Type 2 Diabetes Mellitus Patients with Good or Poor Glycaemic Management: Relationship Between Nitric Oxide, Growth Hormone, Lipid Profile and HbA1c Levels

İyi ve Kötü Glisemik İzlemli Tip 2 Diabetes Mellitus'lu Olgularda, Nitrik Oksit, Büyüme Hormonu, Lipid Profili ve HbA1c Düzeyleri Arasındaki İlişki

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ABSTRACT Objective: In type 2 diabetes mellitus (T2DM), looking for predictive markers for diseases risk as well as glycaemic control (GC) levels is essential for a better management for the disease. The aim of this study is to investigate the possible relationship between nitric oxide (NO) and growth hormone (GH) on lipid profile and HbA1c plasma levels in T2DM patients with good or poor glycaemic management. **Material and Methods:** Twenty seven diabetic patients with good (GC) (HbA1c ≤ 7%), 32 with poor GC (HbA1c ≥ 7%), and 17 non-diabetic subjects were included in the study. Serum cholesterol, triglyceride (TG), high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, HbA1c, fasting serum glucose (FSG), NO and GH levels were determined. **Results:** Of the risk parameters, only GH was lower in good GC group, FSG, HbA1c, TG, LDL-cholesterol and NO were significantly higher ($p< 0.05$) and HDL-cholesterol and GH were lower ($p< 0.05$) in poor GC group compared with control group. FSG, HbA1c and NO were significantly higher in poor glycaemic control group compared with good GC group ($p< 0.05$). **Conclusion:** This study investigated the possible relationship between NO and GH levels and GC in T2DM patients. Results indicated a relation between GC, NO and GH. In poor GC group, high NO level may be a "predictive marker" for early diagnosis of vascular dysfunction. However, in T2DM with and without CVD large scale studies on new parameters are needed for the prediction of CVD.

Key Words: Diabetes mellitus, type 2; nitric oxide; growth hormone; hemoglobin A1c protein, human; lipids; cardiovascular diseases

ÖZET Amaç: Tip 2 diabetes mellitus'ta (T2DM), hastalığın daha iyi izlemi için glisemik kontrol (GK) düzeyleri kadar, prediktif belirteçlerin hastalık riskleri için izlenmesi gereklidir. Bu çalışmanın amacı, kötü veya iyi glisemik izlemli T2DM olgularında, HbA1c ve lipid profili ile nitrik oksit (NO) ve büyüme hormonu (BH) arasındaki olası ilişkiyi araştırmaktır. **Gereç ve Yöntemler:** Çalışmaya iyi GK'li (HbA1c ≤ %7) 27 olgu, kötü GK'li (HbA1c ≥ %7) 32 olgu ve 17 diyabetik olmayan olgu dahil edilmiştir. Serum kolesterol, trigliserid (TG), yüksek yoğunluklu lipoprotein (HDL)-kolesterol, düşük yoğunluklu lipoprotein (LDL)-kolesterol, HbA1c, açlık serum glukoz (ASG), NO ve BH düzeyleri değerlendirilmiştir. **Bulgular:** Risk parametrelerinden iyi GK grubunda, kontrol grubu ile karşılaştırıldığında, yalnızca BH düşük, kötü GK grubunda ASG, HbA1c, TG, LDL-kolesterol ve NO belirgin olarak daha yüksek bulundu ($p< 0.05$) ve HDL-kolesterol ve BH daha düşük bulundu ($p< 0.05$). Kötü GK grubunda, iyi GK grubu ile karşılaştırıldığında, ASG, HbA1c ve NO belirgin olarak daha yüksek bulundu ($p< 0.05$). **Sonuç:** Bu çalışma T2DM olgularında NO ve BH düzeyleri ve GK arasında olası bir ilişkiyi araştırmıştır. Sonuçlar GK, serum NO ve BH arasında bir ilişkiyi göstermiştir. Kötü GK grubunda yüksek NO düzeyi vasküler bozukluk için bir "prediktif belirteç" olabilir. Bununla birlikte bu yeni parametrelerin, kardiyovasküler riski tahmini için daha geniş çalışmalar gerekmektedir.

Anahtar Kelimeler: Tip 2 diabetes mellitus; nitrik oksit; büyüme hormonu; hemoglobin A1c; lipidler; kardiyovasküler hastalıklar

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Diabetes mellitus (DM) is a multifactorial disease characterized by hyperglycaemia. The disease is becoming increasingly common in various populations and being considered as a public health problem. While having its own complications, DM also increases the risk of various other health problems that decrease the quality of life and sometimes lead to early death.

Epidemiologic studies showed us that cardiovascular diseases (CVD) are an important cause of death in T2DM patients.[1] The risks and the causes are still under investigation. To be able to foresee the risks is crucial for better management of DM.[2] This is generally accepted that the people with T2DM have abnormal atherogenic lipid profiles; therefore lipid profiles should be monitorized. But the field studies showed that lipid profiles are not sufficient for predicting the risk of CVD in T2DM. Novel predictive markers are needed for better management of the disease.[3,4]

NO is a gas that is continuously synthesized in endothelial cells and executes multiple functions that maintain vascular homeostasis.[5] Nitrite has the potential to be a major intravascular NO storage molecule in humans that is capable of transducing NO bioactivity distal to its site of formation.[5,6] Serum nitrite has been described as an index of nitric oxide synthase (eNOS) activity in the regional and systemic circulation in humans and various mammals.[6-8]

Many of the studies have shown that poor GC is associated with the vascular complication of the disease.[9] NO, is thought to have an important role on vascular system, especially on endothelial cell functions in diabetic patients.[10-12]

The well-known effects of GH on energy, lipid metabolism, insulin and cardiac functions made hypothalamic-pituitary axes one of the targets of risk reducing treatments and management. There are studies on short and long term administration of GH especially in obese patients with type 2 diabetes.[13-16]

In adults decreased GH is beneficial in increasing the amount of visceral fats, and may therefore result in improvements in insulin resistance, atherosclerotic risk factors and dyslipidaemia, decreased exercise capacity due to decreased cardiac output, decreased energy due to decreased metabolic rate and abnormal body composition.[17,18]

The present study was to evaluate the relationship of NO and GH on lipid profile and HbA1c levels in T2DM patients with good or poor glycaemic management.

MATERIAL AND METHODS

The study was an observational, cross sectional case-control study of T2DM patients with good and poor glycaemic management and healthy controls. All participants gave informed consent to participate in the study, which had been approved by the local Ethics Committee (Ministry of Health Tepecik Education and Research Hospital, local ethics committee, 30.03.2007/67:10).

The study groups were consisted of 17 control patients [Age (y); 41.94 ± 19.09; 14 female and 3 male subjects] 27 T2DM patients with good glycaemic management [Age (y); 62.37 ± 12.19; 11 female and 16 male subjects] and 32 T2DM patients with poor glycaemic management [Age (y); 58.72 ± 8.71; 16 female and 16 male subjects]. All participants of groups were under Mediterranean diet which is famous of being nitrites free (e.g salami, sausage).

T2DM patients continued their regular laboratory visits for HbA1c determination in every 3 months. All participants provided a medical history and were investigated for any cardiovascular diseases. T2DM patients with poor glycaemic management who had two successive measurement of HbA1c levels greater than 7%. Control subjects were apparently healthy and T2DM patients were not on insulin therapy. For this study, participants who smoke and body-mass index greater than 30 kg/m^2 were excluded. Diabetes was diagnosed according to the International Diabetes Federation criteria.[19] All the experimental studies were realized in Ministry of Health, Refik Saydam National Hygiene Center, İzmir Regional Institute of Hygiene laboratories.

Blood samples were obtained between 8.^{00} a.m -10.^{00} a.m early morning following an overnight fasting (12 hours). Serum samples were centrifuged at 1500 *g* for 10 min and were stored frozen at -20°C, until thawed for further analysis. The levels of serum glucose, total cholesterol, TG, HDL-cholesterol and LDL-cholesterol were determinated by Konelab 60i auto analyzer (Dialab, Austria). Glycoheamoglobin (HbA1c) was quantified in whole blood by Tosoh automated Glycoheamoglobin Analyzer (Tosoh Corp., Japan) based on high performance liquid chromatography. GH levels were determined in serum by Tosoh AIA Systems analyzer (Tosoh Corp., Japan) based on two-site immunoenzymometric assay. The minimal concentration of GH was estimated to be 0.1 ng/mL.

Nitrite is generated by the rapid oxidation of NO. Nitrate plus nitrite (NOx) was measured by the Griess method. The minimal concentration of NOx was estimated to be 2.5 μM. To assay nitrite we used a modification of a previously published method.[20] Aliquots of 100 μL samples were mixed with 100 μL of equal volumes of Griess Reagent mixture (A: naphthyethylene- diamine dihydrochloride, 0.1%, B: sulphanilamide in o-phosphoric acid, 1%) in a 96-well microtitre plate (Maxisorb Immunoplate, NUNC). After 10 minutes of incubation at room temperature, the absorbance (540 nm) was measured in a micro plate reader (Reader Model 230S; Organon Technica, Microwell System, Holland). A range of 2-fold dilutions of sodium nitrite (0-128 μM) in PBS was run in each assay to generate a standard curve.

Data were expressed as means ± standard deviation of mean. A two-tailed model was used and a p value < 0.05 was considered to be statistically significant. A statistical significance was tested by Wilcoxon and post-hoc testing performed by Mann-Whitney U test. Relationships among variables were determined by Spearman's correlation analysis. Multiple linear regression analysis was performed in stepwise selection to identify independent factors and to estimate the final predictors of their variability. All statistical analysis was performed by SPSS/Windows version 10.0, SPSS inc., Chicago, IL, USA.

RESULTS

The fasting biochemical profiles and demographic data of T2DM patients are presented in Table 1. The lipid profile parameters did not significantly differ in T2DM patients with good glycaemic control (GGC) with respect to control group. Fasting serum glucose (FSG), HbA1c, TG, LDL-cholesterol and serum NO were significantly higher in T2DM patients with poor glycaemic control (PGC) with respect to control group while levels of HDL cholesterol and GH were significantly lower in this group. The levels of FSG, HbA1c and serum NO

TABLE 1: Fasting biochemical profiles presented in type 2 diabetes mellitus (T2DM) with poor (16 male, 16 female subjects) and good (16 male, 11 female subjects) glycaemic management; and controls without T2DM (3 male, 14 female subjects).

	Control subjects (n= 17)	Good glycaemic management (n= 27)	Poor glycaemic management (n= 32)
Age (y)	41.94 ± 19.09	**62.37 ± 12.19[a]**	**58.72 ± 8.71[a]**
Fasting serum glucose (mg/dL)	89.12 ± 8.62	**129.37 ± 22.74[b]**	**196.63 ± 81.27[a,b]**
HbA1c (Hb %)	5.27 ± 0.29	**6.03 ± 0.48[b]**	**9.07 ± 1.93[a,b]**
Serum total cholesterol (mg/dL)	186.59 ± 22.22	211.63 ± 33.91	205.81 ± 39.02
Serum triglyceride (mg/dL)	96.94 ± 26.69	142.41 ± 74.08	**147.00 ± 64.96[a]**
Serum HDL cholesterol (mg/dL)	56.76 ± 14.17	48.77 ± 10.68	**48.00 ± 9.82[a]**
Serum LDL cholesterol (mg/dL)	115.31 ± 22.26	135.32 ± 30.76	**141.95 ± 24.89[a]**
Serum Nitrite (NOx) (μmol/L)	10.26 ± 3.88	**11.72 ± 6.83[b]**	**15.38 ± 4.16[a,b]**
Growth hormone (ng/mL)	3.17 ± 3.30	**0.69 ± 0.63[a]**	**0.88 ± 1.23[a]**

Values are means + SD. The mean difference is significant at the 0.05 level. A statistical significance was tested by Wilcoxon and post-hoc testing performed by Mann Whitney U test.

[a] Statistical comparison with control subjects,

[b] Statistical comparison with patient subjects.

were significantly higher in T2DM patients with PGC with respect to T2DM patient with GGC. There was no significant difference in the levels of serum total cholesterol between both of diabetic patients and control subjects (Table 1).

Correlation analyses were performed between HbA1c and biochemical markers of current and suggested CVD risk predictors in the T2DM patients. In T2DM patients; FSG, TG and serum NO levels positively correlated with HbA1c (r= 0.695, r= 0.419, r= 0.371 respectively; for all p< 0.01), and FSG and serum NO levels positively correlated with HbA1c (r= 0.701, r= 0.458 respectively; for all p< 0.01) in T2DM male patients. Only FSG levels positively correlated with HbA1c (r= 0.719; p< 0.01) in T2DM female patients. GH levels correlated negatively with HbA1c (r= -0.332; p< 0.05) in female patients (Table 2).

There was a significant difference in the CVD positivity between both of diabetic patients (in poor management group 80% and in good management group 44, 4%) and control subject (any person) for all p< 0.01. Correlation analyses were performed between CVD positivity and HbA_{1c}, NOx, GH in the T2DM patients. In T2DM patients, HbA1c and serum NOx levels positively correlated with CVD positivity (r= 0.631 r= 0.207 respectively; for all p< 0.05), and serum GH levels negatively correlated with CVD positivity (r= -0.321; p< 0.01) in T2DM patients (Table 3).

TABLE 2: Pearson's correlation coefficients between HbA1c and biochemical variables for (of) current and suggested VD risk predictors in the type 2 diabetes mellitus patients.

	HbA1c in male subjects	HbA1c in female subjects
Age	0.024	0.221
Fasting serum glucose	**0.701****	**0.719****
Serum total cholesterol	0.029	0.052
Serum triglyceride	**0.419****	0.079
Serum HDL cholesterol	-0.122	-0.244
Serum LDL cholesterol	-0.120	0.100
Serum Nitrite (NOx)	**0.458****	0.291
Growth hormone	0.147	**-0.332***

Values are pearson's correlation coefficients (r).
* pearson correlation is significant at the 0.05 level (2-tailed).
** pearson correlation is significant at the 0.01 level (2-tailed).

TABLE 3: Pearson's correlation coefficients between patient with CVD and HbA1c, NO, GH in type 2 diabetes mellitus patients

Biochemical parameters	CVD (+)
HbA1c (Hb %)	**0.631****
Serum Nitrite (NOx)	**0.207***
GH	**-0.321****

CVD: Cardiovascular diseases, GH: Growth hormone
Values are Pearson's correlation coefficients (r).
* Pearson correlation is significant at the 0. 05 level (1-tailed).
** Pearson correlation is significant at the 0.01 level (1-tailed).

Multiple linear regression analysis of association among glycaemic levels and predictors in the T2DM patients are shown in Table 4. In female patients, GC levels were significantly correlated with HbA1c, age, NO, and LDL-cholesterol (p< 0.05). This model explained 76.8% of the variance in GC levels while only HbA1c association (53.6% of the variance in GC levels) in male patients was significant (p< 0.05).

DISCUSSION

DM has many complications that lower the quality of life, and some of these end up with early mortality. According to the epidemiologic studies, the most common cause of death is CVD in T2DM patients.[21] In order to identify the diabetic patients that carry this risk, many studies were done with controversial results. Haffner et al found that the risk of CVD is similar in type 2 diabetic patients compared with non-diabetic patients, although general belief is that the T2DM patients have atherogenic and abnormal lipid profiles.[22,23] In this respect, major guidelines which have addressed the area recommend a full serum lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) as a guide to therapy.[24]

On the other hand, in some studies such as Framingham, UKPDS and NICE, the estimated risk was found to be 2 to 3 times underestimated than the real risk.[3,4] Similarly, in our study in the group with good metabolic management, the increase in total cholesterol LDL-cholesterol and TG levels and the decrease in HDL cholesterol level were similar with the control group. But in the PGC group that

TABLE 4: Multiple linear regression analysis of association among glycaemic management levels and predictors.

	Male		Female	
	β	p	β	p
Age	0.007	NS	0.330	0.000
Fasting serum glucose (FPG)	0.088	NS	0.140	NS
HbA1c	**0.741**	**0.000**	**0.639**	**0.000**
Serum total cholesterol	-0.062	NS	0.059	NS
Serum triglyceride	-0.129	NS	0.129	NS
Serum HDL chlosterol	-0.131	NS	-0.074	NS
Serum LDL cholesterol	0.039	NS	**0.161**	**0.046**
Serum Nitrite (NOx)	-0.018	NS	**0.230**	**0.007**
Growth hormone (GH)	-0.196	NS	-0.070	NS
Predictor in the model (Constant)	**HbA1c (H)**		**HbA1c(H),age, NOx, LDL**	
Adjusted r^2 for the model	**53.6**		**76.8**	

Dependent variable glycaemic management levels. NS: Not significant

was carrying a higher risk, TG and LDL cholesterol levels were significantly higher and HDL-cholesterols levels were significantly lower than the control group. The only lipid parameter that has an effect on GC was high LDL levels in female subjects. When multiple regression analysis was performed with GC levels and risk predictors; in the model developed with dyslipidemia markers (HbA1c and Cholesterol constant) adjusted r square was found as 60.3 ($p< 0.0001$) (data not show), and in the model developed by adding GH and NO, adjusted r square was calculated as 76.8 in female subjects ($p< 0.0001$). These results supported the idea that the lipid profiles alone will not be predictive for risk assessment. Lipid profile parameters especially in total cholesterol LDL-cholesterol, HDL-cholesterol and TG did not significantly differ from patient with GGC with respect to patient with PGC. As addressed by the authorities dealing with DM, risk assessment is an important problem that needs to be solved.

GH levels were slightly higher (but not statistically) in PGC group compared to the GGC group in our data. In healthy subjects, GH secretion is stimulated by hypoglycaemia, sleep, exercise, stress, and amino acid and is inhibited by hyperglycaemia. Because in hypoglycaemia, production of counter regulatory hormones such as GH increases, the sympathic system becomes stimulated and features of neuroglycopenia appear in order to save the homeostasis.[25] Hypoglycaemic attacks were frequently shown in poor metabolic control with diabetes. Also, NO stimulates growth hormone releasing hormone in the hypothalamus.[26] In our data, statistically significant higher NO levels were found in PGC group compared to GGC group.

A number of clinical studies, have reported beneficial effects of GH replacement on cardiovascular risk and lipid profile.[14-19] GH levels were statistically lower in both PGC and GGC groups compared to the control group in our study which supports this information.

Some epidemiologic studies stated that the connection between GH deficiency and CVD risk is gender-related or gender-specific. There are more reports about GH deficiency-associated CVD risk in female subjects.[27-29] Our study revealed a similar result: GC levels and GH levels showed negative correlation only in female subjects. We believe that GH will be helpful in this risk management.[15]

Vascular endothelial cells play an important role in the pathogenesis of vascular complications of diabetes. Many of the studies have shown a convincing association between NO activity and endothelial dysfunction.[30] However, there are few studies focusing the interaction between NO and current markers of vascular risk especially lipid pa-

rameters, age and gender. In diabetic cases, NOS activation results in high levels of NO which react with metabolically produced excessive super-oxide and produce a more reactive form, peroxynitrite. In addition, up regulation in the NF-κβ/LOX-1 pathway caused by hyperglycaemia increases the formation of peroxynitrite.[12] It is suggested that peroxynitrite is responsible for endothelial damage.[31] Reactive nitrogen species (RNS) and reactive oxygen species (ROS) were suggested as the putative mechanism of vascular injury in diabetic patients.[28] In our study, serum NO levels were significantly higher in T2DM patients compared with control subjects ($p< 0.05$). Furthermore, statistically significant higher NO levels were found in PGC cases compared to GGC cases. High serum NO levels, both in animals and in humans have been demonstrated in previous studies in diabetes.[32-34] Rodriguez-Manas et al clearly showed that in type 1 diabetes vascular NO system has a role in GC.[35] But there is no report about NO as a risk predictor in T2DM for GC levels. Overview of Szabo C is the role of reactive nitrogen species (nitrosative stress) and associated pathways in the pathogenesis of diabetic vascular complications. Increased extracellular glucose concentration, a principal feature of DM, induces a dysregulation of reactive oxygen and nitrogen generating pathways. These processes lead to a loss of the vascular endothelium to produce biologically active NO, which impairs vascular relaxations.[36] Our data indicate that serum NO levels related with HBA1c levels in T2DM patients.

CONCLUSION

We may conclude that glycaemic control level is related to GH and NO in T2DM subjects. In PGC cases, predictive markers that will provide early diagnosis of vascular dysfunction are important for better management of the disease. We believe that GH and NO will be helpful in this respect. Also, studies on gender associated differences in these markers will be worthwhile. As a result, in T2DM with and without CVD large scale studies on new parameters are needed for the prediction of CVD. Furthermore, the studies will be included some information about late diabetes complications (micro-angiopathy, macro-angiopathy, and neuropathy).

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