

Comparison of plasma and salivary meteorin-like protein levels in patients with newly diagnosed Type-2 diabetes and treated with metformin

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Abstract. – OBJECTIVE: The present study compares the plasma and salivary Metrnl levels of patients newly diagnosed with type-2 diabetes who were treated with metformin for three months with those of a healthy volunteer group and immunohistochemically analyzes Metrnl in salivary glands.

PATIENTS AND METHODS: 30 healthy volunteers and 30 newly diagnosed type-2 diabetes patients were included in the study. The newly diagnosed diabetes patients were treated with metformin for three months, and the plasma and salivary metformin levels of both groups were measured at baseline and after the three months of metformin treatment in the patient group. Plasma HbA1c, low-density lipoprotein (LDL-C) and Triglyceride (TG) values of all groups were also measured at baseline following three months of metformin treatment. Biopsies were taken from the parotid and submandibular glands and immunohistochemical staining was performed to show Metrnl immunoreactivity.

RESULTS: Plasma Metrnl, HbA1c, LDL-C and TG levels were higher in the newly diagnosed diabetes group than in the other group, and salivary Metrnl levels were higher than in the control group after three months of metformin treatment. An examination of the immunohistochemical staining of salivary gland biopsies under light microscope revealed Metrnl immunoreactivity in the intralobular and interlobular ducts of the parotid gland, while Metrnl immunoreactivity was observed in the acinar cells in the intralobular striated duct and interlobular ducts in the submandibular gland.

CONCLUSIONS: Plasma Metrnl, HbA1c, LDL-C and TG levels were higher in the newly diagnosed diabetes group than in the other group. Metrnl immunoreactivity was detected in

the parotid and submandibular glands. The relationship between Metrnl and DM should be investigated in larger groups.

Key Words:

Metrnl, Diabetes mellitus, Saliva Metrnl, Immunohistochemistry, Metformin.

Introduction

Type-2 diabetes mellitus (DM) is a leading cause of morbidity and mortality around the world and can lead to many microvascular and macrovascular complications, such as diabetic retinopathy, neuropathy, nephropathy and cardiovascular disease¹.

For patients diagnosed with type-2 DM, the primary recommended treatment includes diet, lifestyle changes and physical exercise. Metformin is one of the most commonly used oral antidiabetic agents for the treatment of type-2 DM due to its insulin-sensitizing effect².

In recent years, a new adipomyokine called meteorin-like protein (Metrnl), the release of which is stimulated by exposure to cold in adipose tissue and by exercise in muscle tissue, has been discovered³⁻⁵. It has been reported³ that Metrnl accelerates browning in white adipose tissue, promotes glucose uptake in skeletal muscle and the heart, positively affects hepatic glucose and lipid metabolism, and improves the function of beta cells in the pancreas. Metrnl accelerates the browning of white adipose tissue by macrophages

in adipose tissue *via* STAT-6, increases the body's energy consumption and positively contributes to glucose tolerance³. Furthermore, overexpression of *Metnrl* has been shown to reduce insulin resistance by activating the PPAR- γ -mediated signaling pathway, which plays an important role in adipocyte differentiation in transgenic mice. These findings raise the possibility of *Metnrl* playing a role in the pathophysiology of type-2 DM and other metabolic diseases^{6,7}.

To assess the potential role of *Metnrl* in the development of type-2 DM, the present study compares plasma and salivary *Metnrl* levels in newly diagnosed type-2 DM patients at diagnosis and after three months of treatment with metformin.

Patients and Methods

Study Design and Setting

30 healthy volunteers and 30 newly identified cases of Type-2 DM were included in the study. Diabetes was diagnosed based on the identification/diagnostic criteria of the American Diabetes Association (ADA), as follows: fasting blood glucose ≥ 126 mg/dL, 75 g of oral glucose tolerance test (OGTT), second-hour blood glucose ≥ 200 mg/dL, hemoglobin A1c (HbA1c) $\geq 6.5\%$ or randomly observed individuals with symptoms of hyperglycemia, whose blood sugar was ≥ 200 mg/dL⁸. Metformin treatment was started in the newly diagnosed type-2 DM patients at a dose of 2,000 mg/day and continued for three months. The exclusion criteria were as follows: history of diabetes, hypertension, any known cardiovascular disease, malignancy, use of immunosuppressive drugs, steroids, or acute/chronic liver disease, acute/chronic kidney failure, infection, and pregnant or mentally retarded patients with socio-cultural communication problems.

Firat University Rectorate Non-Interventional Research Ethics Committee granted approval to the study (date and approval No.: 26.03.2021-29975), and a voluntary consent form was obtained from each participant.

Metnrl Kit

The *Metnrl* levels in the serum and saliva sample specimens, [Human (*Metnrl*) catalogue number: E39441Hu, Bioassay Technology Laboratory Co. Ltd., Shanghai, China) were assessed using the ELISA method, in accordance with the manufacturer's instructions. The measurement range of the Human *Metnrl* ELISA kit was 0.05-15 ng/mL, the

intra-assay CV value was $< 18\%$, the inter-assay CV value was $< 10\%$ and sensitivity was 0.023 ng/mL. An automatic washer, Bio-Tek ELX50 (BioTek, Winooski, VT, USA), was used for the plate washings, and a ChroMate Microplate Reader P4300 device (Awareness Technology Instruments, FL, USA) was used for absorbance readings. The test results were given in ng/mL. The *Metnrl* kit was shown to measure asprosin in saliva sample specimens⁹, and we also measured the saliva sample specimens using this method.

Immunohistochemistry

Sections taken from paraffin blocks with a thickness of 5-6 mm were applied to polylysine slides. The deparaffinized tissues were passed through graded alcohol series and boiled in a citrate buffer solution at pH=6 in a microwave oven (750 W) for 7+5 minutes for antigen retrieval. After boiling, the tissues were kept at room temperature for about 20 minutes to cool and then washed with PBS (Phosphate Buffered Saline, P4417, Sigma-Aldrich, USA) for 3x5 minutes and incubated with a hydrogen peroxide block solution for 5 minutes to prevent endogenous peroxidase activity (Hydrogen Peroxide Block, TA-125-HP, Lab Vision Corporation, USA). After applying an Ultra V Block (TA-125-UB, Lab Vision Corporation, USA) solution for 5 minutes to prevent the background staining of the tissues washed for 3x5 minutes with PBS, the *Metnrl* primary antibody (*Metnrl* Polyclonal Antibody, PAH662Ra01, Cloud-Clone Corp., Houston, USA) was diluted 1/200 in a humid environment for 60 minutes at room temperature. After the primary antibody application, the tissues were washed with PBS for 3x5 minutes and incubated with a secondary antibody (anti-mouse/rabbit IgG, biotinylated Goat Anti-Polyvalent, TP-125-BN, Lab Vision Corporation, USA) for 30 minutes at room temperature in a humid environment. The tissues were washed with PBS for 3x5 minutes after the secondary antibody application and incubated with Streptavidin Peroxidase (TS-125-HR, Lab Vision Corporation, USA) for 30 minutes in a humid environment at room temperature and then taken into PBS. After 3-amino-9-ethylcarbazole (AEC) Substrate + AEC Chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) solution was dripped onto the tissues and the image signal was obtained under the light microscope, the samples were washed with PBS. The tissues, counterstained

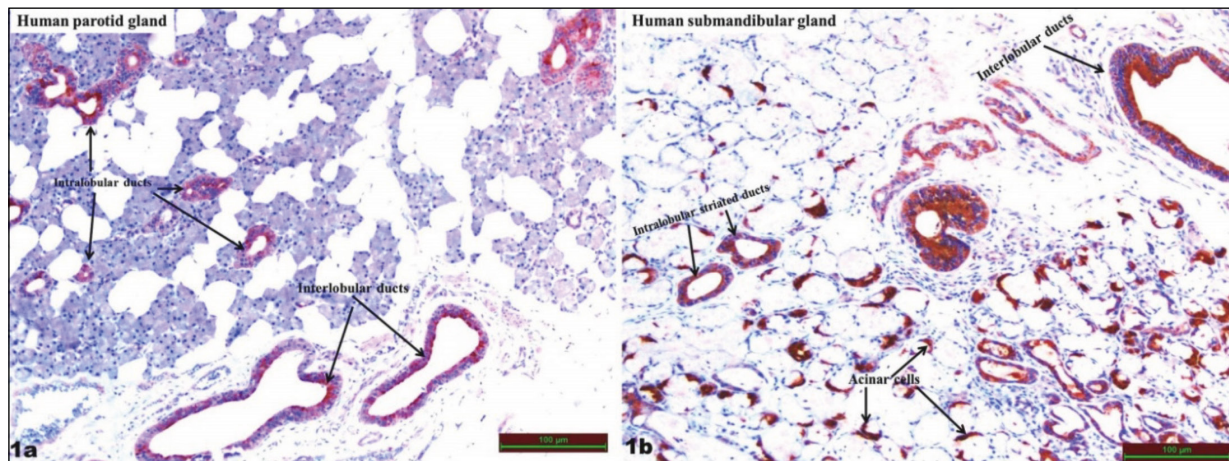


Figure 1. MetrnI immunoreactivity. Intralobular and interlobular ducts in the submandibular (a) and parotid (b) glands (x 100 µm).

with Mayer’s hematoxylin, were then passed through PBS and distilled water and closed with the appropriate closure solution (Large Volume Vision Mount, TA-125-UG, Lab Vision Corporation, USA). The preparations were examined and photographed using a Leica DM500 microscope (Leica DFC295, Danaher, Wetzlar, Germany).

Statistical Analysis

The data were analyzed using IBM SPSS Statistics (Version 22.0., IBM Corp., Armonk, NY, USA). A Kolmogorov-Smirnov test was used to analyze the normality of the data. In line with the characteristics of the variables, percentage, mean, standard deviation, Wilcoxon, Mann-Whitney U, Spearman correlation and Receiver operating characteristic (ROC) tests were performed. A Kruskal-Wallis H test was used for the comparison of more than two independent groups, and a Dunn-Bonferroni post-hoc test was used for binary comparisons to determine the source of the difference. Statistical significance was accepted as $p < 0.05$.

Results

Of the 60 participants, 52.2% (47) were male and the mean age of the patients was 45.33 ± 10.33 years. The Plasma MetrnI and saliva MetrnI levels are presented in Table I.

An investigation of immunohistochemical staining for MetrnI immunoreactivity under light microscopy revealed MetrnI immunoreactivity in the intralobular and interlobular ducts in the parotid gland (Figure 1a), while MetrnI immunoreactivity was observed in the acinar cells in the

intralobular striated duct and interlobular ducts in the submandibular gland (Figure 1b).

There was no significant difference between the age of control and DM groups. A comparison of the control, newly diagnosed type-2 DM, and after 3-months of treatment groups revealed saliva MetrnI laboratory levels to be lower in the control group than in the 3-months treatment group ($p = 0.041$). The newly diagnosed DM group’s plasma MetrnI, HbA1c and LDL-C levels were higher than those of the other groups (Table II).

There was no correlation between saliva MetrnI and plasma MetrnI (Spearman Correlation test, $p = 0.571$). The ROC analysis performed to determine the diagnostic efficacy of saliva MetrnI and plasma MetrnI in DM revealed a Plasma MetrnI value above 2.278 to be diagnostic for T2DM with 55.0% sensitivity and 56.7% specificity [$p = 0.013$, Area Under Curve (AUC) = 0.662], while a Saliva MetrnI value above 2.841 was diagnostic for T2DM with 63.3% sensitivity and 63.8% specificity ($p = 0.027$, AUC = 0.645).

Discussion

Type-2 DM affects many organs and is a chronic disease that occurs due to a decrease in the amount of insulin secreted from the pancreas

Table I. Saliva MetrnI and plasma MetrnI levels of the participants.

	Mean	SD
Saliva MetrnI	2.70	0.914
Plasma MetrnI	3.13	2.847

Table II. Age, and laboratory data of the groups included in the study.

	Groups						p
	Control		Newly DM		3-months treatment		
	Mean	SD	Mean	SD	Mean	SD	
Age	42.10	8.8	54.63	10.8	-	-	0.317
Saliva Metrnl	2.39 ^a	1.044	2.69 ^{a-b}	0.958	3.03 ^b	0.564	0.041
Plasma Metrnl	2.19 ^a	0.852	4.41 ^b	3.252	2.80 ^a	2.114	0.044
Hb A1c	5.88 ^a	0.789	7.64 ^b	1.602	6.26 ^a	1.235	<0.001
LDL-C	113.6 ^a	28.583	153.43 ^b	54.243	123.34 ^a	32.834	0.017
TG (mg/dL)	212.45	79.954	259.56	112.985	209.76	8.567	0.141
AST (U/L)	21.56	10.456	23.13	12.984	22.36	10.651	0.686

Htc: Hematocrit, Hb: Hemoglobin, LDL-C: Low-density lipoprotein cholesterol, AST: Aspartate aminotransferase TG: Triglycerides.

and/or resistance to the effect of insulin¹⁰. Metrnl, known also as subfatin, is a newly discovered adipomyokine that is primarily secreted by white adipose tissue and skeletal muscle⁶. Rao et al³ reported that augmented circulating Metrnl stimulated thermogenesis in brown adipose tissue in mice, increased the body's energy expenditure and positively affected glucose tolerance. In line with these findings, the use of plasma Metrnl levels as a marker in the early diagnosis and treatment of type-2 DM can be considered worthy of study.

None of the previous studies investigating the relationship between Metrnl and type-2 DM have focused on the relationship between salivary Metrnl levels and type-2 DM. To the best of our knowledge, this is the first study to investigate the changes in plasma Metrnl and salivary Metrnl levels after 3 months of metformin treatment in newly diagnosed type-2 DM patients. It is also the first study in literature to demonstrate Metrnl immunoreactivity in the salivary gland immunohistochemically, setting it apart from other studies.

As Metrnl is a recently discovered adipomyokine, there are only limited studies investigating the relationship between type-2 DM and plasma Metrnl levels, and their results are conflicting. Lee et al¹¹ and Zheng et al¹² both reported low plasma Metrnl levels in type-2 DM patients in their studies, while Wang et al¹³, Chung et al¹⁴ and Chuan et al¹⁵ all reported high serum Metrnl levels in type-2 DM patients. In the present study, plasma Metrnl levels were found to be higher in the diabetic group than in the control group. Furthermore, saliva Metrnl levels were found to increase in the diabetic group after 3 months of treatment when compared to the control group. There was no correlation between salivary Metrnl and plasma Metrnl levels. A ROC analysis was performed

to determine the diagnostic efficiency of salivary and plasma Metrnl levels in the diabetic group, revealing a plasma Metrnl value of 2.278 and a salivary Metrnl value of over 2.841 to be diagnostic for diabetes, although this finding should be investigated in larger groups.

The reason for the association between high plasma Metrnl levels and diabetes has yet to be fully explained. Löffler et al¹⁶ reported that an overexpression of Metrnl in human adipocytes inhibited the differentiation of adipocytes and decreased the expression of PPAR- γ , thus contributing to insulin resistance and hyperinsulinemia. This suggests that Metrnl may play a role in the etiology of diabetes.

For the treatment of type-2 DM, lifestyle changes and diet are primarily recommended in the guidelines of diabetes societies such as the American Diabetes Association (ADA). As the initial drug therapy, metformin is recommended if there are no contraindications⁸. Accordingly, in the present study the newly diagnosed diabetes group were treated with metformin for 3 months. To avoid heterogeneity, a single commercial form of the drug was used at a dose of 2x1,000 mg in the study. It was determined that plasma Metrnl levels in the type-2 diabetes group decreased after metformin treatment and approached the plasma Metrnl levels in the control group. Wang et al¹³ reported a positive correlation between a high serum Metrnl level and insulin resistance. In the present study, the decrease in plasma Metrnl levels recorded after metformin treatment can be explained by the fact that insulin resistance started to improve after the administration of metformin and led to a decrease in the serum Metrnl level. It was a stimulating and unanticipated finding that salivary Metrnl levels increased slightly after

metformin treatment when compared to the baseline value, though larger groups should be studied to confirm these findings.

The expression of Metrnl by adipose tissue and skeletal muscle is reported for the first time in the present study. Li et al¹⁷, Zheng et al¹⁸ and Miao et al¹⁹ all reported Metrnl expression to be present in such tissue as the liver, heart, spleen, macrophages, stromal cells and the central nervous system. In the present study, Metrnl immunoreactivity was demonstrated in the salivary gland, in the intralobular and interlobular ducts of the parotid gland, and in acinar cells in the intralobular striated duct and interlobular ducts of the submandibular gland. Metrnl immunoreactivity was detected in the salivary gland for the first time and so encourages future studies of Metrnl expression in different tissues.

Limitations

This study has some limitations. Initially, each group in the study consisted of 30 people. Second, only newly diagnosed diabetes patients were included in the study, and type-2 DM patients with different prognoses (such as with and without complications) were excluded. Third, the study was conducted with patients of a single ethnic origin.

Conclusions

Plasma Metrnl levels were found to be higher in newly diagnosed type-2 DM patients when compared to other groups. Furthermore, the diagnostic predictive values of plasma and saliva Metrnl levels were determined. This is the first time in literature that the immunoreactivity of Metrnl in the parotid and submandibular glands has been demonstrated immunohistochemically. The presented findings may serve as the basis for further studies.

Conflicts of Interest

The authors declare no conflicts of interest related to this study.

Ethics Approval

Firat University Rectorate Non-Interventional Research Ethics Committee granted approval to the study (date and approval No.: 26.03.2021-29975).

Informed Consent

A voluntary consent form was obtained from each participant.

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References

- 1) Schlienger JL. Type 2 diabetes complications. *Presse Med* 2013; 42: 839-848.
- 2) Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, Peters A L, Tsapas A, Wender R, Matthews DR. Management of hyperglycemia in type 2 diabetes. 2015: a patient-centered approach. Update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetologia* 2015; 58: 429-442.
- 3) Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, Jedrychowski MP, Ruas JL, Wrann CD, Lo JC, Camera DM, Lachey J, Gygi S, Seehra J, Hawley JA, Spiegelman BM. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell* 2014; 157: 1279-1291.
- 4) Eaton M, Granata C, Barry J, Safdar A, Bishop D, Little JP. Impact of a single bout of high-intensity interval exercise and short-term interval training on interleukin-6, FNDC5, and Metrnl mRNA expression in human skeletal muscle. *J Sport Health Sci* 2018; 7: 191-196.
- 5) Saghebjo M, Einaloo A, Mogharnasi M, Ahmadabadi F. The response of meteorin-like hormone and interleukin-4 in overweight women during exercise in temperate. warm and cold water. *Horm Mol Biol Clin Investig* 2018; 36: 20180027.
- 6) Zheng SL, Li ZY, Song J, Liu JM, Miao CY. Metrnl: a secreted protein with new emerging functions. *Acta Pharmacol Sin* 2016; 37: 571-579.
- 7) Li ZY, Song J, Zheng SL, Fan MB, Guan YF, Qu Y, Xu J, Wang P, Miao CY. Adipocyte Metrnl antagonizes insulin resistance through PPAR gamma signaling. *Diabetes* 2015; 64: 4011-4022.
- 8) American Diabetes Association. Pharmacologic approaches to glycemic treatment: standards of medical care in diabetes-2018. *Diabetes Care* 2018; 41: 73-85.
- 9) Aydin S. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides* 2015; 72: 4-15.

- 10) Gastaldelli A. Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Diabetes Res Clin Pract* 2011; 93: 60-65.
- 11) Lee JH, Kang YE, Kim JM, Choung S, Joung KH, Kim HJ, Ku BJ. Serum Meteorin-like protein levels decreased in patients newly diagnosed with type 2 diabetes. *Diabetes Res Clin Pract* 2018; 135: 7-10.
- 12) Zheng SL, Li ZY, Zhang Z, Wang DS, Xu J, Miao CY. Evaluation of two commercial enzyme-linked immunosorbent assay kits for the detection of human circulating Metrnl. *Chem Pharm Bull (Tokyo)* 2018; 66: 391-398.
- 13) Wang K, Li F, Wang C, Deng Y, Cao Z, Cui Y, Xu K, Ln P, Sun Y. Serum levels of meteorin-like (Metrnl) are increased in patients with newly diagnosed type 2 diabetes Mellitus and are associated with insulin resistance. *Med Sci Monit* 2019; 25: 2337-2343.
- 14) Chung HS, Hwang SY, Choi JH, Lee HJ, Kim NH, Yoo HJ, Seo JA, Kim SG, Kim NH, Baik SH, Choi KM. Implications of circulating Meteorin-like (Metrnl) level in human subjects with type 2 diabetes. *Diabetes Res Clin Pract* 2018; 136: 100-107.
- 15) Wang C, Pan Y, Song J, Sun Y, Li H, Chen L, Hou X. Serum Metrnl Level is Correlated with Insulin Resistance, But Not with β -Cell Function in Type 2 Diabetics. *Med Sci Monit* 2019; 25: 8968-8974.
- 16) Löffler D, Landgraf K, Rockstroh D, Schwartz JT, Dunzendorfer H, Kiess W, Körner A. Metrnl decreases during adipogenesis and inhibits adipocyte differentiation leading to adipocyte hypertrophy in humans. *Int J Obes (London)* 2017; 41: 112-119.
- 17) Li ZY, Zheng SL, Wang P, Xu TY, Guan YF, Zhang YJ, Miao CY. Subfatin is a novel adipokine and unlike meteorin in adipose and brain expression *CNS Neurosci Ther* 2014; 20: 344-354.
- 18) Zheng SL, Li ZY, Song J, Liu JM, Miao CY. Metrnl: a secreted protein with new emerging functions. *Acta Pharmacol Sin* 2016; 37: 571-579.
- 19) Miao ZW, Hu WJ, Li ZY, Miao CY. Involvement of the secreted protein Metrnl in human diseases. *Acta Pharmacol Sin* 2020; 41: 1525-1530.