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Alterations in Plasma Proteome Pattern and Oxidative Stress in Patients with Type 2 Diabetes

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Abstract

Background: *Free radical production, changes in the proteome, and lipid peroxidation are the consequences of hyperglycemia in diabetes. This study aimed to examine the changes in plasma proteome along with oxidative status in type 2 diabetes.*

Methods: *Thirty type 2 diabetic patients and 25 healthy subjects participated in this cross-sectional study. Fasting blood sugar, glycated hemoglobin (HbA1c), and lipid profile was measured in all subjects. To measure oxidative stress parameters, malondialdehyde (MDA) and total antioxidant capacity (TAC) were assayed. Plasma proteome pattern was determined using two-dimensional gel electrophoresis. Visual analysis of gels was performed using software (Image Master).*

Results: *MDA level was higher in the diabetic group compared to the healthy group (4.10±0.57) vs. (3.2±0.10) nmol/ml, (Pvalue<0.01). TAC reduced in diabetic patients (17.85±1.2) vs. (38.60±2.4) mmol/ml, (Pvalue=0.01). Some changes were observed in the 2-D gel electrophoresis pattern in diabetic patients comparing to those of the healthy group.*

Conclusions: *The results indicated the presence of oxidative stress in type 2 diabetes patients. Also, different proteome patterns showed the presence of different or modified proteins in diabetic patients that can be due to changes in the glycation of proteins or may be induced as a response to oxidative stress.*

Keywords: Diabetes, Plasma proteome, Malondialdehyde, Total antioxidant, Two-dimensional electrophoresis.

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Introduction

Diabetes is a chronic disease that gradually affects many different organs of the body. The disease progresses gradually and its symptoms occur several years after the onset of disease.[1]

Diabetes is a treatable disease; however, when it is not under control the risk of serious and irreversible complications such as retinopathy, nephropathy, neuropathy, and vascular damages are expected.[2] Moreover, physical inactivity is another risk factor for cardiovascular disease and insulin resistance. Although chronic hyperglycemia is an important factor in inducing complications of diabetes, the mechanisms that disrupt the organ's function remains unknown.

three main theories explain how hyperglycemia affects chronic complications in diabetes. One hypothesis is that increased intracellular glucose produces advanced glycation end-products (AGEs) through non-enzymatic glycosylation in cellular proteins. Non-enzymatic glycosylation is the result of glucose reaction with amine groups or proteins. The level of AGEs in serum levels is related to blood glucose concentration. On the other hand, the accumulation of these substances reduces the glomerular filtration rate. The second hypothesis in complications of diabetes is that hyperglycemia increases glucose metabolism through the sorbitol pathway.[3] Most intracellular glucose metabolizes by glycolysis, but when the intracellular glucose increases, it is converted to sorbitol by aldose reductase. Increasing the concentration of sorbitol affects cellular physiology, causes cellular dysfunction and plays a role in the development of retinopathy, neuropathy, and nephropathy. Finally, the third hypothesis states that hyperglycemia increases the formation of diacylglycerol, which in turn activates protein kinases, affects a range of cellular events that cause complications of diabetes.[3]

Humans spend the majority of their time in the postprandial state, which is hypothesized to be both pro-inflammatory and pro-oxidative. This may directly affect insulin resistance in skeletal muscle that contributes to the development of type 2 diabetes.[4] Furthermore, research has shown that T2DM is associated with inflammation, oxidative stress, and vascular dysfunction.[5]

The production and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (NRS) causes oxidative stress in the different tissues of the body, especially pancreatic beta cells.[2,6]

Oxidative stress is caused by the imbalance between the production of free radicals and reactive oxygen species on the one hand and the antioxidant defense system on the other. In other words, in aerobic biologic systems, antioxidant defense mechanisms are designed to counteract free radicals and reactive oxygen species to neutralize or minimize the harmful effects of these invasive factors. Some components of this defense system, such as superoxide dismutase, glutathione peroxidase, and catalase enzymes, as well as, bilirubin and the molecules containing the thiol group are made inside the body, but others such as vitamin C, vitamin E, and beta-carotene should be supplied via diet. In oxidative stress, many macromolecules are damaged, and the process of oxidation of

proteins, lipid peroxidation (MDA), inactivation of enzymes, and malfunction of various membranes occur.[7,8]

Assays for total antioxidant capacity (TAC) in plasma differ in their type of oxidation source, target and measurement used to detect the oxidized product.[9] Glycated hemoglobin (HbA1c), is an indicator for overall glucose exposure integrating both fasting and postprandial hyperglycemia, even though their relative contribution is undefined.[10]

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Study the oxidative status along with alterations in plasma proteome pattern in diabetic patients can help know the mechanism of cellular and molecular dysfunction in this disease.

By definition, all proteins expressed in a cell at a specific time are called the proteome. Since the proteome is expected to be different in T2M patients comparing to healthy people, studying these changes along with oxidative markers can be useful in the identification of molecular damages that are involved in diabetes complications.[11]

The plasma proteome represents an important subproteome as it harbors proteins secreted from almost all tissues. This wide dynamic range of protein abundance makes plasma proteome a challenging proteome to analyze. Plasma proteins undergo various types of post-translational modifications such as glycosylation, which add to their complexity. Moreover, plasma protein glycation that occurs in diabetes may change the physicochemical properties of proteins that can affect the electrophoresis pattern of plasma.[12] Hemoglobin (Hb) binds to four oxygen atoms, when Hb undergoes glycation its molecular weight increases; in this case, the oxygen supply is reduced, and instead of entering the cell, it moves out of the cell, and the risk of micro-vascularity increases.[13]

According to the mentioned findings and with increasing glycated proteins, the proteome pattern in diabetic patients is expected to be different from those of healthy subjects. Therefore, this study aimed to determine the pattern of plasma proteome in type 2 diabetic patients and compared it with that of healthy subjects. Furthermore, oxidative stress indices (MDA and TAC) were measured and compared in these two groups; also, their correlation with glycemic control indices (HbA1c) was assayed and discussed.

Materials and Methods

Thirty type 2 diabetic patients participated in this study. The mean (age±SD) was (50±5) years and they had diabetes for 8±2 years. The patients were selected from the referred individuals to Imam Hossein hospital laboratory (Shahrood, east of Iran). The patients were non-alcoholic and non-smoker. They did not have any other illnesses such as thyroid and autoimmune disorders and high blood pressure. They did not receive any other medication except blood glucose-lowering agents. Twenty-five healthy age and sex-matched subjects who were referred to the laboratory for checkups were selected as a control group. They had normal blood glucose and HbA1c. All participants gave informed consent and this study was approved by the ethics committee of the Islamic Azad university, Shahrood branch (Ethics ID: IR.IAU.SHAHROOD.REC.1399.019).

Blood samples for preparing serum and plasma were collected after 10-12 hours of fasting. HbA1c, fasting blood sugar, and lipid profile were measured using an auto-analyzer and commercially available kits (Pars Azmun, Iran).

Malondialdehyde as a marker for lipid peroxidation was measured using Buege and Aust method based on thiobarbituric acid reaction.[12] Total antioxidant capacity was determined using Benezi et.al. method; it was based on the ferric reducing ability of plasma (FRAP).[13]

Because of the high frequency of proteins present in the plasma, including albumin and gama-globulin, which make up about 80% of plasm proteins, their removal was essential. To remove albumin and gama-globulin, the Trichloroacetic acid/Acetone method was used.[14,15] Total protein concentrations were measured using the Bradford protein assay kit.

To study plasma proteome two-dimensional gel electrophoresis was used. Isoelectric focusing (IEF) that separates proteins due to differences in their isoelectric points (PI) was used as a first direction. 150 µg of total protein was loaded onto 17 cm nonlinear pH gradient (IPG) strips (pH 3-10) and was allowed to be actively rehydrated for 12 hr at 50 V using protean IEF (Bio-Rad, USA). Rehydrated IPG strips, then, were focused according to the protocol until 50,000 Vh for 12 hr. For the second dimension, electrophoresis was performed using 12% SDS-poly acrylamide gels. Briefly, the equilibrated IPG strips were placed on top of an SDS-PAGE and sealed with agarose (0.5%, w/v). Gels were run in Tris-glycine running buffer (pH 8.3) using POWER/PAC 3000 (BioRad) at 100 V for 5 hr, and then at 150 V until the bromophenol blue dye reached the bottom of the gel. For colloidal Coomassie blue staining, the gels were rinsed 5 times with distilled water for 10 min; then they were stained with 0.02%,w/v colloidal Coomassie solution containing G-250 (5%, w/v), aluminum sulfate, ethanol (10%, v/v), orthophosphoric acid (2%, v/v) while rocking for 12 hr. High resolution according to protein size or weight (Mw) in the second direction was achieved by using SDS polyacrylamide gel electrophoresis (SDS-PAGE). Plasma proteome patterns in the 2D gels were recorded using an image Scanner III, Lab Scan 6 (Epson, Japan). Spots detection and image matching were performed with image Master Platinum 6.0. software.[16-19]

The results were analyzed using SPSS (version 19). Normal distribution of data was examined and parametric and non-parametric factors were compared between two groups using t-test and Mann-Whitney U-Test, respectively. The correlation between obtained factors was assessed using Pearson test. The significant level was set at 0.05.

Results

Characteristics of demographic and biochemical parameters in diabetic and healthy subjects are shown in table 1. Among the common biochemical factors, glucose, total cholesterol, LDL-C in the diabetic patients showed a significant increase (Pvalue<0.01) compared to healthy subjects; while, HDL-C decreased significantly (Pvalue<0.01) in the diabetic group.

The total antioxidant capacity that was measured by the FRAP assay showed a significant reduction in diabetic patients (Pvalue<0.01). MDA level as an indicator of lipid peroxidation was higher in the diabetic group (Pvalue<0.01) compared to the healthy group (Table 1).

Correlation between glucose and HbA1c with other factors was evaluated using the Pearson test and the results are shown in table 2. An increase in HbA1c in diabetic patients was directly related to an increase in MDA (r=0.90, Pvalue<0.01); moreover, there was a reverse relationship between HbA1c and TAC (r=-0.88, Pvalue<0.01). Moreover, there was a correlation between FBS and triglycerides and cholesterol (Pvalue=0.04) in diabetic patients.

After the plasma specimen was separated using 2-D gel electrophoresis, visual analysis of gels was performed using software (Image Master) and the obtained pattern was compared between healthy and diabetic subjects. The pattern of 2-D gel electrophoresis of plasma proteome in the diabetic group was different from that of healthy subjects (Figure 1). Figure 2 also shows the result of image analysis of serum proteins in healthy and diabetic groups. After examining the spots that indicated different proteins, it was found that the diabetic patient's pattern was different from that of the healthy subject in at least several spots. Comparing these images with a standard pattern of plasm proteins we identified some of these spots that are presented in table 3. Moreover, the results of the analysis of two spots (1529, 1550) are shown in Figure 3, indicating the difference in density of these proteins between diabetic and non-diabetic subjects. The first spot (1529) was identified as ApoA1 that showed an increase in diabetic subjects; the second one (1550) was Hp α2 chain that showed lower density in the diabetic subject.

Table 1. Biochemical parameters of the studied groups

Factor	Healthy (n=25)	Diabetic (n=30)	Pvalue
Age (year)	49.0±7.0	50.11±3.45	0.92
BMI (Kg/m²)	24.90±0.73	26.31±1.33	0.001
HbA1c (%)	4.78±0.58	10.37±1.94	0.001
Glc (mg/dl)	111.68±11.18	237.68±65.75	0.001
Cho (mg/dl)	191.96±40.49	186.07±40.05	0.001
TG (mg/dl)	147.0±96.0	186.50±133.0	0.18
LDL-C (mg/dl)	106.84±22.13	101.57±25.39	0.001
HDL-C (mg/dl)	57.00±10.89	48.50±8.73	0.001
MDA (nmol/ml)	3.23±0.09	4.13±0.34	0.001
TAC (mmol/ml)	38.96±1.32	17.83±0.68	0.001

Data are presented as mean±SD. Pvalues are from Student's T-Test or Mann–Whitney U-test as appropriated

Table 2. Pearson correlation between concentrations of different variables in diabetic patients and healthy subjects.

Factors	Glucose		HbA1c (%)	
	Correlation coefficient (Pvalue)		Correlation coefficient (Pvalue)	
	Diabetic	Healthy	Diabetic	Healthy
Cho (mg/dl)	0.37(0.04)*	0.18(0.37)	0.19(0.32)	-0.06(0.78)
TG (mg/dl)	0.36(0.04)*	-0.16(0.43)	0.21(0.26)	-0.24(0.25)
LDL-C (mg/dl)	0.32(0.08)	0.03(0.89)	0.07(0.70)	-0.16(0.43)
HDL-C (mg/dl)	-0.05(0.76)	0.05(0.81)	0.06(0.75)	-0.28(0.16)
MDA (nmol/ml)	0.05(0.002)	0.4(0.03)	0.89(0.001)*	0.90(0.001)*
TAC (mmol/ml)	-0.45(0.01)	-0.5(0.009)	-0.88(0.001)*	-0.87(0.001)*

* Statistically significant correlation

a) Non-diabetic

b) Diabetic

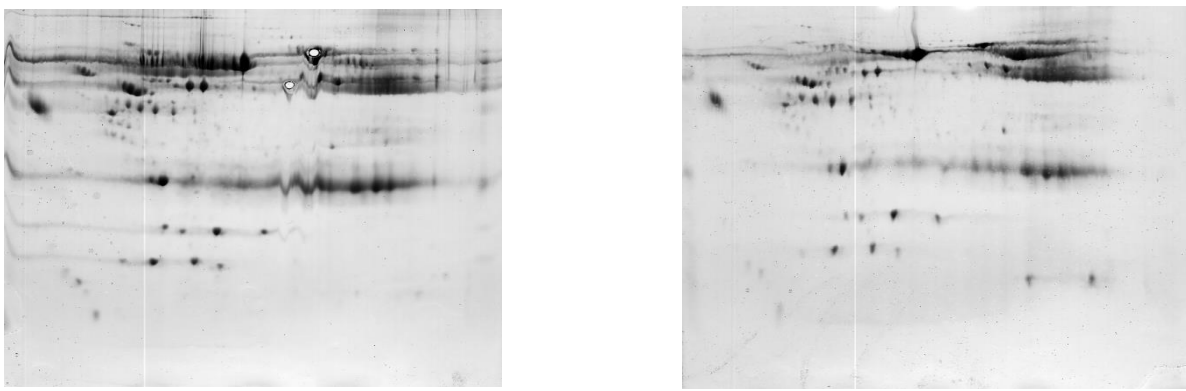
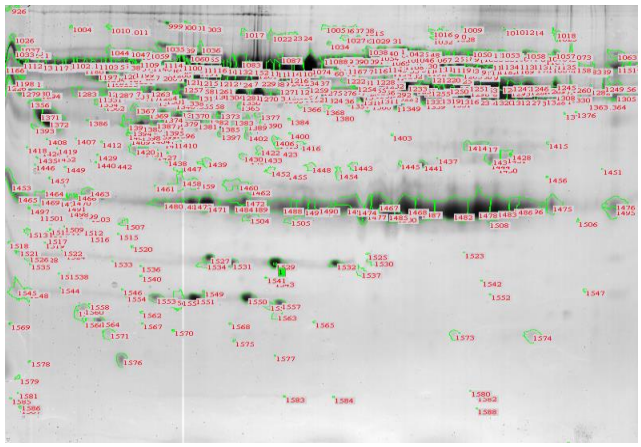


Figure 1. Pattern of 2D-gel electrophoresis of plasma proteome in a healthy subject (a) and a diabetic patient (b)


Healthy

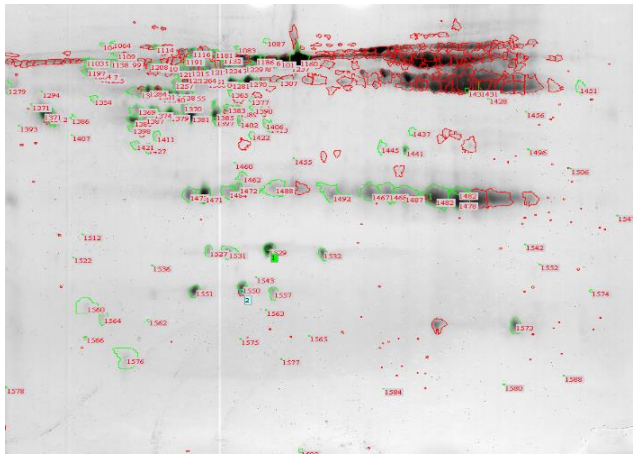
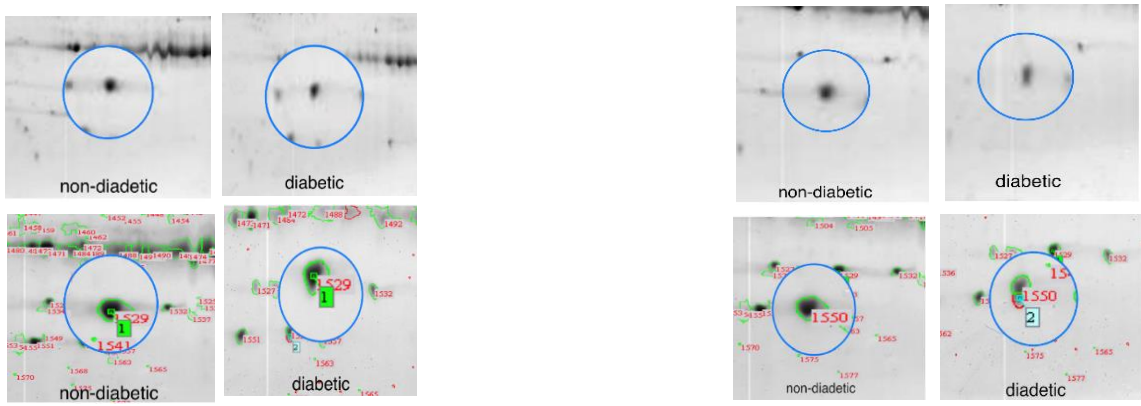
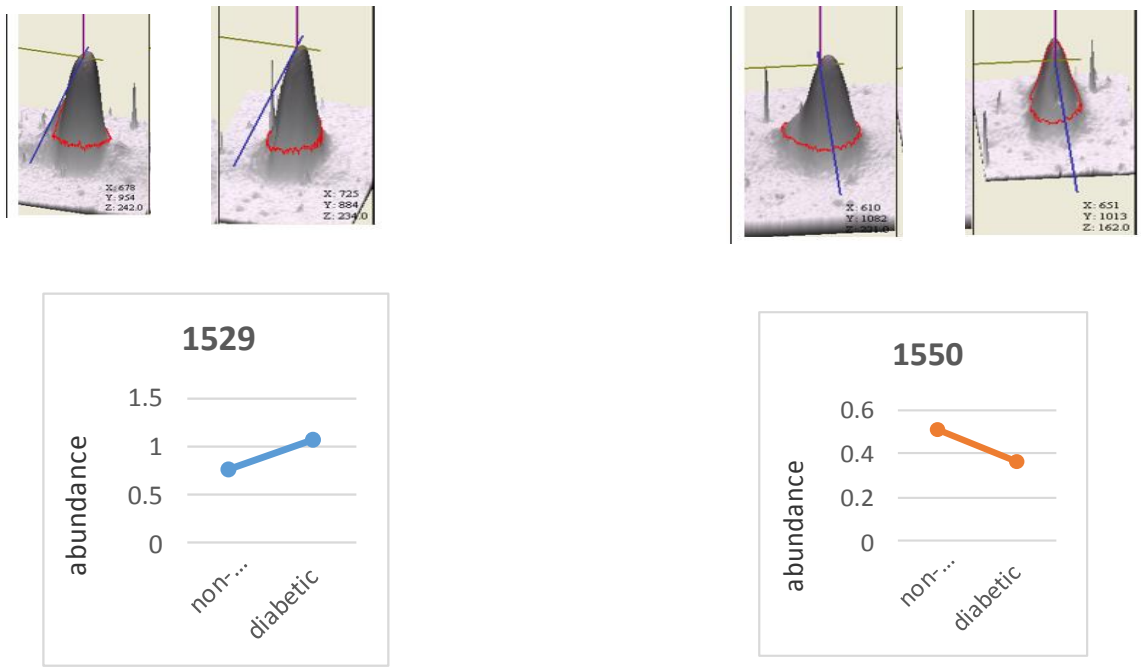

Diabetic

Figure 2. A reprehensive image showing plasma protein pattern of a healthy subjects and a diabetic patient. Spot analysis and density assay was performed using IMage master

Table 3. Optical density of some spots on plasma proteome pattern that were different between diabetic and healthy subjects.

Protein spots	Protein	Healthy	Diabetic	Change
1529	Apo A1	0.770	1.071	increase
1550	Hp α2 chain	0.510	0.362	decrease
1573	Retinol binding protein	0.030	0.520	increase
1294	Unidentified	0.180	0.009	decrease
1469	Ig G light chain	0.871	0.007	decrease
1574	Transthyretin	0.079	0.004	decrease





A(1529) **B(1550)**

Figure 3. The Image view and 3D view showing different relative density of two spots in a diabetic and non-diabetic subject. A 1529 and B 1550; the first one showed higher and the second one lower density in diabetic patient comparing with a healthy subject

Discussion

Proteomic technology has a reliable method for research on diabetes disease-related markers, which could be used in early diagnosis and course confirmation. These plasma molecular markers, mainly low-abundance proteins, are closely related to the disease and promising in diagnosis, accounting for less than 1% of plasma total protein.[20] It is difficult to separate and characterize these proteins; since they are usually covered or interfered with by albumin and immunoglobulin which account for 70% of serum total proteins. Therefore, it is very important to select highly sensitive and reliable proteomic technology when doing plasma proteomic analysis. In this research, we used 2D electrophoresis. The main purpose of this study was to investigate the Plasma protein pattern of type 2 diabetic patients and compare it with those of healthy persons. To study the human proteome, it is possible to examine and analyze the proteins from different tissues such as the liver, kidneys, and various cancer tumors, such as breast and intestinal tumors. However, access to each of these tissues requires surgery, which is costly and dangerous and it can cause many problems for the patients.[21] Therefore, the Plasma sample, which originates from all organs and tissues and is taken through blood vessels, is very easy and inexpensive and does not pose a risk to the patient. It is also repeatable when necessary, which is extremely important.[21] Commercial diagnostic methods used to diagnose diseases are mostly based on ELISA systems (enzyme-dependent fuel safety measurements) that control the presence of specific protein markers in body fluids. In most cases, the invention of these methods has been linked to the accidental discovery of proteins. Protein expression changes significantly during disease, rather than relying on the simultaneous presence of proteins, by comparing protein expression patterns in the Plasma sample of patients and healthy, two-dimensional electrophoresis is used, which can be used to diagnose, prevent, and treat diabetes.[21]

Jantos-Siwy J et al. investigated urinary biomarkers for diabetes, diabetic nephropathy, and non-diabetic renal proteinuria.[22] As a result, this study suggests that the analysis of urinary proteomes may contribute to the early diagnosis of diabetic nephropathy and may provide predictive information.[22] As such, a previous study by Lopez et.al and other researchers indicate that almost no markers alone are enough to predict most diseases, and studies emphasize the need for more than one marker. Simultaneous analysis of several biomarkers, using Plasma or serum samples, can be performed by examining the proteome pattern. That's why this type of analysis has attracted a lot of attention.[11] When oxidative stress increases, certain proteins are produced in the body that has a detrimental effect on the pancreas and other tissues. Therefore, the pattern of Plasma proteome is quite different in diabetic and non-diabetic patients. Pier-H Hung and colleagues who used quantitative proteomic analysis identified new nephropathy markers in Plasma applied in type 2 diabetic patients.[23] Proteomic analysis in human disease usually adopts a comparative method that is defined by the differentiation of proteins in different stages of the disease. They found that subtypes of AMPK-Beta, Carnitine O-palmitoyltransferase I, and 6-phosphofructokinase decreased in type 2 diabetes plasma, indicating that high current regulators could stop AMPK expression thus fatty acid transport is inhibited by mitochondria for beta-oxidation and

carnitine O-palmitoyltransferase I; also the regulation of glycolysis is reduced by inhibiting 6-phosphofructokinase in this disease.[23]

Tea Sundesten et al. use SDS-PAGE and mass spectrometry and found different protein expressions in type 2 diabetic patients compared with the healthy group. Significant differences were observed in transferrin, albumin, and retinol-binding protein between type 2 diabetic patients and healthy individuals.[24] In another study, the Plasma protein pattern was compared to premature insulin response between two groups. They observed lower levels of trans-thyretin in diabetic patients; also 9 proteins containing trans-thyretin and alpha and beta-hemoglobin chains were differed.[24] Increased oxidative stress was seen in people with high levels of MDA and low TAC that can moderate glycosylated protein production.[24] Quantitative plasma proteomics analysis provides a valuable impact on type 2 diabetes research. The quantitative proteomic method has shown earlier reported Plasma markers of type 2 diabetes such as apolipoprotein A-I and phycolin-3.[25] Also, Hong P et al. suggested several indicators indicating that type 2 diabetes may be associated with disease progression and they have the potential to serve as a useful tool for monitoring disease progression.[23] These markers require further research to screen and treat type 2 diabetes.[23] Finding a different pattern in diabetes can be helpful even without knowing the identity of the different proteins in the pattern. Even in some studies, the mass to charge ratio of the identified spots has been only reported. Identifying different points in the two-dimensional model even provides information that can predict whether diabetes is related to genetic or environmental factors.

Diabetes mellitus is one of the most common endocrine diseases and one of the most widespread health problems in the world today.[25] Appropriate strategies have been developed to prevent, treat, control, and reduce the disorders associated with this disease.[26] Increased glucose releases radicals into body tissues, especially pancreatic cells. As a result of the imbalance between the production of free radicals and active oxygen species on the one hand, and the antioxidant defense system on the other, oxidative stress is produced.[27] One of the aims of this research was to study the pattern of plasma proteome in patients with type 2 diabetes and their relationship with oxidative stress indicators.

The results showed a significantly higher level of HbA1c (Pvalue<0.01) in diabetic patients compared with healthy subjects. This was consistent with a study by Inoue et al, that predicted a combined use of FPG and HbA1c levels in people with diabetes or at-risk individuals.[28,29] The results of this study indicated an increase in malondialdehyde due to increased lipid peroxidation in diabetic patients. These results are consistent with the findings of other studies. Ramakrishna V et al. reported that increased lipid peroxidation and protein oxidation in type 2 diabetic patients reduced the levels of enzymatic and non-enzymatic antioxidants.[30] However, diabetic patients are prone to oxidative stress. There is a link between high blood sugar and lipid peroxidation through free radicals production.[31] The positive correlation between FBG and malondialdehyde has been reported in type 1 diabetic patients.[32] According to previous studies, the FRAP method has been used to determine the potency of antioxidants in Plasma, and in some of these studies, Plasma antioxidant levels have been reduced in diabetic patients. In this study, an increase in lipid peroxidation, and a decrease in Plasma TAC in diabetic patients were observed. About malondialdehyde and total antioxidant capacity, the findings were similar to those of Hisalkar, P. et al.[33] They showed a decrease in reduced antioxidant capacity (TAC), which was more severe in patients with type 2 diabetes than in the control group. There was a significant relationship between antioxidant capacity and control of blood sugar.[33]

In this study, serum levels of oxidative stress indicators in diabetic patients and non-diabetic were compared. The level of MDA, which was an oxidant, increased, and the level of TAC, which was an antioxidant, decreased (Pvalue<0.01), affecting the Plasma proteome pattern.

Chronic hyperglycemia in diabetes due to the production and accumulation of reactive oxygen species (ROS) and nitrogen (RNS) radicals causes oxidative stress in body tissues, especially pancreatic beta cells.[34] The formation of these radicals destroys vital large molecules of cells, peroxidation of membrane lipids, and finally leads to cell damage.[35]

Elevated blood glucose affects oxidative stress indices. When antioxidant activity reduces in the body, oxidative stress increases, which in turn inflammatory reactions increase that can affect protein patterns. When intracellular glucose increased, advanced glycosylation end-products (AGEs) are produced, through non-enzymatic glycosylation of cellular proteins that affect the structure, function, and molecular weight of the protein, all of which alter the plasma proteome pattern. In this study, a different proteomic pattern was observed in the two groups of diabetic and non-diabetic. Two-dimensional gel electrophoresis was analyzed using image Master software; different patterns were obtained due to various proteins with different concentrations. Accurate recognition of these proteins required more advanced assays.

The results of our study showed that lipid peroxidation increased in diabetes while total oxidant capacity decreased significantly. Some changes in Plasma proteome can be the result of the glycation process that occurs on proteins in diabetes. The human Plasma proteome promises to revolutionize disease diagnosis and surveillance. Some changes in Plasma protein that were found in this study can be considered as possible or putative biomarkers in type 2 diabetes. The exact identification of these proteins needs a MALTI-TOF analysis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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