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Full Length Research Paper

Carotid intimo-medial thickness [cIMT] and correlation to cardiac risk factors in adolescent type 1 diabetics

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Carotid intimo medial thickness (cIMT) is a sensitive screening tool for cardiac evaluation, but there are limited studies evaluating its role in type 1 diabetes mellitus (T1DM) in developing countries. Thirty diagnosed adolescent type 1 diabetics on conventional insulin regime were included. Their glycated hemoglobin (HbA1C) and lipid profile were measured. Their cardiovascular function (determined by cIMT) was estimated by echocardiography and was evaluated with the clinical and biochemical profile. The mean HbA1C was 8.01%; 18 patients had HbA1C >8%. The mean serum cholesterol, low density lipoprotein (LDL) and triglyceride were normal. The mean cIMT measured was 0.698 ± 0.233 mm. The mean cIMT was higher in patients with higher HbA1C (0.775 ± 0.21 mm) versus those with normal HbA1C (0.583 ± 0.22 mm); $P = 0.019$. The cIMT correlated significantly to systolic and diastolic blood pressure ($P = 0.039$ and 0.01). cIMT values were significantly related to serum cholesterol ($P = 0.002$) and serum LDL ($P = 0.017$). However, few patients with a normal metabolic profile also had raised cIMT >0.8 mm. cIMT was raised in few patients indicating onset of early cardiovascular changes in adolescence. Cardiovascular screening should be offered to type 1 adolescent diabetics early in the disease, irrespective of the metabolic parameters.

Key words: Type 1 diabetes, carotid intimo-medial thickness (cIMT), cardiovascular screening, lipid profile.

INTRODUCTION

Patients with diabetes have two-four fold risk of death from cardio-vascular disease (CVD) as compared to non-diabetics (Larsen et al., 2002). The metabolic derangements (chiefly glucose and lipid) in diabetes have been implicated as major determinants for CVD (Alemzadah and Wyatt, 1994; Kimball et al., 1994; Jarvisalo et al., 2002; Nathan et al., 2003). However, traditional lipid and non-lipid markers for atherosclerosis do not reflect the

atherosclerotic process at arterial level (Bots et al., 2002). Autopsy findings in children with type 1 diabetes mellitus (T1DM) have revealed the presence of early atherosclerosis in the form of coronary plaques (Abdelgaffar et al., 2005; Jarvisalo et al., 2001). Silent coronary atheromatosis was demonstrated in T1DM by Larsen et al. (2002). Early changes of atherosclerosis are arterial vessel wall stiffening and increased carotid artery intimal-medial thickness (cIMT), which are reversible. These are followed by cardiac dysfunction and left ventricular hypertrophy (Bots et al., 2002; Groner et al., 2006). It was seen that cIMT was a powerful indicator for CVD, stroke and cerebro-vascular accidents in adult studies (O'Leary et al., 1999; Hodis et al., 1998; Simon et al., 2002). Ultrasound examination of carotid arteries has emerged as an alternative, noninvasive method to study the evolution of cardiovascular disease (Salonen

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Abbreviations: cIMT, Carotid intimo-medial thickness; T1DM, type 1 diabetes mellitus; HbA1C, glycated hemoglobin; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; ADA, American Diabetes Association; CVD, cardiovascular disease.

and Salonen, 1991; Heiss et al., 1991; Cobble et al., 2010).

The changes in cIMT in adolescents may not be as a result of local atherosclerosis alone, but it may be an adaptive response to altered blood flow and pressure (Sorof et al., 2003). Also, it is important to realize that adolescents experience poorer glycemic control than adults (Couper et al., 1995).

A study was thus undertaken to evaluate the metabolic profile of adolescent type 1 diabetics and identify any risk factors for CVD. These patients were screened for early atherosclerotic changes using echocardiography and the risk factors were correlated using the same method.

MATERIALS AND METHODS

The study was conducted in the Department of Pediatrics of a large tertiary referral hospital.

Sample characteristics

Type 1 diabetics who were attending the Pediatric Endocrinology Clinic at Lok Nayak Hospital were evaluated. Patients were included if they fulfilled the following inclusion criteria: age 10 to 18 years, regular and compliant visits at follow up clinic. Patients were excluded if they had any evidence of neuropathy or retinopathy on clinical examination, any history of hypertension or intake of antihypertensive or lipid lowering medication, any history of substance abuse or any family history of life threatening cardiac event which occurred before 55 years age. All patients were on pre-meal insulin bolus regimen (short and long acting insulin) administered thrice a day. Informed consent was taken from the parents/guardians. Out of the 45 patients attending the Endocrinology Clinic, 30 fulfilled our study criteria and were included in the study. The study design was cross-sectional and the study was approved by the Institutional Ethical Committee.

Experimental

All pertinent clinical information was obtained through a questionnaire which was completed after reviewing medical records and information provided by the patient and their guardian. The weight was measured on standard weighing scale and height was recorded on stadiometer. The body mass index (BMI) was calculated as weight (kg)/height (m²). The BMI readings were interpreted using World Health Organisation (WHO) charts and recorded to nearest percentile (WHO, 2005). Baseline blood pressure (BP) was recorded using a standard mercury sphygmomanometer using appropriate size cuff in the supine position after a 10 min rest period. An average of two readings was recorded. The BP readings were interpreted against height and age adjusted BP centiles as per recommendations by American Academy of Pediatrics and a value of >95th centile was considered as raised (AAP, 2004). Then, patients were subjected to metabolic screening. Five milliliter of fasting blood sample was collected by venipuncture under sterile conditions. Blood was collected in plain vial for lipids, potassium ethylenediaminetetraacetic acid (EDTA)

vial for glycated hemoglobin (HbA1C) and sodium fluoride/potassium oxalate vial for glucose estimation. Fasting and post-prandial blood glucose values were measured by the glucose oxidase method. HbA1C was measured using immunoturbidimetric method which measured the absorbance of the HbA1C fraction and total hemoglobin fraction at 415 nm. Total cholesterol and serum triglyceride were measured using enzymatic colorimetric estimation. Serum high density lipoprotein-cholesterol (HDL-C) was estimated by an automated direct assay method on an auto-analyzer. Serum LDL-C was calculated by Friedewald's formula (Warnick et al., 1990).

cIMT was assessed using trans-thoracic echocardiography (7 to 12 MHz phased array scanner which was interfaced to an AGILENT SONOS 4500 ULTRASOUND MACHINE). The common carotid artery (below the carotid bulb and 1 cm proximal to bifurcation) was scanned with the neck in hyper-extension, on B mode (real time) and Doppler imaging (Paucillo et al., 1994; Larsen, 2002). The longitudinal section of carotid artery was scanned and its wall was assessed for intimal thickness. The first line was the luminal-intimal interface, while the second was collagen containing upper layer of adventitia. cIMT was measured as the difference between two echogenic lines of the vessel wall (Xiao et al., 2007). Both right and left common carotid arteries were scanned and mean of both sides (recorded thrice) was taken as common final value (Figure 1). The normal limit for cIMT is arbitrary and is influenced by age, gender and population. The definition of abnormal cIMT is less clearly defined in children (Sorof et al., 2003). It is thus interpreted in terms of increased risk rather than statistical distribution; however, a value of >1 mm is definitely abnormal (Bots et al., 2002; Simon, 2002). The data for normative cut-off of cIMT in Indian population was not available; a value of >0.8 mm was taken as abnormal (O'Leary, 1999; Sorof et al., 2003).

All parameters were evaluated by a single experienced vascular sonographer who was blinded to the clinical and metabolic profile of the patients.

Statistical analysis

The results were analyzed using appropriate statistical tests on Statistical Package for Social Sciences (SPSS) software. Quantitative data was expressed as mean \pm 2 standard deviation (SD). Statistical significance of quantitative variables between different categories was analysed using t test. Pearson's correlation coefficient/Spearman's rank coefficient (r) was used to indicate significant linear relationship among quantitative variables and regression analysis was done. A P value <0.05 was considered as significant. Any P value <0.001 was taken as highly significant.

RESULTS

The baseline clinical features of the patients are depicted in Table 1. Equal number of males and females were recruited (15 each). The mean BMI was at 25th percentile as per WHO growth charts for both girls and boys and no patient was found to be overweight or obese. Two patients had increased BP as adjusted to age and height. Both systolic blood pressure (SBP) and diastolic (DBP) were directly associated to duration of diabetes (P = 0.002; r = 0.51 and P = 0.016; r = 0.39 respectively). The mean fasting blood glucose was 223.8 mg/dl and post

Table 1. Baseline clinical characteristics of study population.

Parameter	Mean \pm SD
Age (years)]	14.30 \pm 3.09
Weight (kg)	34.40 \pm 11.14
Height (cm)	140.03 \pm 15.4
BMI (kg/m ²)	17.1 \pm 2.91
SBP (mmHg)	111.46 \pm 12.52
DBP (mmHg)	70.48 \pm 9.16
Disease duration (years)	5.35 \pm 2.94
Insulin dose (U/kg/day)	1.102 \pm 0.303

Table 2. Comparison of clinical and laboratory values of patients with good and poor glycemic control.

Parameter	Good glycemic control (n = 12; HbA1C <8%)	Poor glycemic control (n = 18; HbA1C \geq 8%)	P value (Correlation coefficient = r)
SBP (mmHg)	107.66 \pm 11.36	114.0 \pm 12.27	0.17 (0.25)
DBP (mmHg)	67.33 \pm 10.43	73.11 \pm 7.66	0.047* (0.36)
Disease duration (years)	3.96 \pm 2.92	6.28 \pm 2.70	0.009* (0.44)
Insulin dose (U/kg/day)	0.98 \pm 0.30	1.18 \pm 0.28	0.038* (0.49)
FBG (mg/dl)	155.33 \pm 112.33	269.50 \pm 80.72	0.001 [†] (0.46)
PPBG (mg/dl)	191.25 \pm 112.8	319.0 \pm 85.6	0.001 [†] (0.48)
Serum cholesterol (mg/dl)	142.0 \pm 35.39	159.83 \pm 31.26	0.07 (0.33)
Serum triglyceride (mg/dl)	99.17 \pm 46.78	120.22 \pm 50.93	0.21 (0.23)
Serum HDL (mg/dl)	36.28 \pm 11.17	39.67 \pm 11.63	0.48 (0.13)
Serum LDL (mg/dl)	86.30 \pm 29.34	96.4 \pm 30.12	0.19 (0.24)
c-IMT (mm)	0.583 \pm 0.22	0.775 \pm 0.21	0.019* (0.43)

Values are mean \pm SD. *P < 0.05 significant, [†]P < 0.01 highly significant. SBP: Systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; PPBG: post prandial blood glucose.

prandial was 267.9 mg/dl. The observed HbA1C range was 4-13.1% (mean = 8.01%). The mean values of lipid profile-cholesterol, triglyceride and LDL were 152.70 \pm 33.5, 111.8 \pm 49.61 and 92.39 \pm 29.73 mg/dl, respectively; all within normal range. The mean HDL was 38.31 \pm 11.38 mg/dl, less than the reference of 40 to 60 mg/dl. The mean cIMT measured was 0.698 \pm 0.233 mm (range 0.36 to 1.27 mm). There was no difference in cIMT when compared with age (P = 0.16) or gender (equal male and female subjects). Patients with a longer disease duration had higher cIMT; though, result was statistically insignificant (P = 0.282; r = 0.16). The cIMT did not correlate with BMI (P > 0.05). The cIMT correlated positively with SBP and DBP (P = 0.02; r = 0.38 and P = 0.005; r = 0.46, respectively) (Figure 2) after adjusting for age. The patients were further divided into two groups based on their HbA1C values (below and above 8%), as shown in Table 2. The DBP was significantly higher in those with poor glycemic control as compared to good glycemic control (P = 0.047; r = 0.36). Patients with

longer disease duration had higher HbA1C (P = 0.009; r = 0.44). The mean daily insulin dose was significantly higher in those with poor glycemic control (P = 0.038). Patients with higher HbA1C had higher serum lipids; (p > 0.05). The correlation of HbA1C with cIMT was significant (P = 0.019; r = 0.51; Figure 3). There were 8 patients with high cIMT >0.8 mm; 5 of them (62.5%) had HbA1C \geq 9%. Two variables among lipid profile had a significant association with cIMT-serum cholesterol (P = 0.002; r = 0.48) and LDL (p = 0.017; r = 0.44) (Figure 4). Serum triglyceride and HDL failed to show any correlation with cIMT. However, 4 (50%) of the 8 patients with increased cIMT (>0.8 mm) had normal lipid profile.

DISCUSSION

cIMT has been correlated to cardiac risk factors and established as a cardiac screening tool in studies on high risk adults. Both age and gender are identified as non-modifiable

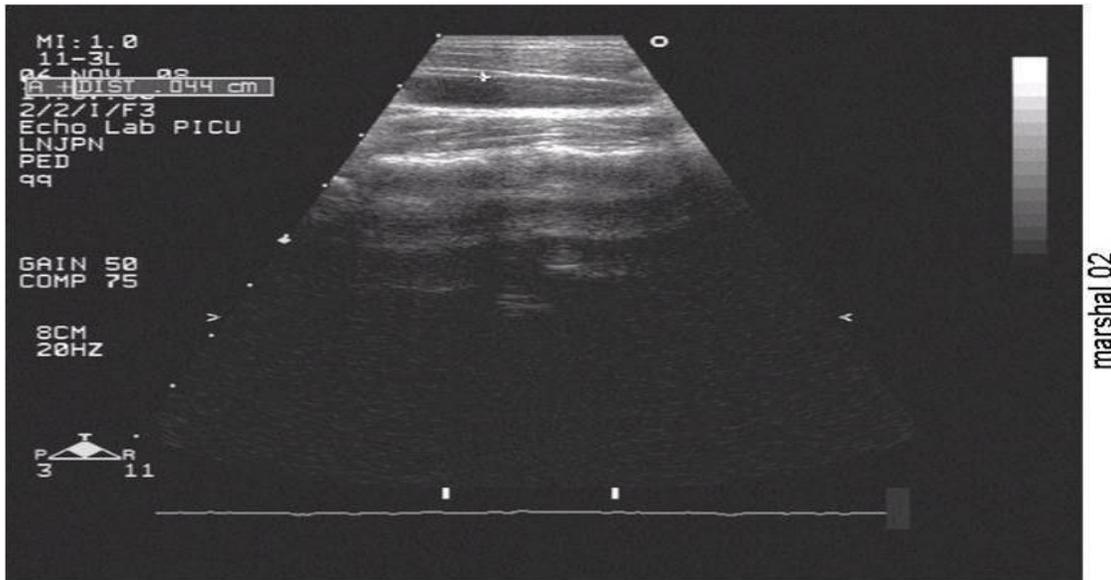


Figure 1. Measurement of carotid intimo-medial thickness on B-mode echocardiography.

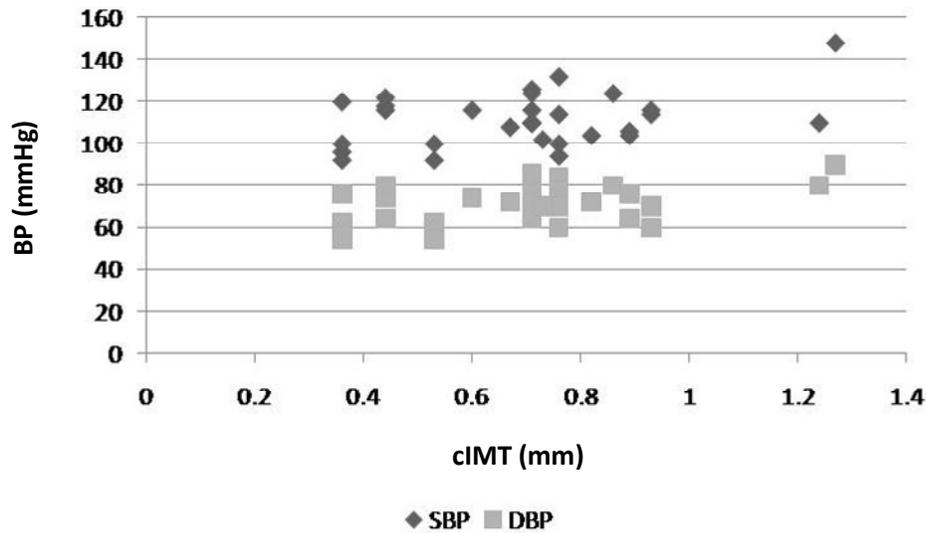


Figure 2. Correlation of cIMT and blood pressure (BP).

atherosclerotic risk factors in adults. However, cIMT is not influenced by age or gender in the pediatric age group (Koivisto et al., 1996; Saas et al., 1998); which is similar to results from our study.

The risk factors predicting cIMT are not well established in pediatric diabetic patients (Paucillo et al., 1994; Hodis et al., 1998; Parikh et al., 2000; Jarvisalo et al., 2002; Rathman et al., 2012). Few follow up studies indicate that progression of cIMT is influenced by factors like HbA1C, BMI, disease duration and serum LDL (Kawamori

et al., 1992; Pozza et al., 2011). Our study established the following as cardiac risk factors affecting cIMT in diabetic adolescents-duration of disease, blood pressure, HbA1C, serum cholesterol and LDL.

The probability of acquiring CVD is increased with prolonged duration of T1DM; more so if HbA1C values remain deranged (Yamasaki et al., 1994; Abdelgaffar, 2005). As the disease progresses, additional biochemical pathways (other than hyperglycemia) contribute and exacerbate disease pathology (Nickerson and Dutta,

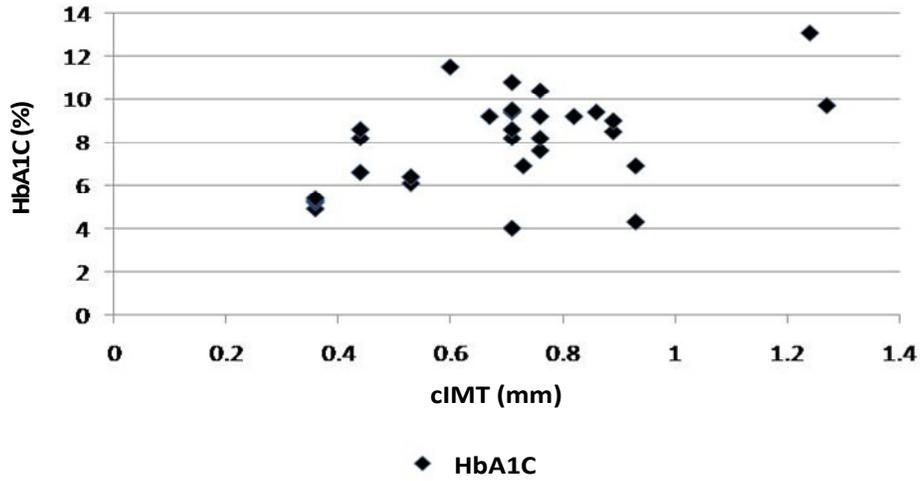


Figure 3. Correlation of cIMT and HbA1C.

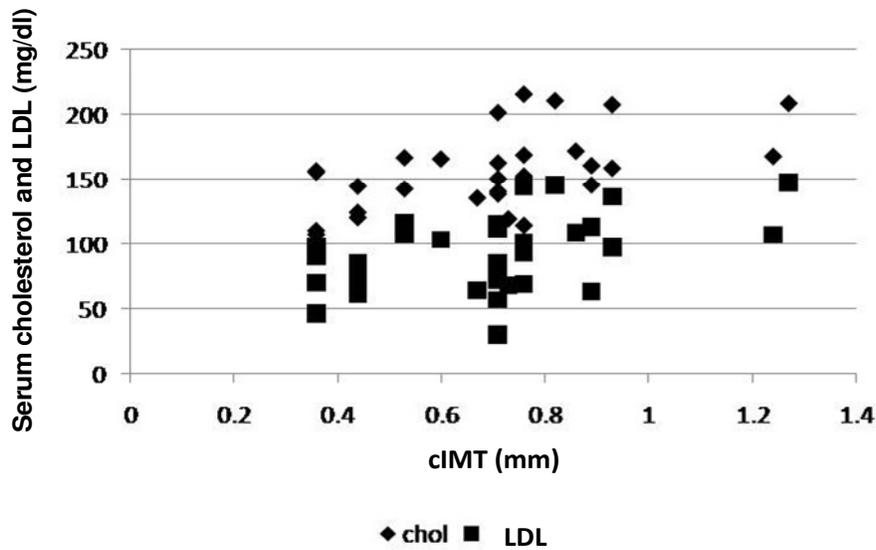


Figure 4. Correlation of cIMT and serum cholesterol and LDL.

2012). However, few authors did not find any correlation between duration of diabetes and cIMT (Yavuz et al., 2002).

Hypertension induces smooth muscle proliferation and exacerbates cardiac structural changes mediated through calcium pathways, predispose to atherosclerosis (Barbagallo et al., 1996). Previously conducted studies have reported a highly significant difference in blood pressure of type 1 diabetics versus controls in respective cohorts (Kimball et al., 1994; Abdelgaffar, 2005; Schwab et al., 2006; Pozza et al., 2011). However, few authors did not find any substantial relation of blood pressure with HbA1C (Peppas-Patrikiou et al., 1998; Parikh et al., 2000).

These variable results can be accounted for by variations in measurement techniques and confounders like race, genetics and environmental factors. A strong association between BMI and cIMT has been established in obese children (Simsek, 2010; Gökçe, 2012) and in T1DM (EDIC, 1999; Pozza et al., 2011). Our cohort belonged to a developing country and mean BMI was influenced by socio-economic factors; thus, was normal and did not correlate with cIMT.

Hyperglycemia is a postulated marker for atherosclerosis and causal factor for macrovascular complications in T1DM (DCCT, 1991; Kimball et al., 1994; Jarvisalo et al., 2002; Danielson, 2013). Conversely, few authors found

no difference in cIMT values of T1DM against controls studied (EDIC, 1999; Parikh et al., 2000; Gunczler et al., 2002). It may be argued that most of the adverse effect of hyperglycemia in atherosclerosis is due to a chronic process and poor glycemic status should not be considered as an unconditional marker for atherosclerosis (EDIC, 1999).

The DCCT research group had found lipid abnormalities more frequently in younger T1DM with relatively higher HbA1C and attributed this occurrence to poor dietary patterns (DCCT, 1991). Loh et al. (1996) reported dyslipidemia in 34 to 60% diabetics (most common aberration found as hypercholesterolemia followed by a mixed pattern and hypertriglyceridemia). Both cholesterol and LDL adversely affected cIMT in the children of this study ($P < 0.05$) similar to results reported earlier by separate authors (Jarvisalo et al., 2002; Pozza et al., 2011). Peppas-Patrikiou et al. (1998) and Yamasaki et al. (1994) did not detect any abnormality between cIMT and lipids in their study on T1DM. The variability in the aforementioned outcomes can be a consequence of various confounding factors which were present in each study like diet, gender, HbA1C level and disease duration.

Though both hyperglycemia and dyslipidemia were established as risk factors in our study; few patients with increased cIMT had a normal metabolic profile. This increases the possible role of other inflammatory markers in the occurrence of CVD and further research is needed to confirm the occurrence.

To summarize, deranged metabolic factors predicted increased cIMT in type 1 diabetics. However, an increase in cIMT was recorded in few others who had relatively normal sugars/lipids, suggesting the role of additional inflammatory factors which contribute to cardiovascular morbidity. Both long disease duration and high blood pressure were identified as adverse factors for increased cIMT. Thus, our study recommends that cIMT should be offered to adolescent T1DM as a screening tool for CVD after minimum of five years disease duration, especially in the presence of deranged metabolic parameters. A healthy lifestyle with good metabolic control is recommended to minimize progression of subclinical atherosclerosis to overt disease.

STUDY LIMITATIONS

The sample size was small and age matched controls were not evaluated. Thus, results from this study cannot be generalized. Also, data for normal range of cIMT in our population was not available. However, our study is one of the first studies which address the role of cIMT and cardiac risk factors from this part of the developing world and results are noteworthy.

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Full Length Research Paper

Obesity and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) polymorphism and their association with pathophysiology diabetes type 2 in Central Indian population

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Diabetes is a metabolic disorder characterized by hyperglycemia (excessive amount of glucose) and is associated with abnormal lipid and protein metabolism. This was the first study done in Vindhyan region. The population size selected in this study was 400 (190 cases of diabetes and 210 healthy control). Anthropometric data was collected during sample collection. Genetic polymorphism study of ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and the observed genotype frequencies, allele frequencies and carriage rates for ENPP1 K121Q polymorphism. We found an overall distribution of ENPP1 K121Q genotypes which was not significantly different in the healthy control (HC) group as compared to the disease group. Type 2 diabetes is a multifactorial disorder; so, we can conclude that in our population, ENPP1 is not associated with type 2 diabetes pathophysiology in Central Indian population. Higher body mass index (BMI) is an indicator of obesity, and in this investigation we found out that BMI is significantly higher in female diabetic patients group as compared to healthy control females ($P = 0.0388$); meanwhile in male patients, BMI was not significantly different in case and control samples. This indicates that obesity could also be associated with type 2 diabetes. Smoking and physical activity data was generated through questionnaire organized during sampling, and the findings suggest that physical activity could be a protective factor which decrease diabetes susceptibility, meanwhile smoking have very little or no effect over diabetes susceptibility.

Key words: Type 2 diabetes, obesity, ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), physical activity.

INTRODUCTION

Diabetes is a metabolic disorder characterized by hyperglycemia (excessive amount of glucose) and is associated with abnormal lipid and protein metabolism. It has become a global health problem having the largest

prevalence worldwide, and now it is the world's sixth leading cause of death. Although, the cause of diabetes are not very much clear, but many habits (life style factors) as well as genetic susceptibility is now known to cause this disease. Type 2 diabetes (T2D) is a complex metabolic disorder resulting from the interplay of both genetic and environmental factors like lifestyle and food habits (McCarthy and Froguel, 2002).

In the past two decades, the genetic analysis with documentation of life style data collection has suggested the possible involvement of the genetic base as well as

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life style factors. Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) encodes for a transmembrane glycoprotein, which interacts with the insulin receptor and inhibits subsequent insulin signaling. ENPP1 interacts with α -subunit of the insulin receptor to interrupt signaling (Maddux and Goldfine, 2000). A missense mutation in 121 codon has been found to be significantly associated with increased diabetes risk, and in many studies this allele has also been found to be associated with obesity and other many related metabolic disorders. K121Q (where a lysine, K, is substituted by a glutamine, Q, at codon 121) predisposes to insulin resistance and related abnormalities. The 121Q variant binds insulin receptor more strongly than the K121 variant. Costanzo et al. (2001) described a K121Q variant in the PC1 gene (rs1044498) and demonstrated that it was strongly associated with insulin resistance in 121 healthy non obese and non diabetic Caucasians in Sicily (Pizzuti et al., 1999).

MATERIALS AND METHODS

Study population

The study population consisted of 400 unrelated subjects comprising of 190 T2D patients and 210 ethnically matched controls of Indo-European ethnicity. In central India, the population is highly matched with North Indian population, because many Hindu, Sikh and Muslims migrated from North India. Cases included consecutive patients of urban region who attended the Department of Medicine, S.S.M.C. Rewa, Ayurveda Medical College Rewa, Ranbaxy Pathology Regional Collection Centre, Rewa. Type 2 diabetes was diagnosed in accordance with World Health Organization (WHO Expert committee, 2003) criteria. Pregnant women, children under the age of 18 years and any patient with type 1 diabetes were excluded from the study. All the participants were asked to fill a detailed questionnaire at the time of recruitment, seeking information regarding individual's age, sex, ethnicity, dietary habits, physical activity, life style, personal and family medical history.

Anthropometry

Height and weight were measured in light clothes and without shoes, in standing position, as per standard guidelines. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in standing position midway between iliac crest and lower costal margin and hip circumference was measured at its maximum. Waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 min, using a standard sphygmomanometer, and the average of the two reading was used.

Biochemical analysis

Biochemical parameters related to type 2 diabetes were estimated for both cases and control subjects. Measurement of serum levels of total cholesterol (TC), triglycerides (TG), HbA1c, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), urea, uric acid, C-reactive protein (CRP) and creatinine were

measured based on spectrophotometric method using automated clinical chemistry analyzer, Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany).

Blood collection and plasma/serum separation

Venous blood samples were obtained from the subjects after 12 h of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min at 4°C.

Deoxyribonucleic acid (DNA) isolation

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller et al. (1988).

Detection of ENPP1 single-nucleotide polymorphism (SNP) via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The K121Q (substitution of A base to C at 121 codon) polymorphism of ENPP1 gene was amplified by PCR. This polymorphism is a functional polymorphism causing change of amino acid from lysine to glutamine. Primer sequences oligonucleotide sequence (primers) was designed to amplify the gene; a wild type gene is as a result of lack of restriction site for Avall enzyme, but mutant allele contains a restriction site.

PCR mix

The PCR was carried out in a final volume of 25 μ l containing 100 ng of genomic DNA (4 to 5 μ l), 2.5 μ l of 10 \times Taq polymerase buffer (10 mM Tris HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1 \times ; Genetix Biotech Asia Pvt. Ltd., India), 1 μ l of 10 mM deoxyribonucleotide triphosphates (dNTPs) (Bangalore Genei, Bangalore, India), 1 μ l of 25 pmol/ μ l of forward and reverse primers specific for and 1 μ l of unit of 1 U/ μ l Red Taq DNA polymerase (Bangalore genei).

PCR thermal program

After an initial denaturation of 5 min at 94°C, the samples were subjected to 35 cycles at 94°C for 1 min, at 55°C for 40 s, and 72°C for 40 s, with a final extension of 10 min at 72°C in a thermal cycler. A 100 bp ladder with amplified product was done under 1% agarose gel electrophoresis and a 238 bp product was generated after PCR.

Restriction digestion

The 238 bp product was digested with Avall enzyme (New England Biolabs, Beverly, MA) for 16 h at 37°C. The wild-type genotype (KK) was not digested, whereas the mutated homozygous genotype (QQ) was cut as a doublet of 148 and 90 bp. The heterozygous genotype (KQ) was represented as 3 fragments of 238, 148, and 90 bp. Samples were analyzed by electrophoresis using 2.5% agarose gels to analyze the genotype pattern of the gene.

Statistical analysis

Statistical analysis was done by using Prism 5.0, San Diego, USA

Table 1. Comparison of anthropometric parameters of diabetic patients and controls.

Characteristic	Case	Control	P-value
n (Men/Women)	190 (126/64)	210 (114/96)	
Age (years)	52.5±12.5	53.0±14.2	0.7100
Height (m)	160.50±13.40	162.2±12.000	0.1815
Weight (kg)			
Women	62.5±5.70	60±4.50	0.0024**
Men	68±5.60	66.0±7.1	0.0157*
BMI (kg/m²)			
Women	26.4±3.1	25.1±4.3	0.0388*
Men	24.6±4.7	24.1±5.1	0.4301
Waist circumference (cm)			
Women	92.5±6.2	84.5±6.7	P<0.0001***
Men	90.0±7.0	89.0±6.0	0.2383
Hip (cm)			
Women	95.0±5.0	96.5±6.0	0.178
Men	91.0±4.0	90.5±5.5	0.4183
WHR			
Women	0.97±0.05	0.88±0.08	P<0.0001***
Men	0.99±0.05	1.00±0.03	0.0147*

(www.graphpad.com).

RESULTS

Anthropometric results

The descriptive data and comparison of anthropometric parameters of diabetic patients versus controls are presented in Table 1. The diabetic patients had markedly higher levels of weight of women ($P = 0.0024$), men ($P = 0.0157$) and BMI of women ($P = 0.0388$), waist circumference in women ($P < 0.0001$), WHR in women ($P < 0.0001$) and WHR in men ($P = 0.0147$). Other results were not significantly different between case and control group and are tabulated in Table 1.

Biochemical and clinical findings

Biochemical test were performed in the blood sample for all the clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t test, and p-value obtained suggested the level of significant changes here. The descriptive data and comparison of biochemical parameters of diabetic patients versus controls are presented in Table 2. As expected, the

diabetic patients had markedly higher levels of fasting plasma glucose ($P < 0.0001$) and HbA1c ($P < 0.0001$) and post prandial glucose ($P < 0.0001$) as compared to that of control subject. Nominal difference was also observed for LDL-C ($P = 0.0462$), triglyceride ($P = 0.0024$), and systolic blood pressure ($P = 0.0447$). Creatinine value, blood urea level, HDL-C level and diastolic pressure were not significantly different between the two groups and all the clinical test results are tabulated in Table 2.

Detection of genetic polymorphism in ENPP1

ENPP1 K121Q polymorphism (rs1044498) was analyzed by PCR-RFLP method (Table 3). PCR amplification with specific primers gave 238 bp products which were digested with Avall enzyme (New England Biolabs, Beverly, MA) for 16 h at 37°C. The wild-type genotype (KK) was not digested, whereas the mutated homozygous genotype (QQ) was cut as a doublet of 148 and 90 bp. The distribution of the polymorphism of ENPP1 (rs1044498) was consistent with Hardy-Weinberg equilibrium (HWE) in diabetic patients as well as in healthy controls.

No significant level of change has been seen in distribution of ENPP1 K121Q genotypes in healthy control

Table 2. Comparison of biochemical and clinical findings of diabetic patients and controls.

Characteristic	Case	Control	P-value
FPG (mg/dl)	143.3± 17.6	92.44±7.5	P<0.0001***
Post-prandial glucose (mg/dl)	211.7±44.7	108.5±12.1	P<0.0001***
HbA1C (%)	6.9±0.8	5.3±0.6	P<0.0001***
HDL-C (mmol/L)	112.2±14.8	109.8±11.6	0.0705
LDL-C (mg/dl)	42.1±4.3	41.3±3.7	0.0462*
TG (mg/dl)	131.1±13.2	126.9±14.2	0.0024**
Systolic BP (mmHg)	130.20±8.1	128.8±5.7	0.0447*
Diastolic BP (mmHg)	87.1±5.8	86.5±6.0	0.3109
Blood urea (mg/dl)	9.1±1.6	8.8±1.8	0.0801
Creatinine (mg/dl)	1.08±0.14	1.06±0.10	0.0986

Values expressed in mean ± standard deviation (SD) are taken at one point of time during treatment and will not indicate a long life trend of the concentrations in the given patients. *Denotes the level of significant change between case and control. BP = blood pressure, LDL-C = low-density-lipoprotein cholesterol, HDL-C = high-density-lipoprotein cholesterol, FPG = fasting plasma glucose, TG = triglyceride.

Table 3. Fisher exact test values of ENPP1 polymorphism.

ENPP1 genotype	Case (n = 190)		Control (n = 210)		P value	Odds ratio (95% CI)
	n	%	n	%		
KK	121	63.68	146	69.52	0.2427	0.7687 (0.5066-1.166)
KQ	66	34.74	62	29.52	0.2841	1.271 (0.8340-1.936)
QQ	3	1.58	2	0.96	0.6717	1.668 (0.2757-10.10)
Allele						
K	308	81.05	354	84.29	0.2608	0.7976 (0.5524-1.152)
Q	72	18.94	66	15.71		
Carriage rate						
K	187	98.42	208	99.05	0.3689	0.8339 (0.5627-1.236)
Q	69	36.32	64	30.48		

*Denotes the level of significant association between case and control), n = number of individuals in study group % = genotype allele frequency and carriage rate.

(HC) group as compared to diabetic patient group; although, HC group showed little increase in common 'KK' genotype as compared to patients of diabetes type 2 (69.5 versus 63.7%, respectively). Similarly, 'QQ' genotype was present at lesser frequency in diabetes type 2 patients group (1.58%) and also in control group (0.96%). The overall genotype was statistically non significant. Major allele 'K' was found at slightly lower frequency in diabetic group (63.68%) as compared to HC group (69.52%) whereas allele 'Q' was present in slightly higher frequency in the disease group (18.94% in patients and 15.71% in control) but the difference was nominal and not statistically significant ($\chi^2 = 1.461$, $P = 0.2268$).

An odds ratio of 1.254 of rare allele 'Q' showed moderate effect of minor allele in diabetes susceptibility. Carriage

rate of allele 'Q' was slightly higher in diabetic group as compared to healthy control (36.32 versus 30.48%) whereas carriage rate of allele 'K' was approximately similar in both control and disease group and no significant level of change has been seen. Odds ratio of minor allele Q carriage is 1.199, which did not suggest any association of Q allele carriage with disease susceptibility.

Physical activity and smoking habits

Physical activity and smoking habits were asked of both case and control individuals during collection of samples by a brief questionnaire, and the data obtained are depicted in Table 4. Physical activity was decided on the basis

Table 4. Life Style factors selected in present investigation.

Life style factors	Case (n = 190)		Control (n = 210)		χ^2 value (P value)	Odds ratio and CI
	n	%	n	%		
Physical active population (according to CDC 2010)	79	41.57	112	53.33	5.524 (0.0188*)	0.6227 (0.4191-0.9253)
Smoking habits (light and heavy smokers)	49	25.79	47	22.38	0.6354 (0.4254)	1.205 (0.7613-1.908)

n = number of individuals in study group, % = genotype allele frequency and carriage rate, *denotes the significant level of association between case and control, CI = confidence interval.

of the Centre for Disease Control (CDC) (2010) recommendations and criteria. In diabetic case, lower number of physically active persons was seen as compared to control (41.57 versus 53.33%). A significant association of physical activity was seen (Chi square value 5.524, df: -1 and P value 0.0188). An odds ratio of physical activity was 0.6227 (CI = 0.4191 to 0.9253), which indicates that the positive association of physical activity with prevention of diabetes and active life style could be concluded as a very important life style factor which can prevent pathophysiology of diabetes type 2.

Smoking habit data were also collected during questionnaire organized during sample collection and the data obtained are depicted in Table 4. Here, mixed urban as well as rural population were included in the present investigation and we did not discriminate light and heavy smokers, because of irregular smoking quantity of patients. Bidi and cigarette smokers were included as smoking population. The data indicates that the percentage of smokers was not very much different both in case and control population (25.79 versus 22.38%), and no significant difference was seen. Odds ratio of smokers were 1.205 which indicates that possibly, smoking could be a risk factor associated with diabetes type 2 pathophysiology, although the modes of infection are not very much clear.

DISCUSSION

Diabetes type 2 is a well established multifactorial disorder which is strongly associated with genetic as well as life style and environmental factors, that is, neither genetic nor environmental factors alone could be sufficient to cause diabetes type 2, but both of them are needed. Although, etiology of type 2 diabetes is not very clear, many studies and family history suggest the transmission of diabetes type 2 in families, and also due to 100% susceptibility of the diabetes in twins, this strongly supports its genetic base.

BMI indexing is a tool used for documentation of obesity. In our present investigation, we found that BMI was significantly higher in diabetic females; meanwhile, the male group did not show any significant level of change. BMI of diabetic females were 26.4 as compared to healthy females 25.1 (P = 0.0388). WHR was also higher in both male and female. Sedentary lifestyle is a key to the rise in the prevalence of both obesity and diabetes (Blair and Timothy, 2003). In the past decade, we have witnessed an epidemic of both type 2 diabetes and obesity. The prevalence of type 2 diabetes has increased by 33% in the United States, and 62% of Americans are classified as obese (BMI \geq 30 kg/m²) or overweight (BMI 25 to 29.9 kg/m²). The recent increase in the prevalence of obesity is closely

paralleled by the increase in the prevalence of diabetes.

Indeed, this new unprecedented phenomenon has been referred to as "diabesity." There is a clear strong relationship between obesity and the risk for diabetes (Caprio, 2003). In India, National Family Health Survey Report (2007) indicates that on average, 12.1% male and 16% female are obese and the risk of obesity is increasing rapidly. Our data also suggests that obesity and higher BMI can be an important factor which can affect the susceptibility to diabetes type 2 (Third National Family Health Survey, 2006). ENPP1 gene is known to be a susceptible gene, because of its affinity to bind with alpha subunit of insulin receptor and may inhibit the tyrosine kinase activity which is essential for glucose metabolism (Maddux and Goldfine, 2000). Metformin, a biguanide oral antidiabetic agent, was shown to affect insulin resistance by decreasing enzymatic activity of over expressed PC-1 molecules in obese type 2 diabetics. In functional studies, the 121Q allele variant binds more strongly to the insulin receptor and inhibits its protein kinase activity more effectively than the K variant (Costanzo et al., 2001).

The role of genetic polymorphism of ENPP1 gene in susceptibility to diabetes type 2 has been widely studied, but the results are inconsistent in different populations. Many case control studies

had concluded the possible role of ENPP1 gene in genetic susceptibility to diabetes type 2, while some others showed that ENPP1 gene plays a possible role in obesity. Many other studies revealed that ENPP1 is neither associated with diabetes nor with obesity. No particular gene could be defined as universal which can alone cause diabetes type 2, because of the multi-factorial nature of diabetes. Our finding showed that genotype pattern and allele frequency of ENPP1 K121Q was not significantly different between case and healthy controls. With odds ratio of 0.7687, KK genotype may indicate little protective role, but neither KK nor other genotypes were significantly different, although the higher allele frequency of mutant 121Q allele in diabetic population has been seen, but the difference was also not significant. The frequency of homozygous mutant 121Q allele was very low in case as well as in control. Overall, allele 'K' was found when the frequency was a little lower in disease group as compared to HC group, whereas allele 'Q' was present when the frequency was a little higher in the disease group (18.94% in patients and 15.71% in control) but the difference was nominal. An odds ratio of 1.254 of rare allele 'Q' showed little or no effect of mutant allele in diabetes susceptibility. Carriage rate of allele 'Q' was slightly higher in diabetic group as compared to healthy control (36.32% in case versus 30.48% in control).

Previous studies suggested that the frequency of the 121Q allele carriers in the ethnic Chinese study was 18.8%, Caucasians (23.2 to 36.4%), South Asian Indians (specially Chennai population living in Chennai and Dallas, USA (27.5 to 34.2%)), African-Americans (67.0%), and Dominicans (78.4%). Caucasian population of USA and Finland showed the significant association of 121Q allele with diabetes type 2 (Abate et al., 2003, 2005; Kubaszek et al., 2004). In addition to diabetes, the PC-1 Q121 allele has recently also been reported to influence the risk of obesity (Barroso et al., 2003). Many association studies have been done, but the results are different in different race and population.

Other Caucasians, including Sweden and Denmark showed that ENPP1 polymorphism was not associated with susceptibility to diabetes type 2 (Gu et al., 2000; Rasmussen et al., 2000), while the same finding of lack of association has been reported from Chinese population (Miao-Pei et al., 2006), North Indian Sikh population (Bhatti et al., 2010). Another study in Spanish population also showed that ENPP1 K121Q polymorphism was not significantly associated with diabetes type 2 (Gonzalez-Sanchez et al., 2003). Our findings were also consistent with Caucasian and African-American adults (Matsuoka et al., 2006), Oji Cree (Hegele et al., 2001) and Mexicans (Cruz et al., 2010). It is possible that the susceptibility induced by the ENPP1 K121Q gene polymorphism is modulated by interactions with other ethnic specific genetic or environmental factors.

The study in Central Indian population has shown that ENPP1 K121Q polymorphism is not associated with

susceptibility to diabetes type 2 and the results are similar to previous study already done in North Indian as well as many Caucasians, Japanese and Chinese. Heterozygous and minor Q allele homozygous was closer to Caucasians and lower than Sikh population. Although, the Sikh population study showed maximum level of heterozygous, but the statistical difference were not seen. Other studies done before in South Indian Chennai population and some Caucasian population showed that Q allele could be a risk factor and in our study, allele Q was present in higher percentage in the form of heterozygous in diabetes patients, but its effect or association with diabetes type 2 was not significantly established. In studies of complex multi-factorial disorders such as type 2 diabetes, discordant results in genotype-phenotype association are not uncommon. These discordant results suggest that differences in either the genetic and/or environmental backgrounds of the subjects studied or the recruitment procedures of the populations investigated are important factors in these analyses.

It has been established before that with lifestyle modification (weight loss, regular moderate physical activity), diabetes can be delayed or prevented (Tuomilehto et al., 2001; Pan et al., 1997). In our study, we collected the information from case and control individuals and their level of physical activity were decided according to CDC (2010) guidelines and recommendations.

In diabetic case, lower number of physical active persons was seen as compared to control (41.57 versus 53.33%). The significance level was sufficiently strong to reveal the protective association of physical activity ($\chi^2 = 5.524$, P value 0.0188). An odds ratio of 0.62 clearly indicates the positive association of physical activity with prevention of diabetes and active life style which could be concluded as a very important factor which can prevent pathophysiology of diabetes type. Exercise has been shown to increase insulin-stimulated glycogen synthesis through an increased rate of insulin-stimulated glucose transport by GLUT4 glucose transporters and increased glycogen synthase activity (Perseghin et al., 1996), and this may be an important effect of active life style which may decrease the hyperglycemia by stimulating the glucose transport and glycogen synthesis. Many other protective role of active life style has been suggested and widely accepted worldwide. Western culture and sedentary life style with intake of high calorie rich food has increased the risk of obesity and diabetes type 2. A complex interaction between lifestyle factors and disease susceptibility was found in the present study. Physical activity (PA) is an independent life style factor which significantly reduces the risk of hypertension and diabetes. We found out that 20 min/day exercise can reduce the risk of diabetes.

Smoking is an established modifiable risk factor which is associated with many diseases such as cardiovascular disease (CVD) (Burke et al., 1997; Greenland et al., 2003) and cancer. To some extent, the effects in physical

conditions of smoking and diabetes are similar, which brings the question of if there is any association between smoking and diabetes. Many studies evidenced that chronic smokers have a higher risk for insulin resistance and to develop diabetes type 2 mellitus (DM2) (Willi et al., 2007; Carlsson et al., 2004; Eliasson, 2003; Facchini et al., 1992). Our result showed that there was not much difference between the percentage of smokers in case and control and there was lack of statistically significant association, but an odds ratio of 1.205 showed a little higher risk of diabetes type 2 in smokers as compared to non smokers. Our results indicated that smoking may increase the risk of diabetes type 2, but relation with disease susceptibility was not established. It was previously reported in a meta-analysis that heavy smokers (at least 20 cigarettes daily) had a 61% higher risk, while less than 20 cigarettes daily were correlated to a 29% increase of the risk (Willi et al., 2007).

Many other studies also showed that current smokers have a 1.2 to 2.6-times higher risk of diabetes type 2 than non smokers (Rimm et al., 1995, 1993; Manson et al., 2000; Persson et al., 2000; Wannamethee et al., 2001; Will et al., 2001). Our results were consistent with this findings obtained by meta-analysis, but risk of diabetes was lower with smoking in our result as compared to results of the meta-analysis. The reason which could clarify our results is that we did not discriminate smokers in both light and heavy, because the people included in this study were not sure about their daily smoking level. Probably due to this reason, our results showed a lower relative risk of smoking habit in diabetes type 2 in the present investigation. Our study supported all these studies and showed that smoking may be a life style factor responsible for diabetes type 2 pathophysiology, and if people start to quit smoking they can prevent themselves from being diabetic.

In our present investigation, we did not select alcoholism as life style factor, because previous findings existing clearly suggested no effect on diabetes susceptibility. Light-to-moderate alcohol consumption is associated with reduced risk of diabetes. A meta-analysis of 370,000 individuals with 12 years of follow-up showed a U-shaped relationship, with a 30 to 40% reduced risk of the disease among those consuming 1 to 2 drinks/day when compared with heavy drinkers or abstainers. The risk of diabetes among those who consumed three or more drinks/day was similar to that of abstainers (relative risk (RR) 1.04 [95% CI 0.84 to 1.29]) (Koppes et al., 2005).

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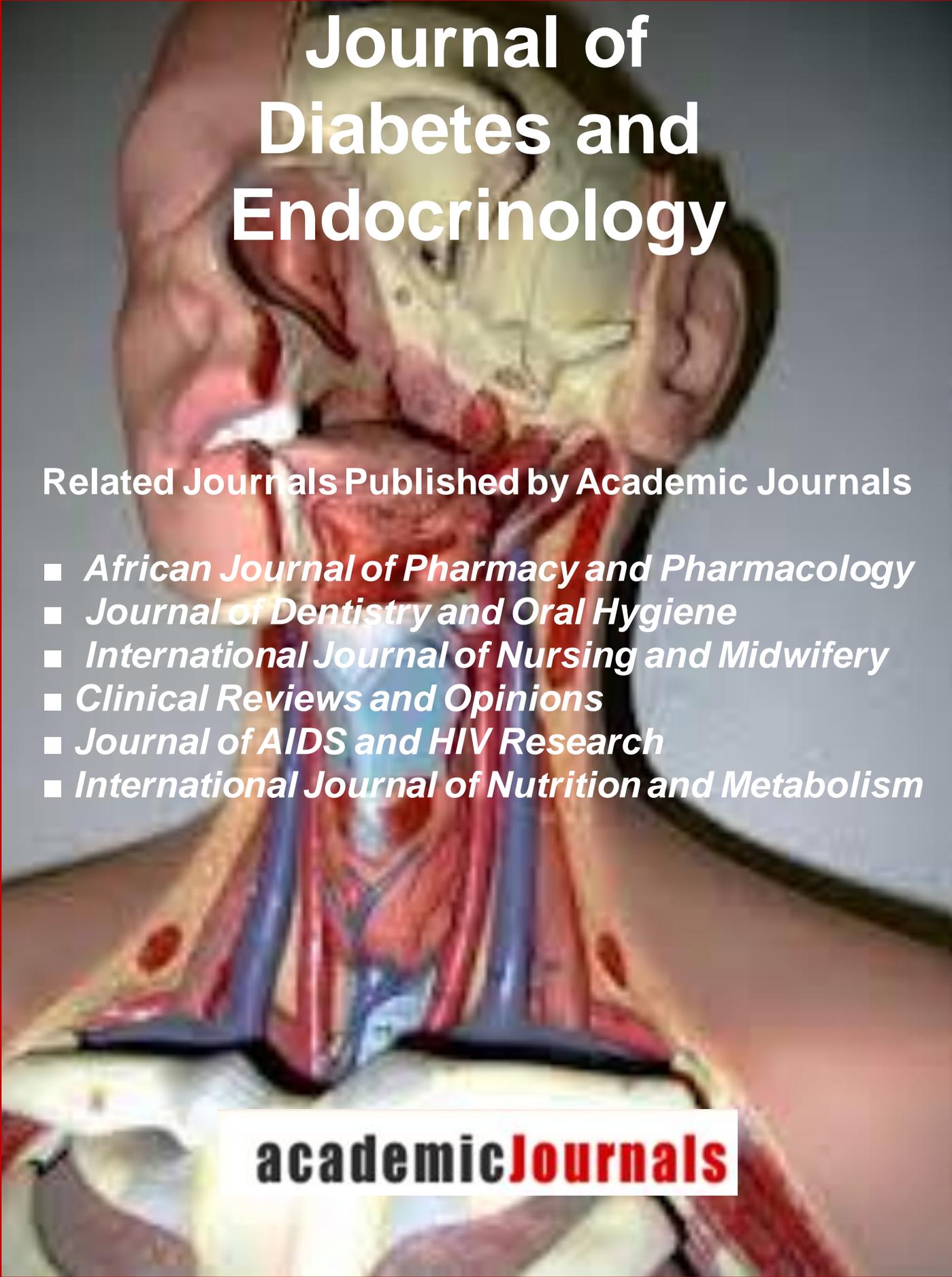
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