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Serum Levels of Protein Z, Laminin Subunit Alpha 2, Mixed Lineage Leukemia 4, and Plexin Domain Containing 2 in Newly Diagnosed and Metformin-Treated Type 2 Diabetes Patients

Sarah Sameer Sami*, Ekhlass M. Taha
Department of Chemistry, College of Science for women, University of Baghdad, Baghdad, Iraq

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Abstract

Diabetes mellitus is a globally prevalent disease with several parameters that may be involved in diagnosis or altered throughout disease progression. In this study, 120 patients with type 2 diabetes and healthy controls were recruited, with ages ranging from 30-65 years old. The research focused on determining levels of Protein Z (PROZ), Laminin Subunit Alpha 2 (LAMA2), Mixed Lineage Leukemia 4 (MLL4), and Plexin Domain Containing 2 (PLXDC2). Participants were divided into four groups: group I (n=30) with long-term metformin treatment, group II (n=30) with short-term metformin treatment, group III (n=30) newly diagnosed without medication, and group IV (n=30) healthy controls. Protein markers were measured by ELISA, while fasting blood glucose, lipid profiles, and HbA1c were determined by spectrophotometry and HPLC. ROC curve analysis revealed PLXDC2 had high sensitivity and specificity as a potential biomarker for diabetes diagnosis. LAMA2, MLL4, and PROZ also distinguished between diabetes patients and controls. Overall, these results identify promising biomarkers for diabetes detection and monitoring.

Keywords: Metformin, Protein Z, Laminin Subunit Alpha 2, Mixed Lineage Leukemia 4, Plexin Domain Containing 2.

قيم (Protein Z, Laminin Subunit Alpha 2, Mixed Lineage Leukemia 4, Plexin Domain Containing 2) في مرضى السكري المشخصين حديثا والمتعاطين لعلاج المتفورمين

ساره سمير سامي* , اخلاص محمد طه
قسم الكيمياء, كلية العلوم للبنات, جامعة بغداد, بغداد,العراق.

الخلاصة

داء السكري هو مرض مرض منتشر عالميا مع العديد من المعلمات التي قد تشارك في التشخيص او قد تتغير مع تطور المرض. في هذه الدراسة , تم تجنيد 120 مريضا يعانون من مرض السكري من النوع الثاني والاصحاء , تتراوح اعمارهم بين 30−65 سنة. ركز البحث على تحديد مستويات البروتين Z (PROZ) , والوحدة الفرعية 2 اللامنين (LAMA2) , سرطان الدم المختلط (MLL4) , و ومجال بلاكسين محتوى2 (PLXDC2). تم تقسيم المشاركين الى اربع مجموعات: المجموعة الاولى (30) فرد من مرضى السكري

*Email: eng.marwa.dawood@uobabylon.edu.iq

المعالج بالمتفورمين لمدة طويلة , المجموعة الثانية (30) فرد من مرضى السكري المعالج بالميتفورمين لمدة قصيرة , المجموعة الثالثة (30) فرد المشخص حديثا بدون دواء, المجوعة الرابعة (30) فرد من الاصحاء. تم قياس علامات البروتين بواسطة ELISA. في حين تم تحديد نسبة الجلوكوز في الدم الصائم وملف الدهون والسكر التراكمي باستخدام طرق القياس الطيفي و HPLC, كشف تحليل منحني لROC أن PLXDC2 لديه حساسية عالية وخصوصية عالية كعلامة حيوية محتملة لتشخيص مرض السكري. كما ميز LAMA2 و MLL4 و PROZ بين مرضى السكري وابضوابط بشكل عام , تحدد هذه النتائج المؤشرات الحيوية الواعدة للكشف عن مرض السكري ومراقبته.

الكلمات المفتاحية :الميتفورمين ، البروتين Z ، الوحدة الفرعية2 Laminin Alpha ، سرطان الدم المختلط 4 ، مجال Plexin الذي يحتوي على .2

1. Introduction

Type 2 diabetes mellitus (T2DM) is a burgeoning global health crisis closely linked to the obesity epidemic. People with T2DM commonly develop microvascular complications including retinopathy, nephropathy, and neuropathy, as well as insulin resistance and hyperglycemia-induced metabolic syndrome [1-2]. The pathophysiological disturbances underlying impaired glucose homeostasis in T2DM arise from a combination of environmental factors like obesity, poor diet, and sedentary lifestyle, as well as genetic factors. Overall, T2DM is a growing concern worldwide fueled by obesity and lifestyle changes, leading to an array of complications through metabolic dysregulation.

T2DM must be treated with a variety of anti-diabetic medications to maintain normoglycemia. To improve insulin sensitivity, treatments are necessary [3-4]. Metformin hydrochloride belongs to the biguanide class and stands as one of the most widely used oral medications for lowering glucose levels. It holds the position of the primary treatment choice for type 2 diabetes (T2DM) in many countries [5]. Numerous research studies have established that metformin demonstrates the ability to improve glycemic control, lower insulin requirements, and act as a deterrent against weight gain.

Clinically, drug therapy for T2DM becomes more complicated with the long duration of the disease. Any medication that is used for DM treatment for a long or short duration will affect the diagnostic tests that are used [6-9]. The most frequently used parameters for the diagnosis of type 2 diabetes are hemoglobin A1C (HbA1c), along with fasting serum glucose (FSG). New methods for diagnosing T2DM and prediabetes have been developed in multiple tries [10-11]. Previous studies evaluated the relationship between T2DM and cytokines [12]. Other parameters like protein Z (PROZ), laminin subunit alpha 2 (LAMA2), mixed lineage leukemia 4 (MLL4), and plexin domain containing 2 (PLXDC2) were mentioned in the review as physiological markers involved in glucose metabolism in Diabetic patients. The function of islet b-cells was reported to be regulated by MLL4's interaction with transcription factors [13]. The LAMA2 mutation has been associated with merosin-deficient congenital muscular dystrophy [14]. PLXDC2 is well known for controlling differentiation and proliferation throughout nervous system development [15]. The primary objective of this study is to investigate the roles of protein Z (PROZ), laminin subunit alpha 2 (LAMA2), mixed lineage leukemia 4 (MLL4), and plexin domain containing 2 (PLXDC2) in the context of type 2 diabetes. Additionally, the study aims to assess the impact of the duration of metformin therapy on these specified parameters.

2. Materials and methods

2.1 Patients and Healthy Subjects

The study received ethical approval from the Committee of Ethics at Al Mustansiriya University's National Diabetes Center for Treatment and Research in Iraq. All participants

gave written informed consent. The research followed the ethical principles outlined in the Helsinki Declaration.

One hundred twenty Diabetes patients and healthy control with an age range between (30-65) years were enrolled in this study to determine PROZ, LAMA2, MLL4, PLXDC2. The selected volunteers were divided into four groups as follows: group I included 30 individuals of T2DM with metformin treatment for a long duration, group II included 30 individuals of T2DM with metformin treatment for a short duration, group III included 30 individuals who were newly diagnosed with T2DM (without medication). These DM patients were without kidney diseases, thyroid or parathyroid disorders, infections, or chronic disorders. Also, smokers and alcohol consumption patients were excluded. The control healthy people included 30 individuals who were chosen as non-diabetic, non-hypertensive, and free from acute illness. Additionally, they were without a history of smoking or consuming alcohol.

2.2 Collection of blood samples

Participants fasted for 10 to 12 hours overnight before blood sampling. Blood was collected using a disposable 10 ml syringe. For each patient and control, 10 ml of blood was drawn and divided into two parts. The first part was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) at 1.5 mg/ml, for serum separation. The collected serum was used to determine levels of FSG, lipid profile, LAMA2, MLL4, PLXDC2, and PROZ. The second part was used without adding EDTA, which used to estimate the HbA1c level.

2.3 Clinical laboratory analysis of groups

Levels of PROZ, LAMA2, MLL4, and PLXDC2 were measured using enzyme-linked immunosorbent test (ELISA). The Kits were purchased from (abbexa., UK, Houston, TX USA, NO.: abx250269), (abbexa., CUSABIO, USA., NO: CSB-EL012726HU), (abbexa., Biosource, USA. NO: MBS75277), and (abbexa., Biosource, USA. NO: MBS281536) respectively. Fasting blood glucose and lipid profile tests were assessed using an automated chemical analyzer (Siemens analyzer, Siemens laboratories, Germany). HbA1C level was assessed via whole blood, using Bio- Rad VARIANT Hemoglobin A1c programmer). The BMI was calculated for all subjects by using the following equation based on weight and height [16]. Eq. (1)

$$\textbf{BMI (Kg/m}^2\textbf{) = Weight (kg) / Height}^2\textbf{ (m}^2\textbf{)} \quad (1)$$

3. Statistical analysis

The data underwent analysis through the statistical software SPSS 25. Descriptive statistics were employed to outline the core findings, while group comparisons were conducted using a one-way analysis of variance test. The association among parameters was examined by Spearman test. Cluster analysis has been used. sensitivity and specificity were calculated for the newly studied parameter and compared it with the routine test using Receiver operating characteristic.

4. Results and Discussion

4.1 Characteristics of patients

Biochemical characteristics were measured in the study population and the results were illustrated in (Table 1).

Table 1: Clinical parameters for the studied population

Variable	GI Mean ± SE	GII Mean ± SE	GIII Mean ± SE	GIV Mean ± SE
BMI kg/m^2	30.55±0.81^a	32.09±1.62^b	33.20±1.57^c	25.97±0.58
HbA1C %	9.08±0.39a,e	8.35±0.40b,f	10.36±0.74 c,e,f	5.21±0.05
FSG mg/dl	210.2±12.04a	191.05±20.12b,f	248.63±32.34c,f	87.53±0.91
Cholesterolmg/dl	213.94±9.02a,d	184.18±11.45b,d	195.33±15.63c	122.77±3.37
TG mg/dl	177.39±10.05a,d	142.62±14.67b,d	179.5±20.91c	93.42±2.30
HDL mg/dl	49.83±4.51	38.01±1.77	52.24±13.70	50.77±0.88
VLDL mg/dl	35.29±1.99a	30.80±3.41b	35.65±4.15c	19.55±0.53

ONEWAY ANOVA/POSTHOC=LSD test at alpha (0.05) were used.
a: significant difference between GIV and G I.
b: significant difference between GIV and GII.
c: significant difference between GIV and GIII.
d: significant difference between GI and GII.
e: significant difference between GI and GIII.
f: significant difference between GII and GIII.

The results from the study groups indicated notable disparities in the investigated parameters such as BMI, FSG, HbA1C, TG, LDL, and VLDL. All of these biomarkers exhibited a substantial elevation across all patient groups in comparison to the control. However, the HDL levels did not exhibit significant differences in the studied groups. Additionally, the outcomes highlighted a significant increase in the HbA1C levels when comparing Group I (T2DM treated with long-duration metformin) and Group III (newly diagnosed without medication). Moreover, the results were showed significant increase between GII (DM treated metformin short duration) and GIII DM newly no drugs) in HbA1C, and FSG.

The levels of protein markers (LAMA2, MLL4, PLXDC2 and PROZ) in studied groups were presented in (Table2). The studied protein markers showed a significant increase in all groups of patients as compared to the control. Also, results showed significant increase between GII and GIII in LAMA2, PLXDC2, and PROZ levels. Moreover, the results were showed significant increase between GI and GIII in PLXDC2.

Table 2: Statistical distribution level of some new markers in the studied population.

Variable	GI Mean ± SE	GII Mean ± SE	GIII Mean ± SE	GIV Mean ± SE
LAMA2ng/ml	11.42±0.73^a	13.36±1.19^{b,f}	9.16±1.30^{c,f}	4.78±0.09
MLL4 ng/ml	2.34±0.14^a	2.61±0.21^b	2.17±0.38^c	1.18±0.06
PLXDC2pg/ml	1263.44±52.00^{a,e}	1283.69±84.70^{b,f}	937.17±72.27^{c,e,f}	702.96±10.78
PROZ ng/ml	22.67±0.80^a	24.94±1.40^{b,f}	19.80±2.01^{c,f}	10.24±0.66

ONEWAY ANOVA/POSTHOC=LSD test at alpha (0.05) were used.
a: significant difference between GIV and G I.
b: significant difference between GIV and GII.
c: significant difference between GIV and GIII.
d: significant difference between GI and GII.
e: significant difference between GI and GIII.
f: significant difference between GII and GIII.

Body mass index (BMI), which is used to determine obesity, has been a serious health problem for individuals around the globe. The worldwide disease burden is now significantly influenced by BMI. Obesity is a chronic condition and a comorbidities risk factor [17-18]. The current study results shown increases in (BMI) ⩾30 kg/m2 in diabetes newly no drugs compared to long duration to metformin this result increased metformin's impact on DM patients (Table1). Numerous studies suggest that metformin can aid patients who are overweight or obese in losing weight [19]. Moreover, metformin can lower BMI for T2DM obesity and ease diabetes symptoms [20]. According to the American Diabetes Association Erratum. (ADA) The first-line oral medication for type 2 diabetes is metformin (T2D) [21]. Additionally, in the current study metformin results showed a stronger impact on people's FBG and HbA1C reductions (Table 1), most likely due to its mechanism of suppressing the synthesis of fasting hepatic glucose [22]. Metformin and lifestyle were equally effective in avoiding diabetes based on A1C, according to resent recognized criteria for the diagnosis of diabetes, about 44 % reduction with metformin and 49 % with lifestyle [23-25]. The outcomes suggested that metformin treatment decreased the individuals' blood glucose levels and may enhanced insulin sensitivity. The primary element of metformin's mode of action is the cell's altered energy metabolism. Metformin appears to inhibit complex I of the mitochondrial electron transport chain inside of a cell, which lowers the cellular energy status [26]. Activated AMPK triggers the enhancement of glycolysis and fatty acid oxidation, while it simultaneously obstructs anabolic pathways such as gluconeogenesis and fatty acid synthesis. In contrast, low concentrations of metformin were observed to elevate complex 1 activity, as noted by Law V. et al., [27].

According to LAI, YEREVANIAN et al, metformin should be taken into consideration for patients with prediabetes, especially those with a BMI of 35 kg/m2 and women who have a history of gestational diabetes. It also shares that metformin was as beneficial as lifestyle interventions [28-29]. According to (Table 2) findings, there was a significant increase. in PROZ, LAMA2, PLXDC2 and MLL4 in a long-treated metformin DM as a compared to control group and newly diagnosed. The increase in LAMA2 may be due to damaged tissues which are considered famous markers for muscular dystrophy is a disorder that causes weakness in muscles function by Bouman, Karlijn, et al, In line with Phielix et al. abnormal skeletal muscle's metabolic function is caused by insulin resistance in type DM patients [30-31]. So, this result of LAMA2 could be a promising marker to diabetes type 2. Current study also shows that increased in PROZ in long-treated and short-treated metformin DM comparation to new DM and control. In a previous study, patients with prediabetes reported a much low level of PROZ than those with normoglycemia according to Bae et al. [32]. Moreover, significant increases in PLXDC2 were reported. Thus, the authors suggested that PLXDC2 may plays important role on developing nervous system according to Sze et al. [33]

4.2 Cluster analysis

4.2.1 Cluster analysis for variables using GI (T2DM patients with long duration of metformin).

The cases' parameters were subjected to hierarchical clustering using the word method, resulting in the classification of the data set into distinct groups. These groupings were determined based on the distances between variables, representing either their similarity or dissimilarity. Notably, MLL4, HbA1C, LAMA2, and PROZ were identified as a cohesive set of variables within a single group. Additionally, a high degree of similarity was observed between VLDL and FSG. The results were supported by fusion algorithms. The studied variables were combined into groups according to the considers segmentation as a homogenous group, At the same time the groups VLDL, and FSG was dissimilar from group of MLL4, HbAIC, LAMA2, and PROZ are shown in Chart 1.

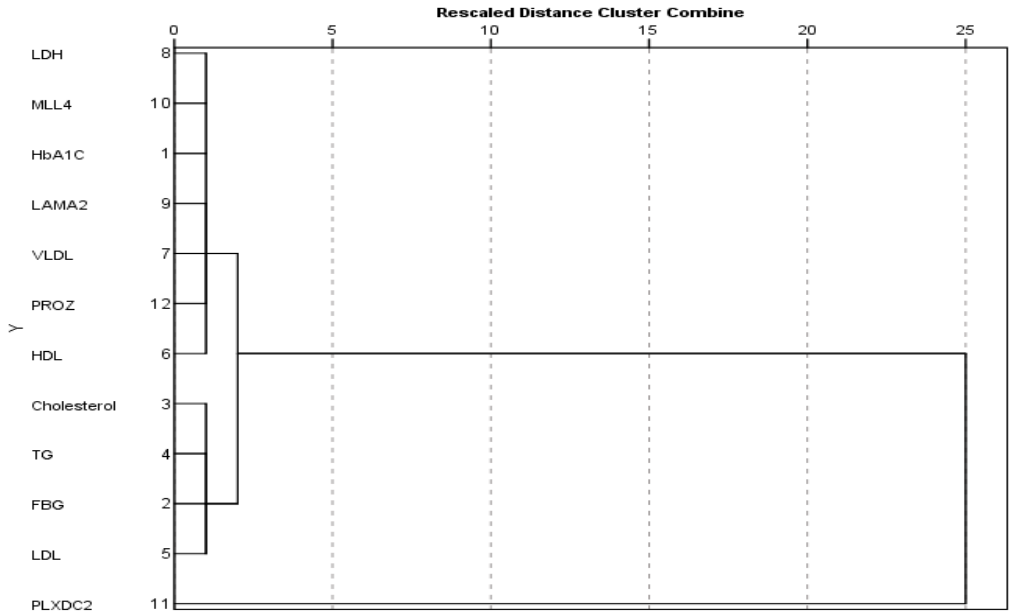

Chart 1: Cluster analysis of studied variables using T2DM patients with long duration metformin therapy as a model.

4.2.2 Cluster analysis of variables Using GII (T2DM patients with short duration metformin therapy).

The congruence of similarity (distance) among variables was verified, revealing that MLL4, HbA1C, LAMA2, FBG, and PROZ share similarity in terms of the designated segmentation variables, thus forming homogenous groups. Additionally, Chart 2 demonstrates a substantial similarity between VLDL and PLXDC2.

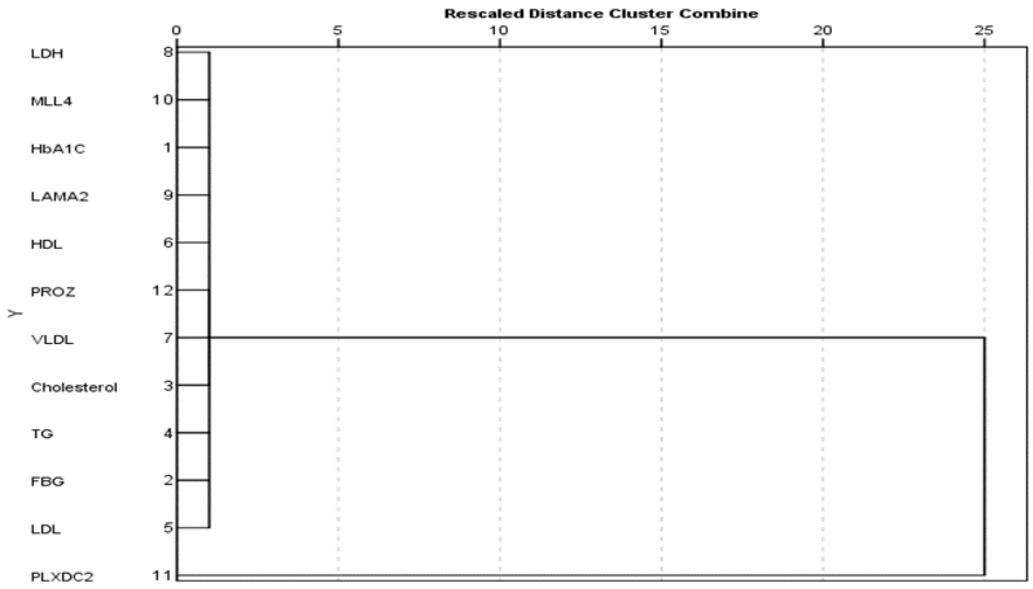

Chart 2: Cluster analysis of studied variables using DM patients with short duration metformin therapy as a model.

4.2.3 Cluster analysis for variables using GIII (newly diagnostic T2DM patients).

The seam pattern has been confirmed in cluster analysis as it was observed in patients with a long duration of metformin. MLL4, HbAIC, LAMA2, and PROZ were similar with respect to the considered segmentation variables (homogenous groups), Chart 3. The similarity in

pattern between patients newly diagnostic and patients with a long duration of metformin therapy may be due to metformin effects.

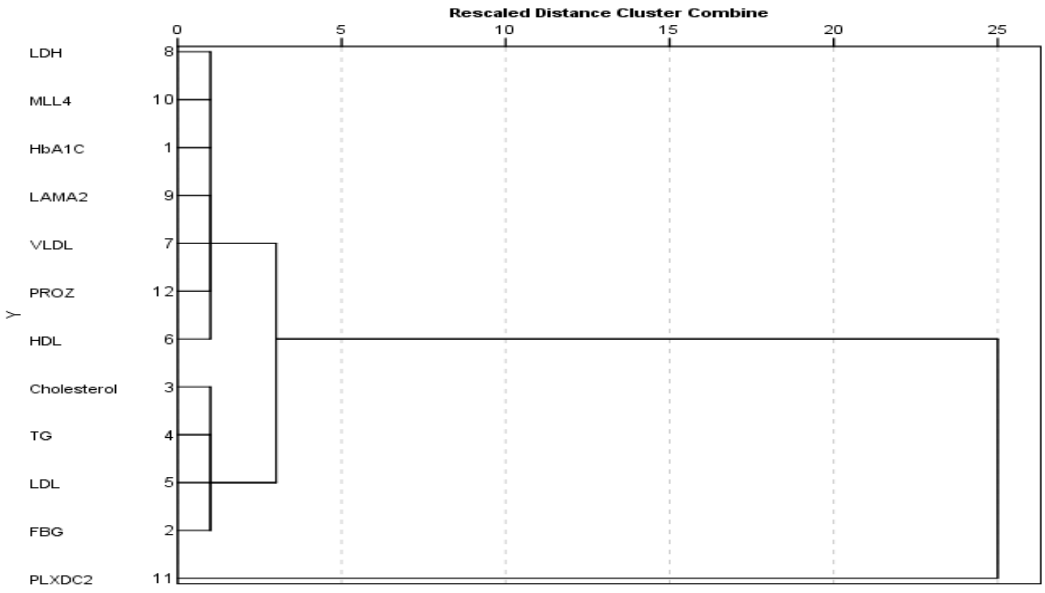

Chart 3: Cluster analysis of studied variables using newly diagnostic DM patients.

The findings of the present study confirmed that levels of MLL4 were elevated in patients from Groups GI and GII (those with type 2 diabetes treated with metformin for both long and short durations) when compared to the healthy control groups. Consequently, MLL4 could potentially serve as a promising biomarker for diagnosing type 2 diabetes patients under metformin treatment.

In GII the results showed that levels of PRO Z were higher than those of the healthy groups. Henes, PROZ blocks activated factor X at the phospholipid surface by blocking blood coagulation factor Previous clinical studies have described the function of PROZ in individuals with cardiovascular disease [34-35]. ischemic stroke, and deep vein thrombosis. S. Fedi et al. supposed that changes in PROZ levels could be related to vascular complications in diabetes type2 newly diagnostic [36]. Further studies are needed to elucidate the role of PROZ in diabetes type2, which may reveal the hidden mechanisms of T2DM.

4.3 Receiver Operating Characteristic (ROC) analysis

Receiver Operating Characteristic (ROC) analysis using patients of DM with long duration of metformin therapy results were presented in Table 6.

Table 6: ROC result analysis of HbA1C, FBG, LAMA2, MLL4, Z protein and PLXDC2 using T2DM patients' long duration with metformin therapy.

Parameters	Area	Sensitivity	Specificity	Cutoff	Asymptotic 95% Confidence Interval	
					Lower Bound	Upper Bound
Z protein	0.89	100%	99%	5.8	0.95	1
LAMA2	0.99	94%	100%	5.8	0.98	1
MLL4	0.89	75%	99%	1.7	0.81	0.96
PLXDC2	0.98	94%	100%	796	0.95	1
FBG	0.99	97%	100%	99	0.99	1
HbA1C	0.98	97%	100%	5.8	0.96	1

Null hypothesis: true area = 0.5

The sensitivity and specificity of Z Protein, LAMA2, MLL4, PLXDC2 were determined using ROC curve. All studied parameters showed very good sensitivity and specificity as compared to FBG and HbA1C, also showed clear cutoff value to distinguished between patients and control, as shown in Figure (1), and Table (6).

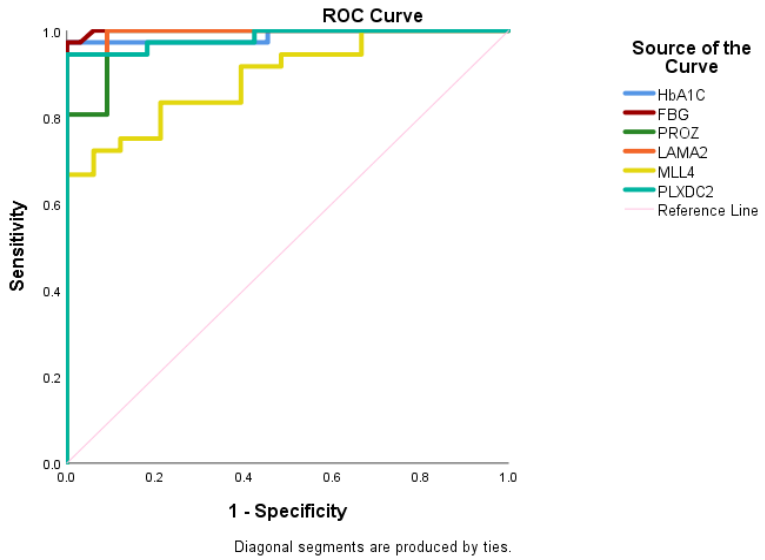

Figure 1: ROC curve analysis of HbA1C, FBG, LAMA2, MLL4, Z protein and PLXDC2 using DM patients' long duration with metformin therapy.

4.3.1 Receiver Operating Characteristic (ROC) analysis using patients of T2DM with short duration of metformin therapy.

The recently examined parameters in patients with type 2 diabetes with a brief history of metformin therapy exhibited notably heightened sensitivity and specificity when compared to traditional markers like FBG and HbA1C. Specifically, the parameters under investigation, namely Z protein, LAMA2, MLL4, and PLXDC2, displayed distinct cutoff values (5.3, 6.4, 1.7, and 735, respectively), as illustrated in Figure 2 and Table 7.

Table 7: ROC result analysis of HbA1C, FBG, LAMA2, MLL4, Z protein and PLXDC2 using DM patients' short duration with metformin therapy.

Parameters	Area	Sensitivity	Specificity	Cutoff	Asymptotic 95% Confidence Interval	
					Lower Bound	Upper Bound
Z protein	0.98	100%	60%	5.3	0.95	1
LAMA2	1	100%	100%	6.4	1	1
MLL4	0.95	88%	99%	1.7	0.89	1
PLXDC2	0.96	94%	98%	735	0.90	1
FBG	0.99	94%	100%	99	0.97	1
HbA1C	0.97	94%	100%	6.1	0.92	1

Null hypothesis: true area = 0.5

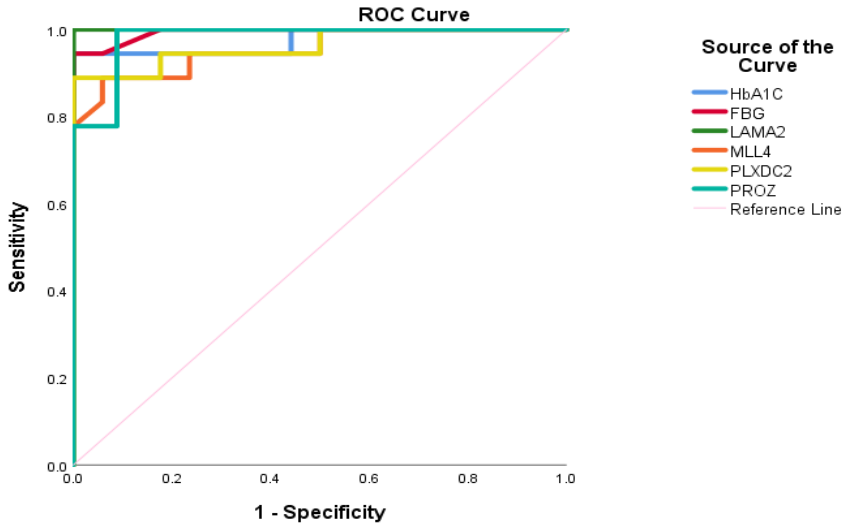

Figure 2: ROC curve analysis of HbA1C, FBG, LAMA2, MLL4, Z protein and PLXDC2 using DM patients' short duration with metformin therapy.

4.3.2 Receiver Operating Characteristic (ROC) analysis using patients of T2DM newly diagnostic.

The new parameters that were studied in patients with T2DM with newly diagnostic appeared very clear sensitivity and specificity as compared to FBG, and HbA1C (100%). The studied parameters were Z protein, LAMA2, MLL4, PLXDC2 showed cutoff value (12.7,6.0,1.3 and 837) respectively Figure (3), and Table (8).

Table 8: ROC result analysis of HbA1C, FBG, LAMA2, MLL4, Z protein and PLXDC2 using newly diagnostic DM patients.

Parameters	Area	Sensitivity	Specificity	Cutoff	Asymptotic 95% Confidence Interval	
					Lower Bound	Upper Bound
Z protein	0.94	88%	99%	12.7	0.85	1
LAMA2	0.96	77%	100%	6.0	0.91	1
MLL4	0.85	89%	98%	1.3	0.70	0.99
PLXDC2	0.84	77%	100%	837	0.63	1
FBG	1	100%	100%	113	1	1
HbA1C	1	100%	100%	6.2	1	1

Null hypothesis: true area = 0.5

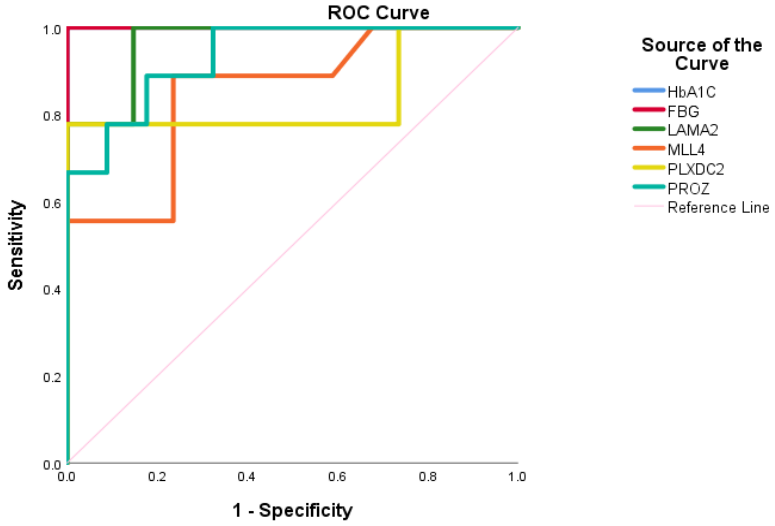

Figure 3: ROC curve analysis of HbA1C, FBG, LAMA2, MLL4, Z protein and PLXDC2 using newly diagnostic DM patient.

The present dataset demonstrates elevated sensitivity and specificity of PROZ, LAMA2, PLXDC2, and MLL4 in diagnosing type 2 diabetes. This applies to patients with both long and short durations of metformin therapy, as well as newly diagnosed patients, when compared to the traditional markers HbA1c and FBG. These results underscore the substantial potential of these parameters in assessing diabetes cases. Importantly, this study pioneers the evaluation of the connection between PROZ, LAMA2, PLXDC2, and MLL4 with type 2 diabetes. These proteins markers may be a promising biomarker to compensate for the limitations of the existing tests, however the previous study showed the correlation of PROZ with prediabetes [37] T2DM is notably accompanied by a high prevalence of related conditions, such as atherogenesis, metabolic syndrome, myocardial infarction (MI), and vascular diseases (cardiac, cerebral, and peripheral), which result in noticeably high morbidity and mortality according to Bahathiq A, et al, [38]. The present research results showed that increase in type2 newly diagnostic DM with LAMA2, MLL4, and PLXDC2, this will assist in investigating the changes linked to protein markers and diabetes complications, as well as in the development of a management program to manage the complications of these abnormalities.

Conclusion

The four parameters under consideration (LAMA2, MLL4, PROZ, and PLXDC2) prove to be effective diagnostic markers for diabetes when compared to the well-established FBG and HbA1c, which also serve as cost-effective diagnostic indicators. Interestingly, metformin treatment did not display any opposing effects on the studied parameters. The four parameters demonstrated significant increases in response to longer durations of metformin therapy. Notably, while PLXDC2 and LAMA2 showcased a discernible correlation when metformin was used for an extended period, cluster analysis outcomes indicated that PLXDC2 diverged from the rest of the parameters. Conversely, MLL4, HbA1C, LAMA2, and PROZ exhibited similarity in their distribution, with their positions being closely situated. The same distribution of cluster analysis was concluded in the case of using metformin therapy for a short time, and in the case of new diagnostics. ROC results support that (LAMA2, MLL4, PROZ, and PLXDC2) are important in DM diagnostic as much as HbAIC, and FBG.

ETHICAL CLEARANCE

This study was approved by The Scientific Ethics Committee of the National diabetes center.AL-Mustansiriyah University for their support to carry out this research work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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