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Serum Cytokine Dependent Hematopoietic Cell Linker (CLNK) as a Predictor for the Duration of Illness in Type 2 Diabetes Mellitus.

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Abstract

Type 2 diabetes mellitus (T2DM) is an endocrine illness associate with various changes in the immune system and adaptor protein levels. Cytokine dependent hematopoietic cell linker (CLNK) is an adapter protein that regulates immune receptor signaling and acts as a regulator of the receptor signaling of T-cells and natural killer T-cell. The role of CLNK in T2DM is not studied previously. In the present study, serum CLNK level was measured and correlated with some sociodemographic and insulin resistance (IR) parameters. This is achieved by performing measurement of CLNK and insulin parameters (glucose, insulin, and HbA1c in addition to the calculation of the functions of IR (HOMA2IR), insulin sensitivity (HOMA%S), and beta-cell function (HOMA%B)) in 60 T2DM patients and 30 controls. The results indicated a significant increase ($p=0.025$) in serum CLNK in patients group in comparison with the controls. Multivariate generalized linear model (GLM) analysis revealed no significant effect of age, BMI, and sex on the CLNK level. The results of tests for between-subjects showed that the CLNK affects diagnosis significantly ($F=7.445$, $p=0.008$, partial $\eta^2 =0.081$) and its effect is approximately the same as the effect of insulin ($F=8.107$, $p=0.006$, partial $\eta^2 =0.087$). The correlation study showed a highly significant positive correlation between CLNK and the duration of disease ($rho=0.420$, $p<0.001$). It can be concluded that the increase CLNK in T2DM revealing the role of the adaptor proteins level in the nature of disease. Elevation of CLNK level may be used as a predictor for diabetes complications, which needs more investigations.

Keywords: Diabetes Mellitus, Insulin resistance, Cytokines, Adaptor proteins, CLNK.

Introduction

Diabetes mellitus (DM) is a prevalent disorder characterized by hyperglycemia and results from the interaction between environmental, genetic, and behavioral risk

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factors (Chen et al. 2012). DM is a major public health concern with a growing prevalence around the world. In 2017, it is estimated that there are 451 million people aged over 18 years with DM worldwide, which expected to reach 693 million by the year 2045 (Cho et al. 2018). It was estimated that almost half of all people living with DM are undiagnosed (Cho et al. 2018). In Iraq, the prevalence of DM had risen significantly from 19.58/1000 in the year 2000 to 42.27 in 2015 (Hussain and Lafta 2019). Diabetes is estimated to contribute to 11.3% of deaths globally (Saeedi et al. 2020). Type 2 DM (T2DM) is the most familiar form of DM described by insulin resistance (IR), hyperglycemia, relative insulin deficiency, and insensitivity (Olokoba et al. 2012). Insulin insensitivity is produced because of declining insulin production, IR, and final pancreatic beta-cell malfunction results in a diminished in glucose transportation into the adipocytes, myocytes, and hepatocytes (Cerf 2013). T2DM is related to chronic inflammation which can be attributed to innate immune system dysregulation and this is a potential link between diabetes and metabolic syndrome (Banerjee and Saxena 2014). T2DM is associated with chronic inflammation in addition to metabolic dysregulation and there is also a link between metabolism and inflammation (Karstoft and Pedersen 2016). Chronic inflammation may be the cause and result of T2DM, and its related complications as an imbalance between proinflammatory and anti-inflammatory cytokines can affect immune functions (Naidoo et al. 2018). Therefore, the study of another biomarker for inflammation in T2DM is important to diagnose and treat low-grade inflammation (LGI) which is responsible for most diabetic complications (Eftekharian et al. 2016; van Diepen et al. 2017). For example, Previous investigations have broadly revealed the relationship of high C-reactive protein (CRP) levels with IR and progression of T2DM (Phosat et al. 2017). While interleukine (IL)-6 plays an exceptional role in the development of T2DM

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and elevated TNF-α levels over time could be a potential predictor of T2DM over time (Lainampetch et al. 2019). In addition to cytokines released in response to several stimuli and immune system dysregulation (Banerjee and Saxena 2012), adaptor proteins are another type, which is a very important constituent of signaling transduction systems both beyond and within the immune system (Verma et al. 2020). Adaptor proteins are molecular platforms that other proteins are accumulated on which (Verma et al. 2020). These adapter proteins control signaling by stabilizing or constraining molecular interactions essential for suitable activation of enzyme and for implementing these key effector molecules correctly inside the cell (Jordan and Koretzky 2010). Many adaptors are expressed in a variety of hematopoietic cell types (Dong et al. 2009; Jordan et al. 2003). The term "hematopoietic cells" describes all mature cell types and their immature precursors, or hematopoietic stem cells, in the blood. Examples of hematopoietic cells include, but are not limited to, hematopoietic stem cells, basophilic myelocytes, basophils, B-cells, etc.(Yu et al. 2018). Cytokine dependent hematopoietic cell linker (CLNK) is a type of adaptor protein Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76) which exist in many lineages of hematopoietic cells including neutrophils, mast cells, macrophages, platelets, T-cells, and natural killer (NK) cells (Jordan and Koretzky 2010). CLNK has been reported to be spoken in cytokine-dependent cell lines of both lymphoid as well as myeloid in addition to some mast cell lines (Goitsuka et al. 2000). Although it can be distinguished in any type of cell, its expression appears to be strictly dependent on continual exposure to cytokines such as IL-2 and IL-3 (Ishihara and Hirano 2002). Furthermore, the temporary expression of CLNK results in increased immune-receptor signaling events in T-cells that are activated by cytokines (Cao et al. 1999). CLNK can act as a modulator to limit extreme NK cell activation in the proceeded stage of immune

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reactions anywhere teeming cytokines are out there (Hidano et al. 2008). NK cells are inhabitant cells of adipose tissue and they have numerous immune regulatory capacities including the release of immunoregulatory cytokines (IL-4 and IL-10), prompt dendritic cells, and expanding the recurrence of T regulatory cells. NK cells number are decreased in obese persond, proposing their contribution in local and sytemic inflammation in obesity (Tard et al. 2015). Obesity leads to IR which is the main feature for the etiology and pathogenesis of T2DM (Rehman and Akash 2016) that is found to be caused by activated immune-inflammatory pathway (Khodabandehloo et al. 2016) as the pro-inflammatory molecules are responsible of the activation of a variety of kinases in IR state (Hameed et al. 2015). There was no study deals with the role of CLNK in T2DM disease. Therefore, the aim of the present study is to examine the role of CLNK in the T2DM as a possible inflammatory marker or a potential predictor for some sociodemographic parameters and IR state parameters.

Subjects and methods

Subjects

The case-control study involved 60 T2DM patients and 30 healthy controls age and sex-matched. The samples were obtained from Al-Sadder Hospital in Najaf Governorate-Iraq from December 2019 to the end of January 2020. T2DM patients were diagnosed according to the World Health Organisation criteria (WHO 2006). They were assessed by full medical history by the physicians. All subjects were non-smokers. All subjects were fasted for at least 12 hours before the aspiration of blood in the morning. All the patients were on one tablet (5 mg) of glibenclamide drugs daily. The control group was confirmed to be normal by clinical and biochemical tests. Written informed consent was taken from all subjects before participation in the current

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study. All procedures were under the established ethical standards. Approval for the study was obtained from the IRB of the University of Kufa (411/2019), which complies with the International Guidelines for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, and International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Exclusion criteria: The present study excluded patients who satisfied the following criteria: patients with serum FBG >25 mmol/L and fasting insulin >57.6 mIU/L based on HOMA calculator software requirements, and patients with evident major diabetic complications, such as heart diseases, liver disease, and renal diseases. We also excluded patients who are receiving metformin and patients with albumin/creatinine ration above 30 mg/g. This exclusion is based on previously mentioned facts (Al-Hakeim and Abdulzahra 2015) that patients using metformin cannot be used for insulin sensitivity studies because of the well-known effect of metformin on IR (Sangeeta 2012) and insulin sensitivity (Pernicova and Korbonits 2014). Serum CRP titer was negative in all samples i.e. less than 6mg/L. CRP test was used to exclude inflammation that causes changes in the acute phase reactant proteins.

Methods

Five milliliters of venous blood samples were drawn from patients and controls. After complete clotting, the blood was centrifuged 3000 rpm for 10 min, and then serum was separated to be stored at -80 C until analyzed. Commercial ELISA sandwich kits were used to measure serum insulin (DRG®International Inc., USA) and CLNK (Bioassay Technology Laboratory ®, China). Fasting serum glucose was measured spectrophotometrically by an enzymatic method (endpoint) using ready for use kit supplied by Biolabo® Co., France. HbA1c percentage in the whole blood was

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determined by ichroma™ HbA1c is a fluorescence Immunoassay (FIA) for the quantitative determination HbA1c. Serum CRP was measured using a kit supplied by Spinreact®, Spain. The test is based on the principle of latex agglutination. Anthropometric measurements weight and height were taken and BMI was calculated by weight in kilograms divided by square of height in meters.

Insulin sensitivity percentage (HOMA%S), percentage of beta-cell function (HOMA%B), and IR HOMA2IR were measured from fasting insulin and serum glucose by HOMA2 Calculator downloaded from (https://wwwdtuoxacuk/homacalculator/).

2.3.Statistical analysis

The distribution types of the variables' results were examined using the Shapiro–Wilk test as a normal statistical distribution. The results were expressed as (mean ± standard deviation) or median (interquartile range). A Chi-square test was used for comparison between nominal variables. The pooled t-test has been used for the comparison between the patients and control groups and among subdivided groups in the measured parameters. Mann-Whitney U test was used for comparison between nonparametric variables. Pearson's correlation coefficients (r) or Spearman's correlation coefficient (rho, ρ) were used to evaluating the relationships among parameters. We used multivariate general linear model (GLM) analysis to delineate the associations between diagnosis (having T2DM or healthy control) and five biomarkers (CLNK, HOMA%B, Insulin, HOMA2IR, and HOMA%S). while controlling for confounding variables including age, BMI, and sex. Consequently, we carried out tests for between-subject effects to delineate the associations between diagnosis and each of the biomarkers. The effect size was estimated using partial eta-squared values. We excluded the FBS and HbA1c from the multivariate GLM analysis because they already

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have a very high correlation with T2DM and will cause a big colinearity of the tests The difference between groups is considered as statistically different when $p<0.05$. All statistical analysis was performed using SPSS Statistics Version 25 (2017) by IBM-USA. While the figures constructed using the Excel program of Microsoft Office 2016.

Results

Socio-demographic and clinical characteristics

The clinical and socio-demographic characteristics of the patients with T2DM and control groups are presented in Table 1. The results are typical for T2DM patients where there is a significant increase ($p<0.001$) in FBS, HbA1c, and HOMA2IR ($p=0.031$), while the secretion of insulin is significantly decreased ($p=0.008$) along with HOMA2%B ($p<0.001$). While HOMA2%S showed no significance between the two groups. Serum CLNK was significantly elevated ($p=0.025$) in patients group in comparison with the control group.

Multivariate GLM analysis

The data were analyzed by multivariate generalized linear model (GLM) analysis with the biomarkers (HOMA%B, Insulin, HOMA2IR, HOMA%S, and CLNK) as dependent variables and diagnosis and the covariates (age, BMI, and sex) as an explanatory variable to examine their effect on the measured parameters were shown in Table 2. We found a highly significant effect of diagnosis ($F=25.048$, $p<0.001$) on the measured parameters with a big effect size of 60.7%, while age, BMI, and sex were not significant ($p<0.05$). We also examined the effects of measured parameters on the diagnosis by using tests for between-subjects. We found that the highest parameter that affects diagnosis significantly is HOMA%B ($F=30.14$, $p<0.001$, partial $\eta^2=0.262$), followed by HOMA2IR ($F=13.164$, $p<0.001$, partial $\eta^2=0.134$), Insulin ($F=8.107$,

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p=0.006, partial η^2 =0.087), and CLNK (F=7.445, p=0.008, partial η^2 =0.081). While HOMA%S has no significant effect on the diagnosis ($p<0.05$). These results indicated that diagnosis (i.e, being patients) have the same effects on the variation of serum CLNK or insulin levels.

Correlation of |CLNK with other parameters

The result of the correlation study showed a highly significant positive correlation between CLNK and the duration of disease in the patients' group (rho=0.420, $p<0.001$) as presented in Figure 1. The results showed that R^2=0.1761, which represents that 17.61% of the variance in the duration of illness is explained by serum CLNK level which is statistically significant ($p<0.001$). To obtain a better view of the dependence of CLNK level on the and duration of the illness, the duration of the disease is distributed into 5 years periods and the average of CLNK for each period was calculated and plotted in Figure 2. The figure presents the increase in the average serum CLNK with increasing the duration of disease. There is no significant correlation between serum CLNK and other parameters.

Discussion

The major finding of the present study is the increase in serum CLNK level in T2DM patients in comparison with the control group as presented in Table 1. Because of the lack of literature about the level of CLNK in the serum of T2DM patients, there is no simple explanation for this result. In the literature, only one research measured the serum CLNK in humans, namely in thalassemia patients (Al-Hakeim et al. 2019). The suggested explanation for the increase of CLNK in thalassemia patients in comparison with healthy controls depends on the reciprocal effects between immune

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system signaling and immature erythrocytes that release soluble receptors and signaling molecules including CLNK in the blood (Al-Hakeim et al. 2019). In our T2DM patients, the factor that may underpin the explanation of increased CLNK is via the LGI associated with T2DM disease (Burhans et al. 2018; Esser et al. 2014). The disease is started with chronic LGI associated obesity, which is involved in the development of IR that increases the risk of T2DM (McGill et al. 2008). When LGI is associated with lipid toxicity, together they appear to be major assaults on insulin sensitivity in insulin-responding tissues (Bilan et al. 2009). hyperinsulinemia also has a bidirectional relationship with chronic LGI, which may be developed into systemic inflammation and systemic inflammation causing IR and eventually compensatory hyperinsulinemia, which increases oxidative stress and inflammation processes (van den Oever et al. 2010). Therefore, it is confirmed that T2DM associated with LGI, IR, and obesity that interact with each other in a multidirectional mechanism (Badawi et al. 2010; Kang et al. 2016). For example, there is strong evidence that activation of inflammatory pathways interferes with normal metabolism and disrupts proper insulin signaling resulting in increased expression of pro-inflammatory cytokines (Gregor and Hotamisligil 2011). These cytokines can attach to their receptors on the cell membrane and produce an inflammatory reaction and exacerbating IR (Solinas et al. 2007).

chronic activation of immune-inflammatory pathways lead to IR, thus rising risk of T2DM (Kan et al. 2013). IR development is generally correlated with pro-inflammatory cytokines-dependent LGI responses in various tissues (Fève and Bastard 2009). These cytokines eventually prevent the activation of insulin signaling receptors in β-cells islets (Rehman and Akash 2016) by activating kinases that phosphorylate serine residues of insulin receptor substrate and causes suppression of insulin signaling (Aroor et al. 2013). IR could be produced by altered recruitment of downstream adaptor

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proteins that CLNK belongs to (Siragusa and Fisslthaler 2017). The CLNK homology adaptor protein SLP-76 interacts physically with signaling molecules, the serine/threonine kinase HPK1(Homeodomain-interacting protein kinase-1) (Jordan et al. 2003), which may cause IR by affecting the insulin sensitivity by modifying the insulin receptors.

The other part that should be confirmed is the correlation between CLNK and inflammation. It is noticed previously that CLNK, as an adaptor protein, plays a crucial role in the T-cell signaling pathway mediated by the T-cell receptor, which is necessary for the adaptive immune response and important for differentiation, proliferation, and cytokine secretion (Ji et al. 2015). It is expressed exclusively in cytokine-stimulated hemopoietic cells, including IL-2-induced T cells and NK cells, and IL-3-induced myeloid cells and mast cells (Boomer and Tan 2005).

Another important finding of the present study is the strong association between CLNK and the duration of T2DM illness. The strongest association comes from the finding that CLNK regulates the IFN-γ production by different immune cells (Sasanuma et al. 2006). CLNK expression in NK cells is needed to improve the subsequent acquired immune responses. At the advanced stage of immune responses, CLNK can restrict excessive NK-cell activation, where abundant cytokines upregulate CLNK expression in NK cells. Since CLNK is needed for complete activation of NKT cells at such an advanced stage of immune responses, activated NKT cells expressing CLNK produce high amounts of cytokines includingIL-4and IFN-π which can ultimately decide the nature of the subsequent acquired immune responses (Sasanuma et al. 2006).

Figure 1 showed the dependence of the duration of illness (17.61% variation change) on the serum CLNK level. This result recalls a certain change in T2DM patients

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that depends on the period of illness. The duration of diabetes reflects the course of the disease (Gupta et al. 2017) and patients with longer illness duration have more complications (Akujuru et al. 2020). Another set of researchers found an association between the duration of diseases and the development of immune system concentrations. In glycemic controlled patients without complications, it is found a significant negative correlation between duration of disease with monocyte RANTES and a significant correlation with various types of neutrophil Toll-like receptors. The nature of correlation (negative or positive) depends on the type of Toll-like receptor, degree of glycemic control, and the presence of complications (Gupta et al. 2017). The duration of diabetes mellitus affects the concentration of IL-1β in both types of diabetes (Amin et al. 2020). Diabetes duration showed strong negative correlations with serum level of brain-derived neurotrophic factor (BDNF) (Li et al. 2016), and vitamin D3 (Aljack et al. 2019; Kishore and Code 2017). These changes with time indicating continuous deterioration of immune system defense with the duration of illness.

The CLNK level increases with the duration of T2DM as seen in Figure 2. The long duration of the disease leads to more complications (Hsu et al. 2015). Many diabetes complications appear with the progress of disease and correlated with the duration of disease including coronary artery diseases, nephropathy, retinopathy, and neuropathy (Kobayashi et al. 2020). As the duration of disease prolonged, the serum level of many biomarkers including uric and microalbuminuria (Latif et al. 2017) indicating the progression of nephropathy with time. Therefore, this is a good point to consider CLNK as a predictor for a complication that needs more investigation.

Another explanation of the increase of CLNK in T2DM with time through the mutual correlation of CLNK and duration of disease with platelet counts and properties. CLNK as an adaptor protein has a role in the activation of platelets (Senis et al. 2014).

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Moreover, the duration of illness in T2DM patients achieved a significant positive correlation with platelet count and volume (Biadgo et al. 2016). Collagen-induced platelet aggregation and granule release are markedly impaired in the absence of SLP-76,.(Clements et al. 1999). These data revealed that SLP-76 expression is required for optimal receptor-mediated signal transduction in platelets as well as T lymphocytes (Clements et al. 1999). The overall of these results underpins a connection between CLNK, platelets, and duration of diabetes.

Conclusion

The present work is the first report about increase CLNK in T2DM revealing the role of the adaptor protein level in the disease naturally. The level of serum CLNK is strongly associated with the duration of illness and not associated with the IR parameters. As the prolonged duration of the disease leads to the development of diabetes complications and deterioration of the immune system, the elevation of the CLNK level may be used as a predictor for diabetes complications, which needs more investigations.

Limitations of the Study

The first limitation is the relatively small sample size. The second limitation is the restricted criteria in choosing the subjects of the study. All subjects were nonsmokers, and the patients were free of obvious complications and on one type of treatment (glibenclamide). These criteria are useful in removing the cofounders affecting the CLNK level but they are ideal and do not represent the real-life state.

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Conflict of interest

The authors declare that there is no conflict of interest .

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Author's contributions

All the contributing authors have participated in the preparation of the manuscript.

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Table 1: Socio-demographic, clinical, and biomarker data in T2DM patients as compared with healthy controls.

Parameter	Control (n=30)	Patients (n=60)	p-value
Sex M/F	20/10	31/29	0.176*
Age Year	50.27±8.25	53.08±7.85	0.056
BMI kg/m2	26.90±2.54	28.65±5.23	0.088
Duration of illness Year	-	7(3-11)	-
HbA1c %	4.69±0.38	8.57±2.06	<0.001
FBS mM	5.20±0.29	10.54±4.58	<0.001
Insulin pM	88.57(52.26-122.96)	73.53(35.99-103.91)	0.008**
HOMA2%B	103.30(79.25-124.25)	42.70(19.8-72.20)	<0.001**
HOMA2%S	75.10(64.00-99.45)	49.40(24.10-106.65)	0.058**
HOMA2IR	1.33(0.99-1.56)	2.09(0.94-4.15)	0.031**
CLNK ng/ml	4.27(2.59-5.08)	4.89(3.67-5.93)	0.025**

-Results expressed as mean±SD or median (interquartile range).

*: Calculated by χ^2, **: Calculated by Mann-Whitney U test, while other p-values were carried out by using pooled T-test.

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Abbreviations: FBS: Fasting blood sugar, BMI: Body mass index, HOMA2R: Homeostatic model assessment of insulin resistance, HOMA2%B: Homeostatic model assessment of beta cells function percentage, HOMA2%S: Homeostatic model assessment of insulin sensitivity percentage, CLNK: Cytokine Dependent Hematopoietic Cell Linker.

Table 2. Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as an explanatory variable while adjusting for extraneous variables.

Tests	Dependent Parameters	Explanatory variables	F	df	P	Partial η^2
Multivariate	HOMA%B, Insulin, HOMA2IR, CLNK, HOMA%S.	Diagnosis	25.048	5/81	<0.001	0.607
		Sex	0.735	5/81	0.599	0.043
		Age	0.501	5/81	0.774	0.030
		BMI	1.027	5/81	0.408	0.060
Tests of Between-Subjects Effects	HOMA%B	Diagnosis	30.140	1	<0.001	0.262
	HOMA2IR	Diagnosis	13.164	1	<0.001	0.134
	Insulin	Diagnosis	8.107	1	0.006	0.087
	CLNK	Diagnosis	7.445	1	0.008	0.081
	HOMA%S	Diagnosis	0.056	1	0.601	0.003

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Abbreviations: BMI: Body mass index, HOMA2R: Homeostatic model assessment of insulin resistance, HOMA2%B: Homeostatic model assessment of beta cells function percentage, HOMA2%S: Homeostatic model assessment of insulin sensitivity percentage, CLNK: Cytokine dependent hematopoietic cell linker.

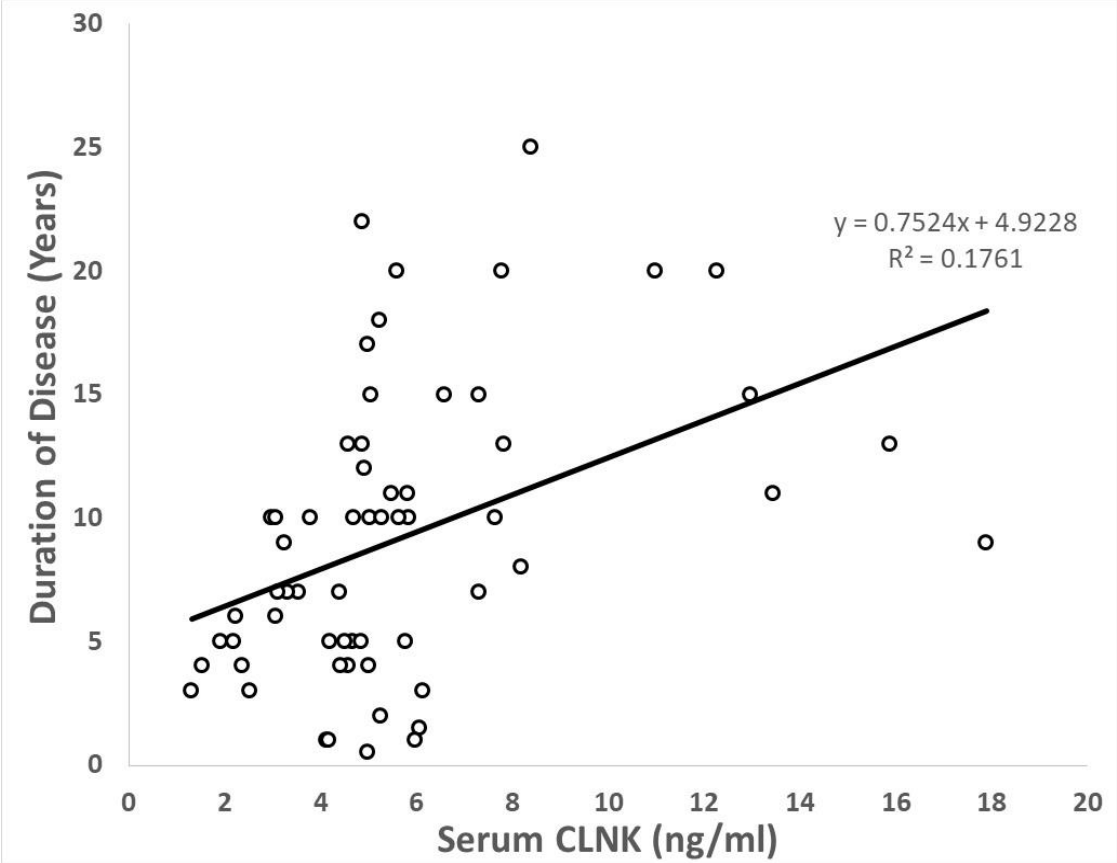

Figure 1. The correlation between CLNK and duration of disease in the T2DM patients' group.

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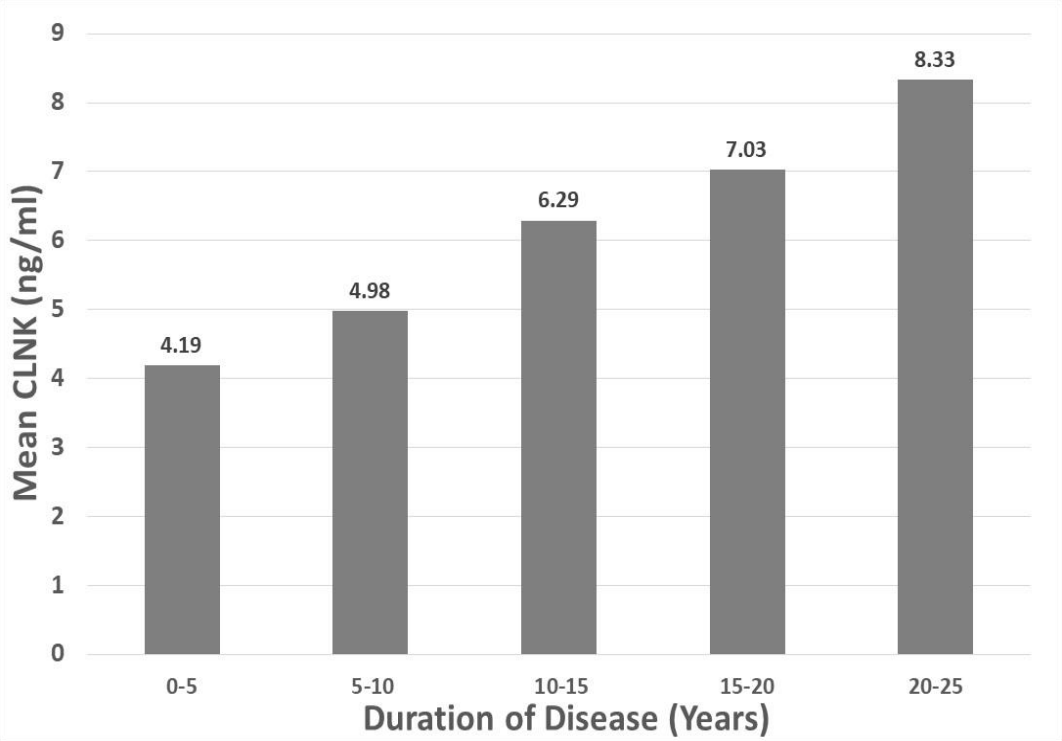

Figure 2. Distribution of mean CLNK according to the periods of the duration of T2DM disease.