Age and gender related differences in free fatty acid levels in patients with type 2 *diabetes mellitus*

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Abstract

Introduction: Several decades of basic science and animal research provided considerable support for significant role of plasma free fatty acids (FFAs) in etiology of Type 2 *diabetes mellitus* (T2DM). Contradicting data related to significance of elevated FFAs in plasma of patients with Type 2 diabetes prompted us to study concentrations of palmitic acid, stearic acid, and linoleic acid, in patients and healthy controls in an attempt to possibly use them as potential biomarkers in progression of the disease. Since aging is associated with increased plasma glucose and insulin levels that are consistent with an insulin resistant state, in this study, age differences in the concentration of the above mentioned acids were tested.

Methods: Progressive changes in their concentrations were followed through a period 6 months. All subjects included in the study were free of evidence of hepatitis B or C viral infection or active liver and kidney damage. Analysis of glucose and glycated hemoglobin levels were performed on BT PLUS 2000 analyzer using standard IFCC protocols, while concentrations of FFAs were analyzed by gas chromatography.

Results: Our data demonstrated significantly higher FFA values in plasma of diabetic patients as compared to healthy controls. There was a trend of correlation of FFAs levels with the blood glucose levels in diabetic patients, which was more prominent in diabetic men than in women.

Conclusion: With aging, levels of free fatty acids significantly increased in plasma of diabetic patients, and this effect was also more profound in male than in female diabetics.

Keywords: Diabetes mellitus, free fatty acids, insulin resistance, biomarkers

Introduction

Diabetes is associated with a variety of derangements manifested through defects in the use of carbohydrates, synthesis and catabolism of proteins, and lipid metabolism. So far, it has been demonstrated that age is a significant risk factor for the development of Type 2 diabetes (T2D) (1). Namely, numerous studies have reported a significantly higher prevalence of impaired glucose tolerance test (IGT), obesity, and type 2 diabetes in people older than 65 years. Moreover, in all studies related to this phenomenon, insulin secretion decreased with age, while insulin resistance and fasting plasma free fatty acids (FFAs) concentrations increased in older subjects (2-4). Free fatty acids represent important nutrients and the key oxidative fuel for the heart, liver, and skeletal muscle. They are thought to be potent signaling molecules (5-9), whose presence in the circulation is a result of dietary intake and endogenous relase from stored fat, primarily adipocytes. Growing evidence indicates that FFAs

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may be involved in pathogenesis of T2D and obesity through mechanisms of insulin resistance (IR) (10-14). An important consequence of IR at the level of adipose tissues is enhanced lipolysis and reduced free fatty acid uptake and esterification, leading to an increased flux of FFA into nonadipose tissues, such as liver and muscle. Although both unsaturated and saturated fatty acids have been linked to insulin resistance, there is evidence that saturated fat intake more effectively induces IR. The data indicate that FFAs cause IR both *in vitro* and *in vivo*. The potent effects of long-chain saturated fatty acids (LCSFA) on IR development were confirmed in adipocytes *in vitro* (10, 15). Furthermore, overnight reduction in FFAs improved insulin sensitivity *in vivo* in obese patients, Type 2 diabetics and nondiabetics. In addition, substantial evidence from both, humans and animals, has indicated that essential fatty acid (EFA) metabolism is also abnormal in diabetes (15, 16). In this study, a potential biomarker role of three most abundant FFAs (palmitic, stearic, and linoleic acid) was examined in T2D patients and respective healthy controls. In addition, the effects of optimal glucose control, patients´gender and age on plasma FFAs were also evaluated in these patients.

Methods

Patients

In this study we have analyzed FFAs levels in a group of 40 patients diagnosed with Type 2 *diabetes mellitus* with a mean age of 61 years and 40 healthy, nondiabetic controls with a mean age of 43 years. All humans subjects involved in this study were patients of General Hospital in Tešanj, BH. All research involving human subjects and material derived from human subjects in this study was done in accordance with the ethical recommendations and practices of the Tešanj General Hospital and complied with ethical principles outlined in World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects (initiated in June 1964, last amendment in October 2000). Subjects included in this study were free of evidence of hepatitis B or C viral infection or active liver and kidney damage. Patients were selected for the study on the basis of presence of history of diabetes for more than five years and were receiving standard drug therapy of 250 mg Metformine. Patients receiving drugs known to influence FFA levels were excluded from this study. Initial diagnosis of T2D was established by a specialist of internal medicine. Nondiabetic controls were of approximately same age (35-87 years old), with normal glucose tolerance (fasting plasma glucose less than 6.2 mmol/l and two hours postprandial glycaemia less than 7.8 mmol/l). They also had no abdominal obesity as a clinical criteria for insulin resistance.

Sample Analysis

Blood samples were obtained from patients and nondiabetic controls subjects in fasting conditions from antecubital vein into siliconized tubes (BD Vacutainer Systems, Plymouth, UK). Analysis of glucose and glycated hemoglobin levels (HbA1c) in plasma were performed by employing BT PLUS 2000-Biotechnic Instruments. Standard IFCC (International Federation for Clinical Chemistry) protocols were used for all analyses. For fatty acid analysis, lipids were extracted with chloroform-methanol 2:1 (vol/vol) than, sample of fatty acid methyl esters (FAMEs) of free fatty acids were prepared according to method self-modifying Lepage and Roy. Samples were analyzed on a Shimadzu QP-5000 GC/MS gas chromatograph equipped with mass spectrometer detector, Shimadzu 20A GC/FID gas chromatograph equipped with a flame ionization detector and capillary column Resterkorp OPTIMA® 120 (30mx0.32x0.25μm film thickness). The identity of each fatty acid peak was obtained by comparing the retention time of the peak with the retention times of referent standards with known fatty acids composition.

Statistical Analysis

Data are expressed as mean ± SEM. The significance of differences among groups was analyzed statistically by ANOVA, followed by Spearman's coefficient correlation and Student's *t* test. Calculations were done using SPSS 17.0 for Windows. Statistical significance was set as $p<0.05$.

Results

Our study analyzed plasma levels of palmitic, stearic and linoleic acid in a group of 40 patients

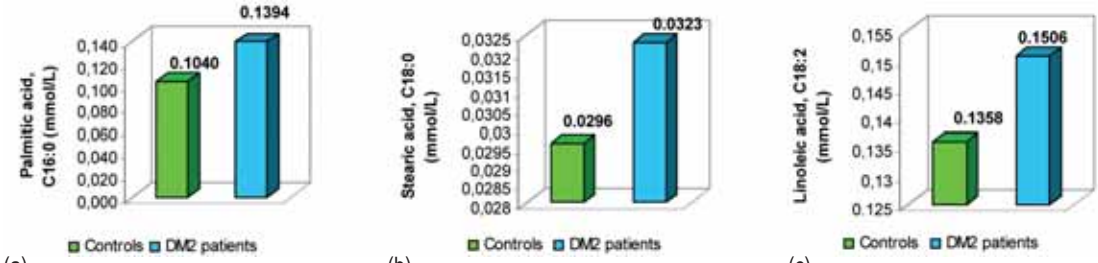


FIGURE 1. Average concentration of palmitic acid (a), stearic acid (b), linoleic acid (c) in plasma of patients with Type 2 dibetes mellitus and control subjects, no statistically significant.

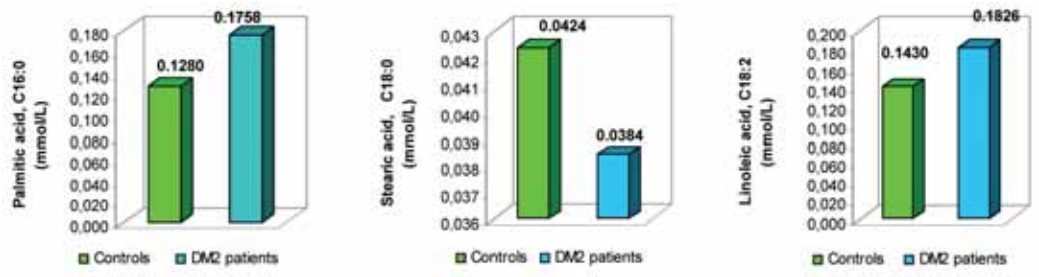


FIGURE 2. Average concentration of palmitic acid (a), stearic acid (b), linoleic acid (c) in plasma of male patients with Type 2 dibetes mellitus and control subjects, no statistically significant.

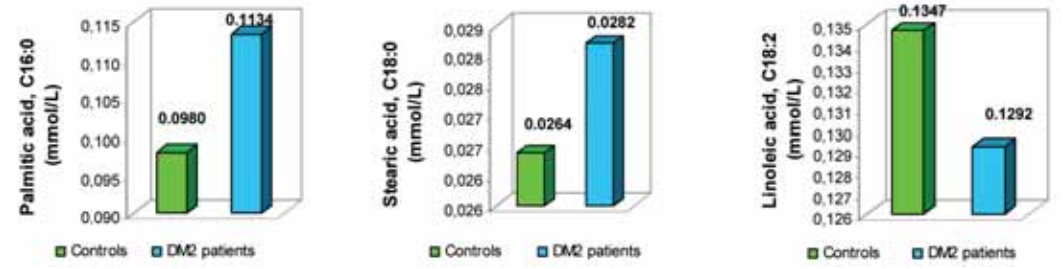
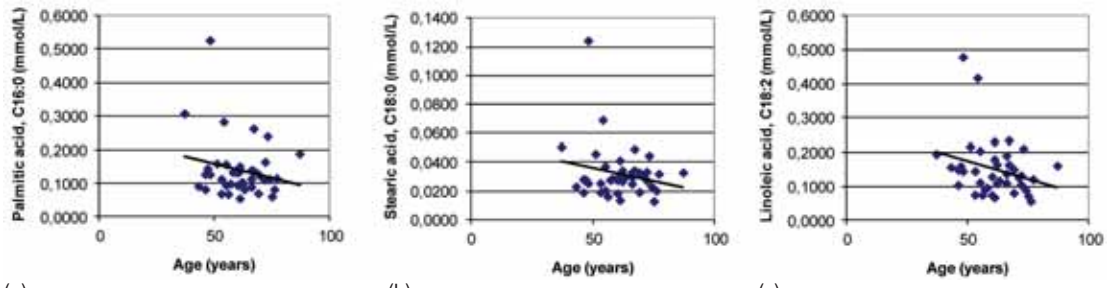


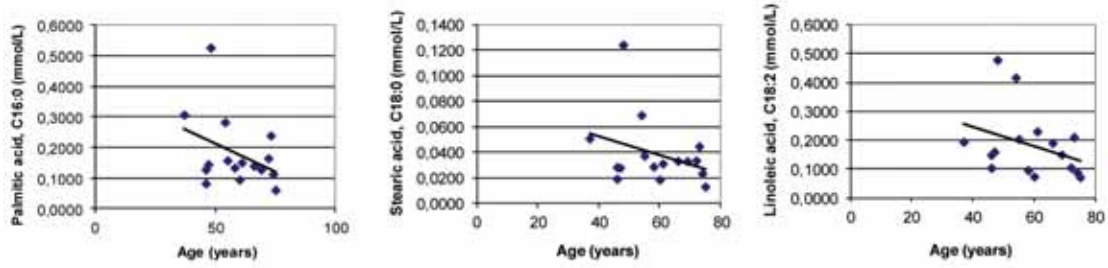
FIGURE 3. Average concentration of palmitic acid (a), stearic acid (b), linoleic acid (c) in plasma of female patients with Type 2 dibetes mellitus and control subjects, no statistically significant.

diagnosed with Type 2 *diabetes mellitus* (average age of 61 years) and 40 adequate controls (average age of 43 years). Strikingly, as shown in Figure 1 the concentrations of examined FFAs were higher in diabetic patients when compared to controls. Plasma levels of individul FFAs and sex differences are presented in Figures 2 and 3. As shown in Figure 2 and 3, levels of palmitic and linoleic acids i.e palmitic and stearic acids were higher in both, male and female diabetic patients. Interestingly, as shown in Figure 2, plasma levels of stearic acid were lower in male T2D patients as compared to nondiabetic subjects, while concentration of linoleic acid was lower in diabetic female patients as compared to control sujects (Figure 3). Our data demonstrated a trend in positive and statis



(a) (b) (c)

FIGURE 4. The correlation between age and plasma levels of palmitic acid (a), stearic acid (b), linoleic acid (c) in the patients with Type 2 diabetes mellitus. Spearman´s rho correlation coefficients ρ =0.040, ρ =0.021, and ρ = -0.135, respectively.



(a) (b) (c)

FIGURE 5. The correlation between age and plasma levels of palmitic acid (a), stearic acid (b), linoleic acid (c) in male patients with Type 2 diabetes mellitus. Spearman´s correlation coefficients ρ = -0.100, ρ = -0.204, and ρ = -0.178, respectively.

tically significant correlation of concentrations of palmitic and stearic acid ($p<0.001$, $p<0.05$; respectively) with the blood glucose levels and glycated hemoglobin in diabetic patients, which was more prominent in male than female patients (Figure 5). As shown in Figures 4 and 5, a significant correlation between plasma FFA levels, age and sex was demonstrated only in diabetic patients.

Discussion

Elevated levels of free fatty acid have been closely associated with insulin resistance, hyperinsulinemia, and diabetes (2-3, 9-10). Diabetic patients who are lipidemic appear to be at increased risk for developing diabetic complications and cardiovascular disease. In Type 2 diabetes, hypertriglyceridemia seems to be associated with the insulin resistance syndrome, impaired glucose tolerance, and an early onset of endothelial dysfunction, while dyslipidemia is associated with nonadequte metabolic control, hyperinsulinemia and a late onset of overt dysfunction of β-cells (4, 11, 13, 17). Multiple disturbances in free fatty acid metabolism, including day-long elevated plasma FFA levels and accelerated rates of lipolysis, are characteristic features of T2D. Elevated plasma FFA concentrations impair glucose metabolism by inhibiting the more proximal steps of insulin action in muscle as well by augmenting basal hepatic gluconeogenesis and impairing the insulin-mediated suppression of hepatic glucose production (8, 16, 19). In addition to increased FFA plasma levels, Type 2 diabetic and obese patients have increased stores of triglycerides in muscle and liver, which correlates closely with IR in these tissues (16, 20-21). Chronic elevated plasma FFAs are also closely linked to the various components of the metabolic syndrome and represent a possible link between fatty acids levels and cardiovascular morbidity and mortality (7-8). A possible link of FFA levels and T2D incidence have been recently reported in different ethnic groups, including Caucasian (22), Chinese

(13), American (23) and Japanese (24) population. In addition to inhibiting insulin action, FFAs also have an important role in the regulation of pancreatic β-cell function (25-28). Acute elevations of FFA produce insulin resistance dose-dependently in diabetic and non-diabetic individuals (1, 5-8). The acute or short-term stimulatory effect of FFAs on glucose-stimulated insulin secretion has been well described both *in vitro* and *in vivo*. Data from recent studies, however, have shown that prolonged (48^h) exposure of rat and human islets to fatty acids decreases glucose-stimulated insulin secretion (9). The effects of prolonged elevation of FFAs plasma levels on insulin secretion in humans remain controversial. Several investigators have reported that 24^h to 48^h elevation of FFAs either increased and decreased insulin secretion (24, 28). Although the increased blood glucose levels could result from the FFA-induced insulin resistance, some studies implied that the amount of secreted insulin was inadequate to maintain normal glucose homeostasis. In line with these results, Belfort et al. recently reported that although a 4-day lipid infusion impaired insulin secretion, did not worsen IR in already insulin-resistant subjects. In addition, the high levels of polyunsaturated fatty acids (PUFAs) have been shown to impair endothelial function and nitric oxide (NO) production (17, 26, 29). There are no data reported related to concentrations of FFAs in diabetic population on Bosnia Herzegovina territory, this is one of the first studies in which concentration of plasma palmitic, stearic, and linoleic acid was evaluated in these patients. Our results demonstrated a profound increase in the concentrations of the above mentioned FFAs in T2D patients as compared to nondiabetic controls. This is in line with data reported in previous studies, in which acute and chronic elevations of FFAs have been associated with the higher risk for developing impaired glucose tolerance and T2D (22, 30). This was complemented with our results, which demonstrated that the FFAs, especially saturated fatty acids (SFAs) and PUFAs levels, were higher in diabetic patients than in controls. IR might represent the link between elevated glucose and FFA levels, however, our group (data not shown) as well as the others did find a significant correlation between these parameters in T2D patients. In our study, correlations between glucose levels and concentrations of FFA (positive correlation with palmitic and stearic acids, negative with linoleic acid) were demonstrated. A positive correlation was found between glycated hemoglobin and concentration of palmitic and stearic acids. Saturated fatty acids, i.e. palmitic and stearic acid, decrease insulin-induced glycogen synthesis, glucose oxidation and lactate production by impairment of mitochondrial function as demonstrated by decrease of both mitochondrial hyperpolarization and ATP generation as reported in numerous studies (23-24). Furthermore, basal glucose oxidation and activation by insulin is also reduced. Interestingly, our data suggest a trend of positive correlation between FFAs and plasma glucose levels with age, which was more profound in male than female diabetic patients. These results complement previous studies in American and French men in which the correlation between plasma FFAs and fasting glucose levels (with aging) was also positive (22, 26, 31), while study in Japanese men did not find a significant correlation between plasma FFAs and glucose levels (24). Based on our results, it appears that in male patients with inadequate glucose control, concentrations of FFA were higher when compared to diabetic patients with adequate glucose control. This is in line with previous reports, where an effect of sex and age on FFA levels in relation to glucose control was also observed in diabetic patients (10). However, the effects of gender on concentrations of FFA in diabetics are still controversial (7-8). Recently, Boden reported that athough elevated FFAs (SFAs and PUFAs) predicted incident Type 2 diabetes in both sexes, their further analysis, stratified by glucose tolerance status, showed that FFAs, especially saturated fatty acids, predicted Type 2 diabetes development in women with impaired fasting glucose, but not in men (8, 32-33). Here we demonstrated an effect of aging on plasma SFAs and PUFAs in diabetic patients, and there was a positive correlation between diabetic patients age, SFA and PUFA levels in plasma, particularly in diabetic men. Plasma FFA levels were significantly higher in older male and female as compared to control subjects, thus, with aging the levels of palmitic, stearic, and linoleic acid increased in plasma of diabetic patients, probably due to impaired glucose tolerance, increased levels of metabolites FFAs (diacylglycerol DAG,

ceramide and fatty acyl CoA) in these patients. Interestingly, our data demonstrated a significant correlation between concentration of palmitic, stearic, and linoleic acid and plasma glucose levels and glycated hemoglobin with aging, as compared to data obtained by other researchers. This is in line with recent report, which has demonstrated that the inverse association between diabetic (or HbA1c level > 7%) and plasma FFA levels was significantly more profound in older men and women. In general, our results demonstrated effects of gender on FFA (SFAs and PUFAs) levels in both, control and diabetic patients. Although there was a trend of increased concentrations of FFA in males as compared to females, this difference was not significant. This is in line with the recent report in which plasma FFA levels were analyzed in larger group of people (about 2400), where increased incidence of elevated FFAs in males as compared to females was reported. Since estrogen promotes FFA excertion, this could explain higher incidence of dyslipidemia in men and increased levels of plasma FFA in postmenopausal women (23, 32-35). This may also explain, at least partially, our findings that plasma concentrations of FFA (SFA, PUFA) in diabetic patients increased significantlly with aging. Furthermore, recent study by Pankow et al. (5) identified SFA (palmitic and stearic acids), and PUFA (linoleic acid) that are associated with FFA plasma concentrations, with profound gender specific effects of specific gene(s) on free fatty acids regulation. Therefore, it appears that there are strong genetic, age, and gender effects on FFA levels in diabetic patients. On the basis of these findings, it would be pertinent to perform more studies on genetic variations and their effects on FFA levels (35-36).

Conclusions

In summary, this study showed significantly elevated plasma levels of free fatty acids, especially saturated fatty acids, in patients with Type 2 diabetes as compared to nondiabetic controls. This is in line with previous studies, which suggest a possible link between FFA levels and diabetes. Interestingly, this phenomenon seemed to be more profound in male diabetic patients, who also demonstarted more prominent effects of an optimal glucose control on FFAs clearance than their female counterparts. Since, to our knowledge, this is one of the first studies in Bosnia and Herzegovina addressing free fatty acids and their role in diabetic progression, more emphasis should be put on their potential use as a risk factor for developing metabolic and cardiovascular diseases in BH clinical practice. Additional genetic studies addressing gender and ethnospecific effects on FFA levels in T2D patients appear also to be justified. Thus, considering the potential link of elevated concentrations of free fatty acid with insulin resistance, impaired glucose tolerance, and progression of diabetes, further research should attempt to determine wheather it is effective to utilize FFA levels as a predictor in prevention of Type 2 diabetes development.

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Competing Interest

Authors received no grant for this study and have no conflict of interest to declare.

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