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Relation of Gut *Lactobacillus acidophilus* and Atherosclerosis in a Sample of Egyptian Type 2 Diabetic Patients

Salwa S. Hosny, Rania S. Abdelbaky, Yara M. Eid, Rana H. El attary*, Mark N. Bios and Nagwa R. Mohamed

Department of Internal Medicine and Endocrinology, Ain Shams University, Cairo, Egypt

Abstract

Background: Atherosclerosis is a major burden of modern society; ischemic heart disease and cerebrovascular stroke are the top leading causes of death worldwide. There is increase in the number of diabetics in the coming years. Microbiota is linked to development of type 2 DM in numerous ways; increased energy harvest, insulin resistance, chronic low-grade inflammation and modulation of gut-derived peptide secretion. There are accumulating studies that revealed that type 2 diabetic patients showed an altered intestinal microbiota.

Aim of the work: To study the association of gut *Lactobacillus acidophilus* and the presence of atherosclerosis in type 2 diabetic patients.

Patient and methods: A case control study was conducted on 64 patients divided into two groups.

• Group I: 32 type 2 diabetic patients with atherosclerosis.

• Group II: 32 type 2 diabetic patients without atherosclerosis.

They were subjected to full detailed medical history taking, clinical examination including anthropometric measurements of body weight, height, Body Mass Index (BMI) and peripheral pulsations. Serum creatinine, liver enzymes, fasting plasma glucose, fasting insulin, HOMA-IR, HbA1c, total cholesterol, triglycerides, high density lipoproteins, low density lipoproteins levels were assessed. Identification of stool *Lactobacillus acidophilus* by Polymerase Chain Reaction (PCR) semi-quantitative technique. Atherosclerosis was identified by measuring carotid artery Intima Media Thickness (IMT) using the carotid arterial duplex.

Results: On comparing the two studied groups we found a significant difference regarding two hours Post-prandial Plasma Glucose (2 hrs PPG), glycosylated Hemoglobin (HbA1C) (P-value<0.05), highly significant difference regarding insulin resistance model (HOMA-IR) (P-value=0.010), Intimal Medial Thickness (IMT) (P-value<0.001) and *Lactobacillus acidophilus* bacteria PCR Cut-off Threshold (PCR CT) in PCR positive cases among the two studied groups (P-value=0.016) being higher in group I. There was a significant positive correlation between *Lactobacillus acidophilus* PCR cut-off threshold and two hours Post-prandial Plasma Glucose (2 hrs PPG) (r=0.0319), glycated Hemoglobin (HbA1C) (r=0.328) in all studied subjects. However *Lactobacillus acidophilus* PCR cut-off threshold correlated positively with Intimal Media Thickness (IMT) only in diabetics with atherosclerosis (Group I).

Conclusion: Diabetic patients with atherosclerosis have lower level of *Lactobacillus acidophilus* with higher level of *Lactobacillus acidophilus* PCR cut-off threshold, and on correlation there was a positive correlation between *Lactobacillus acidophilus* PCR cut-off threshold and IMT in atherosclerotic group which provide a clue about protective effect of certain strains of GUT microbiota (mainly *Lactobacillus acidophilus* in this study) against the development of atherosclerosis in diabetic patients and may recommend the use of probiotics containing Lactobacillous acidophilous to protect against atherosclerosis.

Keywords: Type 2 diabetes; Intestinal microbiota; Insulin resistance; Atherosclerosis

Abbreviations:

ADA: American Diabetes Association; ANOVA: Analysis Of Variance; AUC: Area Under Curve; BMI: Body Mass Index; CVD: Cerebrovascular Disease; CHOL: Total Cholesterol; CAD: Coronary Artery Disease; ELISA: Enzyme-Linked Immuno Sorbent Assay; FBG: Fasting Blood Glucose; HbA1c: HemoglobinA1c; HDL: High Density Lipoprotein; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; IMT: Intima Media Thickness; LDL: Low Density Lipoprotein; PAD: Peripheral Arterial Disease; PCR: Polymerase Chain Reaction; PCR CT: Polymerase Chain Reaction Cut-off Threshold; ROC: Receiver Operating characteristic Curve; SD: Standard Deviation; SPSS: Statistical Package for the Social Sciences; TG: Triglycerides; TMAO: Trimethylamine-oxide; WHO: World Health Organization

Introduction

Atherosclerosis is a major burden of modern society and according to the 2018 report by the World Health Organization (WHO), ischemic heart disease, a major complication of atherosclerosis; is the leading cause of death worldwide [1].

***Corresponding author:** Rana H. El attary, Department of Internal Medicine and Endocrinology, Ain Shams University, Cairo, Egypt, Tel: (20)01067112217; E-mail: ranattary@gmail.com

Raymond C. Harris, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, Tel: (615) 322-2150 ; E-mail: ray.harris@vumc.org

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Diabetes is considered an important risk factor for the development and severity of all forms of atherosclerosis, including Peripheral Arterial Disease (PAD), Coronary Artery Disease (CAD), and Cerebrovascular Disease (CVD) [2].

There have been several studies suggesting that microbes may play a role in the development of atherosclerosis, and recently, colonic bacteria were considered as agents activating chronic inflammatory mechanisms. Many studies suggesting a link between the gut microbiota, inflammation and autoimmunity [3].

Search on animal models suggested that obesity, insulin resistance and the metabolic syndrome are associated with alterations of the composition and the functional properties of the gut microbiota [4].

A direct connection between microbiota and atherosclerosis has been established through directly atherogenic compounds like Trimethylamine-oxide (TMAO) which is produced by the action of gut microbiota [5].

Mechanisms also have been postulated for their athero-protective role including: increasing colonic short chain fatty acids decreasing Low Density Lipoproteins (LDL) and cholesterol synthesis, lowering inflammatory cytokines i.e, Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumor Necrosis Factor alpha (TNF-α) [4]. We conducted our study to detect the association of *Lactobacillus acidophilus* and atherosclerosis in type 2 diabetic patients.

Aim of the work

To study the association of gut *Lactobacillus acidophilus* and the presence of atherosclerosis in type 2 diabetic patients.

Materials and methods

A case control study was conducted on 64 patients attending the internal medicine and endocrinology outpatient clinics at Ain Shams University Hospital. It was conducted from February 2017 to October 2018. Before inclusion, an oral consent was obtained from each patient after full explanation of the study. Patients were divided into two groups.

- Group I: 32 type 2 diabetic patients with atherosclerosis.
- Group II: 32 type 2 diabetic patients without atherosclerosis.

Exclusion criteria included patients with hepatitis, renal impairment, recent myocardial infarction, recent cerebrovascular stroke or recent infection, chronic pulmonary disease or any history of intake of drugs that alter normal bacteria flora in the last month. Full medical history was taken from all subjects, emphasizing on the duration of diabetes mellitus, history of diabetic complications and other co-morbid conditions. Thorough clinical examination including blood pressure measurement, weight, height, BMI (kg/m^2) and peripheral pulsations examination.

Laboratory studies

Basic laboratory work: Ten ml of venous blood were collected by venipuncture (at the morning after 6-8 hours fast) using sterile plastic syringe, for measuring glycated hemoglobin and was stored at 4C to be carried out within one week which was measured by quantitative colorimetric determination of glycol-hemoglobin in blood. Another two ml sample was collected by venipuncture, two hours after intake of an oral glucose load of 75 gms for two hours post prandial blood glucose level and Fasting Plasma Glucose (FPG), were measured using an automated glucose oxidase method using Behring diagnostics reagents (SVR Glucose Test; Behring; La Jolla; CA) and fasting serum insulin (using Enzyme-Linked Immuno Sorbent Assay (ELISA)). Calculating the hemostatic model (HOMA-IR) as an estimate for insulin resistance (fasting plasma glucose (mg/dL) × fasting plasma insulin (mIU/mL)/405. Normal value <1 in adults, <3 in children [6]. High sensitivity C-reactive Protein (CRP) (by commercially available Human ELISA kit).

Assessment of atherosclerosis

Laboratory measures: Two ml of venous blood were collected by venipuncture (at the morning after 12 hours fast) using sterile plastic syringe for lipid profile (Total Cholesterol (TC), Triglycerides (TG) and High Density Lipoprotein (HDL-c) by quantification colorimetric and fluorometric methods.

Imaging: Atherosclerosis was identified using carotid arterial duplex done at Ain Shams university radiology department for measuring carotid artery Intima Media Thickness (IMT), where values>1.1 mm are considered to indicate the presence of an atherosclerotic plaque (Stein, et al. 2008).

Microbiota detection: Identification of stool *Lactobacillus acidophilus* by Polymerase Chain Reaction (PCR) semi-quantitative technique using QIAamp DNA stool kit.

Statistical analysis: Data was analysed by SPSSV17 2012, IBM Corporation, USA. The quantitative data were presented as mean, standard deviation and range, while qualitative data were presented as number and percentages. Chi-square test used compare between two independent groups with qualitative data. Independent t-test used to compare between two independent groups with quantitative data. One Way Analysis of Variance (ANOVA) followed by post hoc analysis using LSD test used to compare between more than two independent groups with quantitative data. Spearman correlation coefficients were used to assess the correlation between two quantitative parameters in the same group. Non-parametric distribution was done by using Mann-Whitney test. Receiver Operating Characteristic Curve (ROC) was used to assess the predictive value of PCR CT with its sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Area Under Curve (AUC). The confidence interval was set to 95% and the margin of error accepted was set to 5%. P>0.05: Non Significant (NS), P<0.05: Significant (S) and P<0.01: Highly Significant (HS).

Results

This study was conducted on 64 type 2 diabetic patients, with mean age (52.33 ± 4.60) years old. Divided into two groups.

- Group I: 32 type 2 diabetic patients with atherosclerosis.
- Group II: 32 type 2 diabetic patients without atherosclerosis.

We found a significant difference between the 2 groups regarding two hours Post prandial Plasma Glucose (2 hrs PPG), glycosylated Hemoglobin (HbA1C) (P value<0.05) and a highly significant difference regarding insulin resistance model (HOMA-IR) (P-value=0.010), Intimal Medial Thickness (IMT) (P value<0.001) being higher in group one. On comparing PCR value for *Lactobacillus acidophilus* between the two groups, the results of our study showed: 68.8% of cases (22 cases) had positive PCR for *Lactobacillus acidophilus* bacteria in group I while, 75% of cases (24 cases) had positive PCR for *Lactobacillus acidophilus* bacteria in group II, although its higher in group II but still with no significant statistical difference (P value=0.578). While there was a high statistically significant difference regarding *Lactobacillus acidophilus*

bacteria PCR Cut-off Threshold (PCR CT) in PCR positive cases among the two studied groups, being higher in group one (P value=0.016) as shown in Table 1.

There was a significant positive correlation between *Lactobacillus acidophilus* bacteria PCR cut-off threshold and two hours Post prandial Plasma Glucose (2 hrs PPG) (r=0.0319), glycated Hemoglobin (HbA1C) (r=0.328) in all studied subjects. There was a significant positive correlation between PCR cut-off threshold and two hours Post prandial Plasma Glucose (2 hrs PPG) (r=0.0581), glycated Hemoglobin (HbA1C) (r=0.424), Intimal Medial Thickness (IMT) (r=431) in group I as shown in Table 2.

The curve shows that *Lactobacillus acidophilus* bacteria PCR cut-off threshold has an excellent predictive value for development of atherosclerosis in type 2 diabetes patients (P value <0.0001, AUC 0.723). The best cutoff value for PCR cut-off threshold is >26.82 which had sensitivity 72.73% and specificity 70.83% as shown in Table 3 and Figure 1.

Discussion

The current study showed that 68.8% of diabetic patients with atherosclerosis (group I) were positive for PCR stool examination for Lactobacillus acidophilus, compared to 75.0% in diabetic patients without atherosclerosis (group II). P-value was (0.578), which make the higher percent of *Lactobacillus acidophilus* in group II of no statistical significance. There was a statistically significant difference among the two studied groups Regarding the *Lactobacillus acidophilus* PCR Cut-off Threshold (PCR CT) being higher in group I Which in turn denotes a higher concentration of *Lactobacillus Acidophilus* in-group II than in group I. This is in agreement with Salma, et al. who studied PCR for *Lactobacillus acidophilus* among controlled, uncontrolled diabetics and normal control subjects. There was no statistical difference between the studied groups, but Salma et al, didn't study its relation with atherosclerosis [7]. Also concordant with meta genome wide association study of gut microbiota by Qin, et al. who examined type 2 diabetes-related dysbiosis in gut microbiota. They found a degree of

Variable	Group I no=32	Group II no=32	Test value	P value
4 Age (yrs) Mean ± SD	51.91 ± 5.08	52.75 ± 4.10	0.731•	0.467
Duration of DM (years) Median (IQR)	6 (2-11)	10 (4-15)	-1.469‡	0.142
BMI (kg/m^2) Mean ± SD	29.11 ± 3.35	29.67 ± 2.43	-0.765•	0.447
Hypertension Non HTN HTN	17 (53.1%) 15 (46.9%)	17 (53.1%) 15 (46.9%)	0.000*	1.000
4 Cholesterol (mg/dl) Mean ± SD	218.56 ± 33.20	200.81 ± 42.96	1.849•	0.069
TG (mg/dl) Mean ± SD	180.81 ± 28.44	162.09 ± 67.94	1.438•	0.156
LDL (mg/dl) Mean ± SD	138.91 ± 33.54	135.56 ± 27.93	0.433•	0.666
HDL (mg/dl) Mean ± SD	39.38 ± 10.42	35.47 ± 8.73	1.626•	0.109
Alb/creat ratio (mg/dl) Median (IQR)	15.50 (10.5 25.5)	14.50 (10-27)	-0.121‡	0.904
FPG (mg/dl) Mean ± SD	182.41 ± 52.73	157.84 ± 47.72	1.954•	0.055
2 hrs PPG (mg/dl) Mean ± SD	260.09 ± 62.40	224.75 ± 49.32	2.514•	0.015*
HBA1c (%) Mean ± SD	9.50 ± 1.54	8.71 ± 1.15	2.332•	0.023*
F.insulin (miu/L) Median (IQR)	7 (5-13)	6.25 (5.5-8)	-1.246‡	0.213
HOMA-IR Median (IQR)	3.84 (2.22-5.77)	2.31 (1.88-3.44)	-2.572‡	0.010*
IMT (mm) Mean ± SD	1.54 ± 0.48	1.18 ± 0.21	3.855•	0.001*
PCR Negative Positive	10 (31.3%) 22 (68.8%)	8 (25.0%) 24 (75.0%)	0.309•	0.578
PCR Cut-off threshold Mean ± SD	29.77 ± 6.17	25.34 ± 5.82	2.507•	0.016*
P-value>0.05: Non Significant (NS); P-value<0.05: Significant (S); P-value<0.01: Highly significant (HS); •: Independent t-test; ‡: Mann Whitney test; *: Significant values.				

Table 1: Comparison of all parameters between group I and group II.

PCR CT						
Variable	**All Cases**		**Group I No=32**		**Group II No=32**	
	r	P value	r	P value	r	P value
Age (yrs)	-0.052	0.733	-0.090	0.689	-0.042	0.844
Duration of DM	-0.281	0.059	-0.262	0.239	-0.394	0.057
BMI (kg/m^2)	-0.151	0.317	-0.193	0.390	-0.122	0.569
Systolic blood pressure (mmHg)	-0.048	0.749	-0.422	0.051	-0.474	0.019
Diastolic blood pressure (mmHg)	0.021	0.890	-0.292	0.187	0.392	0.058
Cholesterol (mg/dl)	0.100	0.510	0.027	0.904	0.146	0.495
TG (mg/dl)	0.063	0.679	0.005	0.984	-0.057	0.791
LDL (mg/dl)	-0.110	0.468	-0.358	0.102	-0.096	0.656
HDL (mg/dl)	0.105	0.489	0.205	0.360	-0.128	0.552
FPG (mg/dl)	0.025	0.871	-0.145	0.519	-0.148	0.489
2 hrs PPG (mg/dl)	0.319*	0.031*	0.581*	0.005*	-0.026	0.905
HbA1c (%)	0.328*	0.026*	0.424*	0.049*	0.010	0.963
Alb/creat ratio (mg/dl)	0.054	0.723	0.015	0.948	0.111	0.605
F. insulin (mIu/L)	-0.143	0.342	-0.339	0.123	0.000	0.999
HOMA-IR	-0.068	0.653	-0.196	0.382	-0.097	0.654
IMT (mm)	0.228	0.128	0.431*	0.045*	-0.159	0.459
P-value>0.05: Non Significant (NS); P-value<0.05: Significant (S); P-value<0.01: Highly Significant (HS); *: Significant values.						

Table 2: Correlation between Lactobacillous acidophilus bacteria PCR cut-off threshold and the other studied parameters in all patients, group I and group II.

Parameter	Area under curve	Cut of point	Sensitivity	Specificity	PPV	NPV
PCR CT	0.723	>26.82	72.73	70.83	69.6	73.9

Table 3: Receiver Operating Characteristic Curve (ROC) for PCR cut-off threshold as a predictor for atherosclerosis in type II diabetic patients.

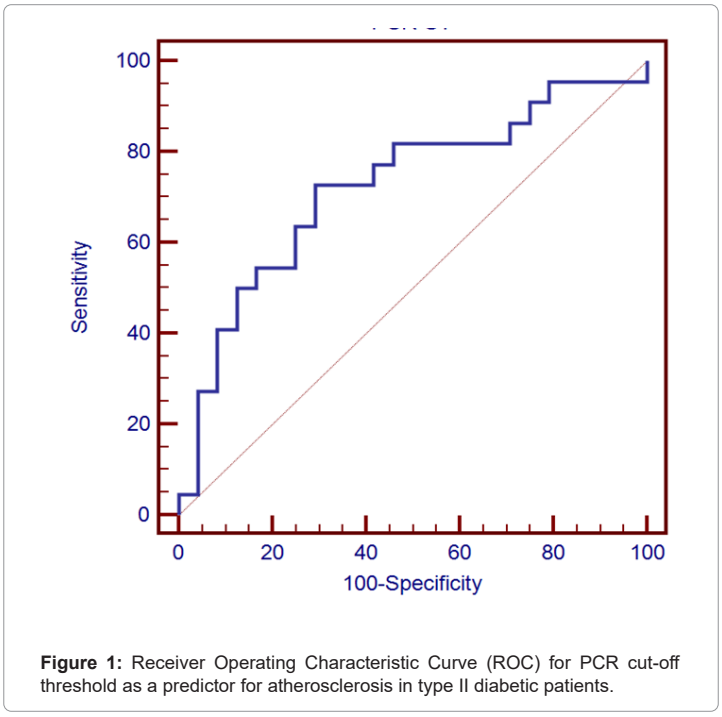

Figure 1: Receiver Operating Characteristic Curve (ROC) for PCR cut-off threshold as a predictor for atherosclerosis in type II diabetic patients.

moderate dysbiosis with a decrease in the abundance of some universal butyrate-producing bacteria and an increase in various opportunistic pathogens; however, they didn't observe a significant difference in within sample diversity between type 2 diabetes and control groups [8].

Our study also shows a statistically significant positive correlation between *Lactobacillus acidophilus* PCR Cut-off Threshold (PCR CT) and Intima Media Thickness (IMT), 2 hrs PPG, HbA1c in diabetic patients with atherosclerosis group I. And since a higher cut-off threshold means a lower concentration of *Lactobacillus acidophilus* bacteria. Therefore accordingly, patients with a lower concentration of *Lactobacillus acidophilus* are more associated with atherosclerosis which agrees with our postulate of the protective value of *Lactobacillus acidophilus* bacteria against atherosclerosis. This is in agreement with Dong-Mei, et al. who studied the probiotic role of *Lactobacillus plantarum* against atherosclerosis; he introduced a novel strain of *Lactobacillus plantarum* isolated from naturally fermented mustard into high-fat-diet-induced hyperlipidaemic rats, which resulted in a more obvious lipid-lowering effect and significant decrease in total cardiovascular risk, atherosclerosis index [9]. Lihua Chin, et al. in his study showed that the introduction of *Lactobacillus acidophilus* ATCC 4356 daily for 12 weeks into mice caused a decrease in TNF-α and improvement in atherosclerosis index which were used as surrogates for atherosclerosis. They also found an increase in High Density Lipoproteins (HDL), decrease in Low Density Lipoproteins (LDL) [10].

This also agreed with Yao, et al. who conducted a meta-analysis aimed to summarize the effect of probiotics on glucose and lipid metabolism and C-reactive Protein (CRP) from 12 Randomized Controlled Trials (RCTs) in 684 patients. The effect of probiotics (including Lactobacillus acidophillus) was significant on reducing HbA1c level, fasting insulin level, and HOMA-IR [11].

Also Moroti, et al. reported that the administration of a synbiotic beverage called shake, which contained *Lactobacillus acidophilus*, Bifidobacterium bifidum and oligofructose to patients with total cholesterol>200 mg/dL; triglycerides>200 mg/dL and fasting glycemia>110 mg/dL. All patients were taking glucose-lowering medications however, drug treatment was not able to normalize blood glucose levels in them. Over a total test period of 30 days markedly increased the plasma HDL cholesterol and decreased the condition of fasting glycemia in elderly T2DM patients [12].

Conclusion

In summary, our study concluded that diabetic patients with atherosclerosis has lower level of *Lactobacillus acidophilus* with higher level of *Lactobacillus acidophilus* PCR cut-off threshold, and on correlation there was a positive correlation between PCR cut-off threshold and IMT in atherosclerotic group. Therefore accordingly, patients with a lower concentration of *Lactobacillus acidophilus*, are more associated with atherosclerosis, this provide a clue about protective effect of certain strains of GUT microbiota, exclusively here *Lactobacillus acidophilus* against the development of atherosclerosis and macrovascular disease in diabetic patients. We recommend further studies with larger number of subjects and studying the same effect of other strains of our GUT microbiome on development of atherosclerosis.

Conflict of Interest

The authors declare no conflict of interests.

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