistent low grade infection. The Apolipoprotein A1 (ApoA1) has an anti-inflammatory role and therefore can influence the chronic inflammation associated with the DFU. Polymorphisms of ApoA1gene have been implicated as determinants of plasma High-Density Lipoprotein cholesterol (HDL-C) and Apo A1 levels. However, the influence of ApoA1 polymorphism on susceptibility to DFU has not been studied.

Aim: To study the distribution of ApoA1 polymorphism (G-75A and C+83T) and association between the polymorphism and the risk of DFU in patients with Type 2 Diabetes Mellitus (T2DM) so that timely detection and prevention of DFU can be done.

Materials and Methods: This was a hospital based observational study on 80 patients of DFU, 80 diabetes mellitus without ulcers and 75 normal controls. ApoA1 polymorphism (G-75A and C+83T) was detected by Real Time Polymerase Chain Reaction (RT-PCR) technique and plasma ApoA1 by immunoturbidimetric assay using blood collected in EDTA. Data was analysed using IBM® Statistical Package for Social Sciences (SPSS) 21.0 software. A $p<0.05$ was considered as statistically significant.

Results: The GA and CC were the most predominant genotype in all the groups. HDL and ApoA1 were significantly lower in GG ($p=0.009$, $p=0.03$) and CT ($p=0.03$, $p=0.002$) compared to GA and CC. The APOA1-75A allele and +83C allele were associated with raised levels of HDL and ApoA1 in T2DM and DFU ($p<0.05$).

Conclusion: The two polymorphism G-75A and C+83T were found to be equally distributed across the study populations. These polymorphisms were associated with serum levels of ApoA1 and HDL in the DFU patients.

Keywords: Allele, Anti-inflammatory, Dyslipidaemia, Genotype, High density lipoprotein

INTRODUCTION

The DFU is one of the most severe complications of Diabetes Mellitus (DM), with complications affecting over 30% diabetic patients, and DFU being the leading cause of nontraumatic amputation among adults in the working age group [1]. In countries like India, where there is wide spread diffusion of diabetes at an alarming rate, the burden of DFU is also exponentially increasing the economic load on the patient. According to the International Diabetes Federation, the average global prevalence of diabetic foot complications is 6.4% with the prevalence being higher in males and among people with T2DM [2]. The prevalence of diabetes is varied ranging from 5.3% in central India to 13.6% in Northern India [1].

To contain the huge cost and challenge, detection and effective management of DFU at early stages is essential. Diabetes mellitus with its multitude of factors like microvascular and macrovascular complications, peripheral neuropathy, duration of diabetes, control of plasma glucose and trauma serve as risk factors for DFU [3]. The multifactorial process involved in DFU calls for a multidisciplinary approach for treatment where along with control of infection, debridement, adequate perfusion, newer wound dressings like growth factors and tissues from bioengineering have been included [4]. The pathogenesis of DFU is unclear and the association of various genes related to inflammatory can play an important role in the development of DFU [5,6]. The early onset of ulcers is due to the influence of environmental factors, and their modifying effects on the early disclosure of gene factors. Therefore, genetic testing could potentially be used to identify patients more vulnerable to early development of DFU.

Type II Diabetes mellitus is associated with dyslipidaemia characterised by high triglycerides level and low High Density Lipoprotein (HDL) [7]. Evidence is evolving that ApoA1, the major lipoprotein of HDL has anti-inflammatory, anti-infective and endotoxin neutralising effects. It inhibits monocyte inflammatory function in peripheral blood mononuclear cells [8]. A persistent low grade inflammation is associated with the pathogenesis of DM, which lowers the HDL and further the ApoA1 level, however, all patients of DM do not develop DFU. At the start, the wound in DFU passes through acute inflammatory phase followed by a phase of remodeling and healing. The chronicity of inflammation with infection may result in the DFU to progress to amputation. The chronic inflammation that accompanies DFUs suppresses the focused acute inflammatory response to injury that is needed for normal wound healing which results in impaired leukocyte function and aberrant expression and activity of inflammatory cytokines [9]. Two Single Nucleotide Polymorphisms (SNPs) of ApoA1 gene {-75 G/A (rs 1799837) and +83 C/T (rs 5069)} of ApoA1 gene have been identified to affect HDL and ApoA1 by altering their levels [10-12]. It has also been found that ApoA1 -75 G/A and +83 C/T genotypes are associated with DM but with inconclusive findings [13]. The relation of ApoA1 and the genetic association of -75 G/A and +83 C/T polymorphisms has not been studied in DFU.

Therefore, the aim of the present study was to find the distribution of ApoA1 polymorphism (-75 G/A and +83 C/T) and association between the two genetic variants with susceptibility to DFU and correlation with plasma levels of HDL and ApoA1, so that timely detection and prevention of DFU can be done.

MATERIALS AND METHODS

It was a hospital based observational study. Patients were recruited from the Outpatient Clinics of Medicine and Surgery of the Institute over a period of 12 months from January to December 2019 at AIIMS, Chhattisgarh, India. A total of 235 adults which included 80 DFU, 80 T2DM without DFU and 75 age matched healthy controls were enrolled for this parallel group. Institutional Ethics Committee approval (Ethical clearance obtained vide IEC Proposal No. AIIMSRPR/IEC/2018/128) was obtained before initiation of research work and written informed consent was obtained from all participants.

Sample size calculation: Sample size for frequency in a population was calculated at 97% Confidence Interval (CI), 90% power, ratio of controls to cases being one, and proportion of cases with exposure being 13.6, the sample size was 74. All patients were enrolled after evaluation by the clinical co-investigators and detailed history was recorded with help of a case proforma.

Inclusion criteria: Patients of DFU who were defined as diabetic patients with 'ulceration, infection, or destruction of deep tissues located in the lower limb below the ankles were included in the study [14].

Exclusion criteria: Patients with ulcers on both feet, or with acute inflammation, hepatic, cardiac or renal failure, varicose veins, malignancy and psychiatric disorders were excluded from the study.

Sample Collection

Venous blood (5 mL) was collected from all participants under aseptic conditions in Ethylenediaminetetraacetic Acid (EDTA) vacutainers for polymorphism studies as well for measurement of ApoA1. Genomic Deoxyribonucleic Acid (DNA) was extracted using commercially available kit Qiagen kit (QIAEN Inc., Valencis, CA, USA) as per manufacturer's instructions and the extracted DNA was stored at -20°C for analysis of polymorphisms. Plasma collected was also stored at -20°C for determination of ApoA1.

Detection of Polymorphisms

The polymorphisms of ApoA1, G-75A (rs1799837) and C+83T (rs5069), along with allelic discrimination were analysed using pre-validated TaqMan based human Single Nucleotide Polymorphisms (SNPs) genotyping RT-PCR assay (Helini Biomolecules, Chennai, India). The assay has two unlabelled primers, forward and reverse primers, along with Fluorescein Amidites (FAM) and Hexachloro-Fluorescein (HEX) dual-labeled probes to detect both alleles (Supplementary Table-1). The PCR reaction set up was composed of Taq enzyme activation for 15 minutes at 95°C, followed by denaturation at 95°C for 20 seconds, annealing at 58°C for 20 seconds and extension at 72°C for 20 seconds as per manufacturer's instructions. Forty such cycles of denaturation, annealing and extension completed the reaction.

Biochemical Investigations

Detailed investigation reports with regard to plasma glucose, Haemoglobin A1c (HbA1c), lipid profile and renal function test were obtained from the patient records. The level of ApoA1 was determined in Beckman AU680 analyser by immunoturbidimetric immunoassay method at 340 nm using commercially available kits from Randox Laboratories Ltd., (UK). The assay was carried out according to the protocol described in the kit manual. The assay range for ApoA1 is 5.78-234 mg/dL, intra assay and inter assay CV% is 3.08 and 2.04 respectively. The minimum detectable level was determined as 5.78 mg/dL.

STATISTICAL ANALYSIS

Data was checked for normality distribution after which continuous variables were reported as means with Standard Deviations (SD). For qualitative data, proportions were summarised. The genotype frequencies of the two polymorphisms were tested for Hardy-Weinberg Equilibrium (HWE) using Goodness of fit, Chi-square test. Genotype and allele frequency between controls and cases were analysed using Chi-square test and confidence interval. Comparison of data in three groups was done using analysis of variance (ANOVA) and post-hoc Tukey's test. Logistic regression analysis was performed to estimate the Odds Ratio (OR) and 95% confidence interval for strength association. Data was analysed using IBM® SPSS 21 software. $p<0.05$ was considered as statistically significant.

RESULTS

Characteristics of study groups: The demographic details of the study population is depicted in [Table/Fig-1] showed no significant difference in age between control, patients of T2DM and those of DFU. A higher number of males were observed in DFU group compared to T2DM and control groups (p=0.03). The authors assume that exposure to the different risks like trauma and plantar pressure was more in men as they had more outdoor activities than females. Obesity is a well known risk factor for diabetes and its complications and this study depicted that the DFU group was significantly heavier and had lesser height in comparison to the healthy controls (p=0.03) and with a higher BMI when compared to T2DM and healthy controls, p=0.002, p<0.001 respectively. Longer periods of the diabetic state was observed in DFU group than T2DM (p=0.005).

Parameters	Controls (n=75)	T2DM (n=80)	DFU (n=80)	p-value
Age (years)	50.3±7.9	50.9±9.74	52.7±9.48	0.23[a]
Female/Male n (%)	21/54 (28,72)	29/51 (36.3,63.7)	17/63 (21.3,78.7)	0.03[b]
Weight (kg)	66.7±12.5	69.8±10.57	71.2±10.34	0.03[a]
Height (m)	1.71±0.46	1.68±0.1	1.6±0.08	0.03[a]
BMI (kg/m^2)	23.9±5.12	24.5±4.28	26.9±4.25	0.0001[a]
Duration of diabetes (years)	-------	7.23±5.02	8.78±6.9	0.005[b]

[Table/Fig-1]: Demographic patterns of study population.
a Analysis of variance; b Student's t-test; BMI: Body mass index; T2DM: Type 2 diabetes mellitus; DFU: Diabetic foot ulcer, Values are entered as mean±SD

Clinical complications and biochemical analysis: The clinical complications and biochemical analysis are presented in [Table/Fig-2]. The duration of diabetes and associated complications like neuropathy, retinopathy, dyslipidaemia were the clinical risk factors along with family history of diabetes for developing foot ulcers. While considering the biochemical analysis, markers of glycaemic control [Fasting Plasma Glucose (FPG), Post Prandial Plasma Glucose (PPPG), HbA1c], renal function tests and lipid biomarkers were significantly altered in DFU group (p<0.001) justifying the clinical risk factors. These results show that poor plasma glucose control and dyslipidaemia could be linked to delayed wound healing.

ApoA1 polymorphisms, lipids and Diabetic Foot Ulcers (DFU): The RT-PCR curves of APoA1 polymorphisms rs1799837 and rs5069 are presented in (Supplementary Figure-2a,b). The frequency of Apo A1 genotypes for G-75A and C+83T polymorphism for HWE in study population and the expected and observed frequency for these Apo A1 variants are depicted in (Supplementary Table-3). The distribution of C+83T genotype as well as G-75A did not follow HWE. The authors assume that this deviation from HWE is less probable due to genotyping errors as RT-PCR technique was used instead of Restriction Fragment Length Polymorphism (RFLP). This departure from equilibrium could be due to its proximity to an allele affecting a phenotype on which the sample is ascertained, or by chance. It is likely to be a selection bias introduced in the present study population because it is a hospital based study and due to the predefined disease profile of patients.

Parameters	T2DM (n=80)	DFU (n=80)	p-value	Odds ratio (Confidence interval)
Obesity (BMI >29.9 kg/m^2)	9/71 (11.2, 88.8)	3/77 (3.2, 96.3)	0.13	0.30 (0.08, 1.18)
Duration of diabetes (>5 years)	33/47 (41.2, 58.8)	48/32 (60, 40)	0.02	2.12 (1.08, 4.21)
Neuropathy	7/73 (8.2, 91.3)	39/41 (48.8, 51.2)	<0.001	9.77 (3.87, 28.29)
Retinopathy	12/68 (15, 85)	29/51 (36.2, 63.8)	0.003	3.19 (1.42, 7.59)
Dyslipidaemia	21/59 (26.3, 73.7)	46/34 (57.5, 42.5)	<0.001	0.26 (0.13, 0.51)
Hypertension (>140/90 mmHg)	15/65 (18.8, 81.2)	20/60 (25, 75)	0.44	1.44 (0.63, 3.32)
Active smoking	9/71 (11.2, 88.8)	17/63 (21.3, 78.7)	0.13	2.11 (0.82, 5.80)
Family history of diabetes mellitus	22/58 (27.5, 72.5)	36/44 (45, 55)	0.03	2.14 (1.06, 4.41)
FPG (mg/dL)	143.41±7.17	194.21±7.04	<0.001	-
PPPG (mg/dL)	209±9.92	266.92±9.15	<0.001	-
$HbA1_c$ (%)	8.68±0.19	9.43±0.20	<0.001	-
Urea (mg/dL)	29.7±1.75	32.65±2.92	<0.001	-
Creatinine (mg/dL)	1.01±0.23	1.23±0.04	<0.001	-
Uric acid (mg/dL)	5.71±0.15	6.77±0.24	<0.001	-
Total cholesterol (mg/dL)	172.58±4.88	189.31±5.80	<0.001	-
Triglycerides (mg/dL)	83.8±3.52	105.69±10.27	<0.001	-
HDL (mg/dL)	41.46±0.73	33.15±0.84	<0.001	-
LDL (mg/dL)	91.3±4.98	112.87±5.94	<0.001	-
ApoA1 (mg/dL)	99.56±2.69	74.76±3.15	<0.001	-

[Table/Fig-2]: Clinical complications, risk factors {number of patients, yes/no (%) and biochemical parameters (mean±SD) in T2DM and DFU patients.
a p-value obtained after Fisher-exact test and Student's t-test FPG: Fasting plasma glucose; PPPG: Post prandial plasma glucose; HDL: High density lipoprotein; LDL: Low density lipoprotein

Analysis of genotypic and allelic frequency distribution of Apo A1 polymorphism, G-75A C+83T, in [Table/Fig-3] showed that amongst the genetic parameters, the most predominant genotype at G-75A was GA with highest frequency in T2DM followed by DFU and controls. The GA genotype was significantly more in T2DM than in DFU from controls (p=0.03). There was one individual with AA genotype in T2DM group. At the C+83T site the predominant genotype was wild type CC with highest frequency in T2DM followed by DFU and controls. Frequency of both the CC and CT genotypes in the patient groups were not significantly different from that in the control group. The mutant genotype GA was predominant whereas the wild genotype CC was more

Genotype		Control (n=75)	T2DM (n=80)	DFU (n=80)	Odds ratio (95%CI)	p-value
rs 1799837 (G-75A)	GG	23 (30.7)	12 (15)	19 (23.75)	1.00 (Reference)	0.08
	GA	52 (69.3)	67 (83.75)	61 (76.25)	1.7 (0.923-3.325)	
rs 5069 (C+83T)	CC	49 (65.3)	61 (76.25)	55 (68.75)	1.00 (Reference)	0.393
	CT	26 (34.7)	19 (23.75)	25 (31.25)	0.770 (0.424-1.401)	
Allele		**Control (n=75)**	**T2DM (n=80)**	**DFU (n=80)**	**Odds ratio (95%CI)**	**p-value**
G		98 (65.3)	91 (56.9)	99 (61.9)	1.00 (Reference)	0.217
A		52 (34.7)	69 (43.1)	61 (38.1)	1.2 (0.861-1.930)	
C		124 (82.7)	141 (88.1)	135 (84.4)	1.00 (Reference)	0.607
T		26 (17.3)	19 (11.9)	25 (15.6)	1.140 (0.691-1.882)	

[Table/Fig-3]: Genotype and allele frequency of the G-75A and C+83T polymorphisms in the ApoA1 gene between control, T2DM and T2DM with DFU group. The reference category is control group.
a: The reference category is control group. AA genotype was not used for statistical study; T2DM: Type 2 diabetes mellitus; DFU: Diabetic foot ulcer

prevalent among the study groups however the authors speculate that ApoA1 gene polymorphisms rs1799837 and rs5069 had no obvious association with DFU susceptibility in the population of Chhattisgarh.

In [Table/Fig-4], while assessing the effect of the genotype on lipid profile the HDL and ApoA1 were significantly higher in GA (p=0.009, p=0.03) and CC (p=0.03, p=0.002) genotype compared to GG and CT genotypes, respectively.

Biochemical parameters	Genotype		p-value	Genotype		p-value
	GG (n=54)	GA (n=180)		CC (n=165)	CT (n=70)	
Total cholesterol (mg/dL)	165.73± 54.42	152.37± 40.52	0.09	157.46± 47.88	164.64± 53.25	0.33
Triglycerides (mg/dL)	101.17± 44.89	106.45± 67.16	0.5	107.81± 70.47	98.81± 37.61	0.31
HDL (mg/dL)	36.89± 9.09	40.46± 8.75	0.009	40.58± 8.64	37.97± 9.19	0.03
ApoA1 (mg/dL)	94.72± 30.38	104.54± 28.74	0.03	96.44± 28.08	83.98± 30.84	0.002

[Table/Fig-4]: Comparison of total cholesterol, triglycerides, HDL and ApoA1 in the different genotypes at G-75A and C+83T.
HDL: High density lipoprotein

The lipid profile was further compared in the different genotypes in the three study groups [Table/Fig-5,6]. The HDL and ApoA1 were significantly lower in GG genotype compared with the GA genotype in T2DM and DFU [Table/Fig-5]. A similar analysis at +83bp suggested that heterozygosity at the point had lower levels of HDL and ApoA1

Study group	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	ApoA1 (mg/dL)
Control				
GG (n=23)	127.36±34.1	103±29.5	45.2±8.67	108.5± 21.04
GA (n=52)	121.94±32.5	92.13±24.92	47.7±6.05	118.84±23.15
p-value	0.51	0.10	0.15	0.07
T2DM without DFU				
GG (n=12)	174.75±32.62	137.58±73.16	37.75±6.4	81.9±16.74
GA (n=67)	192.84±54.24	136.17±95.77	42.15±6.5	99.51±25.3
p-value	0.26	0.96	0.03	0.02
T2DM with DFU				
GG (n=19)	170.37±34.34	76.84±10.85	32.84±7.08	60.96±22.13
GA (n=61)	172.23±47.16	85.64±35.53	36.2±5.7	77.19±29.84
p-value	0.66	0.29	0.03	0.03

[Table/Fig-5]: Comparison of lipid profile in different genotypes in cases and controls in ApoA1-75 G > A polymorphism.
T2DM: Type 2 diabetes mellitus; DFU: Diabetic foot ulcer; HDL: High density lipoprotein

Study group	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	ApoA1 (dL)
Control				
CC (n=49)	120.35±29.81	93.29±24.65	47.27±6.04	111.37±21.8
CT (n=26)	128.38±38.08	98.92±29.81	44.77±7.97	101.54±26.27
p-value	0.14	0.25	0.13	0.08
T2DM without DFU				
CC (n=61)	189.0±51.82	138.61±101.55	41.51±5.9	104.5±14.9
CT (n=19)	190.32±53.69	126.32±50.74	37.32±8.7	96.76±11.87
p-value	0.92	0.61	0.01	0.04
T2DM with DFU				
CC (n=55)	177.07±35.21	86.58±36.90	33.58±7.99	79.54±28.52
CT (n=25)	162.72±33.45	77.8±12.29	30.2±4.01	64.2±24.95
p-value	0.09	0.25	0.04	0.02

[Table/Fig-6]: Comparison of lipid profile in different genotypes in cases and controls in APOA1 +83C > T polymorphism.
T2DM: Type 2 diabetes mellitus; DFU: Diabetic foot ulcer; HDL: High density lipoprotein

and that the CC genotype showed highest level of protection in all subjects [Table/Fig-6]. This indicates that the GA and CC genotypes elevate HDL and ApoA1 concentration.

DISCUSSION

The pathogenesis of DFU is complex because it is caused by multiple factors like genetic and environmental which also influence the healing of ulcer. Apart from trauma, infection, neuropathies, altered functioning of white blood cells and regenerating tissue along with bacterial infection contribute to delayed healing [15]. HDL and its apoprotein, ApoA1 have cytoprotective and wound healing effects, which is vital in diabetes mellitus as there is a milieu of altered endothelial function and poor wound healing, thus this lipoprotein, can play a role in healing of DFU [16]. Experiments in mice model have shown reconstituted HDL to improved wound healing containing human ApoA1 protein [17]. Similarly, lower levels of HDL has also been associated with lower extremity amputation and wound related death in patients with DFU [18]. This was the first study where the ApoA1 polymorphism is being studied in DFUs. Since, the polymorphisms G-75A and C+83T are involved in HDL and ApoA1 levels [11], the study aimed to investigate the pattern of this polymorphism and its association with DFUs.

The authors assessed the impact of ApoA1 polymorphism on the risk of the development of DFU. After literature search the authors did not find any published study that had evaluated DFU with respect to ApoA1 polymorphism. The slightly higher age group and male preponderance of DFU observed in the present study is reported by others as well which is probably due to the chronicity of the disease [1,19]. Compared to T2DM patients, the DFU population had a greater duration of diabetes and higher incidence of neuropathy, retinopathy, dyslipidaemia and risk factors like family history of diabetes. There was no statistical difference between the frequency of hypertension or obesity in T2DM and DFU groups. Similar such complications and risk factors were observed by other researchers [20,21]. The renal function tests of urea, creatinine and uric acid were higher in DFU groups, although nephropathy was not observed in T2DM or DFU cases. The lipid profile biomarkers were significantly elevated in DFU patients except for HDL and ApoA1 which were significantly lower. The presence of low HDL and ApoA1 in DFU may indicate inhibition of its anti-inflammatory role which it does by modulating innate immunity as well as adaptive immunity [22].

In diabetes the dyslipidaemia is primarily characterised by increased triglyceride level and lowered HDL level. However, the association of ApoA1 polymorphism with serum HDL and ApoA1 level remains controversial [10,11,23]. Polymorphism of the ApoA1 gene was studied in two sites G-75A and C+83T in the first intron. The genotype GA was found more frequently in controls, T2DM as well as DFU patients than the GG phenotype. The presence of genotype GA was not significantly associated with developing DFU (OR=1.7, CI- 0.923-3.325, p=0.08), dampened by the wide confidence interval. Of the two alleles the G allele was more frequent and was present in similar frequencies in the three groups than A allele. Certain studies of ApoA1 polymorphism involving G-75A, similar findings were obtained [11,24]. In contrast other authors observed significant difference in allelic frequency [10,25]. The authors found a positive association of A allele on HDL and ApoA1 level similar to that of Yangchun Z et al., and Saha N et al., [26,27]. In contrast, Bora K et al., observed no such effect [11]. Individuals with GA genotype had a significant higher value of HDL and ApoA1, which was observed across T2DM and DFU groups also indicating the protective nature. This could be one of the reasons why the authors could not get any history of myocardial infarction, even though the population was of middle age group. Also, there was no previous history of DFU in these patients as high ApoA1 and HDL have anti-inflammatory role. One of the reasons for the elevated ApoA1 and the HDL level is that presence of A allele increases the transcriptional efficiency of the promoter. Further, it decreases the binding affinity of a 90kD factor to the -75bp position and reduces the repression of ApoA1 gene transcription [10].

The genotype CC was found more frequently in controls, T2DM as well as DFU patients than CT genotype, with no difference in the CC genotype amongst the groups. Of the two alleles the C allele was more frequent and it was present in similar frequencies across the three groups than the T allele, indicating no relation with the DFU group. The C allele has been shown to be higher amongst controls in few studies [12,25,28,29], although it has been refuted by few researchers [10,30]. In CC genotype, there was a higher value of HDL and ApoA1 levels in the three groups as compared to the CT genotype. The CT group had a significantly lower HDL and ApoA1 levels in the entire study population across diabetes and DFU groups. Liao BH et al., had found no association between +83C/T and lipids [31]. Further, the presence of CT with OR=0.7 did not confer susceptibility towards developing DFU (CI- 0.424-1.401, p=0.393). The C+83T polymorphism located in the first intron of ApoA1 is a part of Cp nucleotide expressed differently in nonexpressing and expressing tissues like liver. Wang XL et al., and Shemer R et al., had mentioned that C+83T transition results in demethylation of the gene resulting in increased ApoA1 expression and thus high HDL level [30,32]. However, Bora K et al., in his study found no difference in ApoA1 levels in CC and CT genotypes [11]. No TT genotype was observed in the three groups.

The GA and CC were the predominant genotypes equally present in all the three groups of DFU, T2DM and controls. The G and C alleles were the frequent in all the three groups with no difference in the frequency among the three groups. This could be because these polymorphisms at the two sites could be in linkage disequilibrium with each other or with nearby gene. The GA and CC genotype were associated with significantly high levels of HDL and ApoA1 levels in all the three groups. This effect could be as a result of the disease process or various other hormonal, metabolic or environmental factors that could have modulated the genotype effect on HDL and ApoA1 for the development of a complex complication like DFU.

Limitation(s)

The limitations of this study was that diabetes is a polygenic disease, where there are a multitude combination of genes and environmental factors that can influence the outcome. It is difficult to pin point on a single polymorphism, but going by the anti-inflammatory role of HDL and Apo A1 this preliminary study may be helpful. Secondly, the population of T2DM and DFU were on medications and that could influence the lipid profile although the regression analysis took into consideration these parameters during analysis. Also, the populations in different literature quoted are from different ethnic population in comparison to this study. The short term and the observational nature of the study may also limit the findings and statistical power of the study. Since, all patients were recruited from a single hospital, the scope of the present result may be limited.

CONCLUSION(S)

The GA genotype which was more prevalent appeared protective due to higher levels of HDL and ApoA1 whereas the CT genotype individuals had a lower HDL and ApoA1 levels. It is recommended that further progressive research to focus on gene-gene interaction and gene-environment interactions and its relationship with the genotypic variations of ApoA1 gene in DFU patients. Therefore, well designed studies using whole genome sequencing is necessary to divulge extensive level of variation and heterogeneity between individuals can be undertaken with adequate sample size to eliminate bias in candidate gene selection is necessary to carry the work further ahead.

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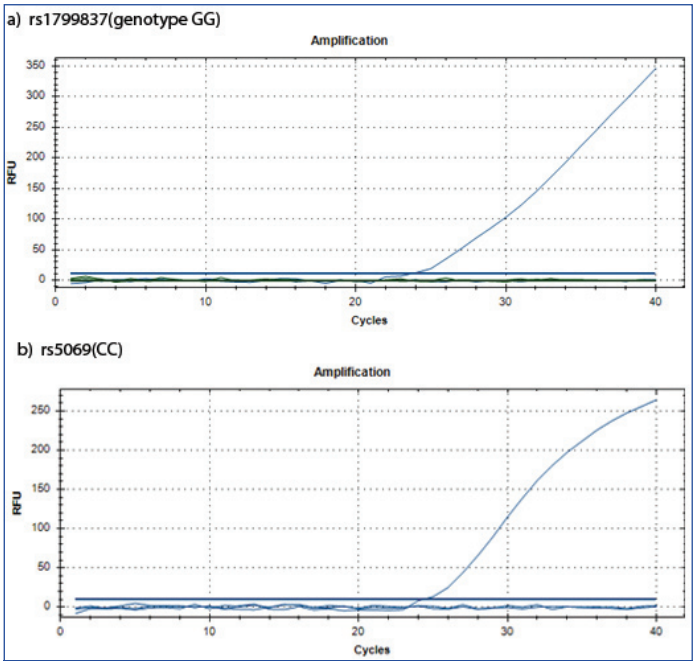
SUPPLEMENTARY TABLES

SNP		Primer sequences
rs1799837	Forward	5"-GCAGCTTGCTGTTTGCCCACTC-3"
	Reverse	5"-ACGCACCTCCTTCTCGCAGTCT-3"
rs5069	Forward	5"GGCCACGGGGATTTAGGGAGAA-3"
	Reverse	5"AGCTGGCTGCTTAGAGACTGCGA-3"

[Supplementary Table-1]: Primer sequences of Apo A1 gene polymorphisms rs1799837 and rs5069.



[Supplementary Figure-2]: Showing the RT-PCR curves of Apo A1 gene polymorphisms(wild type); a) rs1799837(GG); 2b) rs5069(CC).

Genotypes	Expected frequency	Observed frequency	p HWE
CC	169	165	0.007
CT	61	70	
TT	5	0	
GG	87	54	0.000
GA	112	180	
AA	36	1	

[Supplementary Table-3]: Expected and observed frequency for ApoA1 genotypes in study population along with HWE(n=235).
HWE: Hardy weinberg equilibrium