Novel Biomarker Serum Calprotectin for Early Diagnosis of Diabetic Peripheral Neuropathy in Type 2 Diabetes Patients

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ABSTRACT

Background: Calprotectin was identified as an endogenous activator of Toll-like receptor 4 (TLR4) and as receptor for advanced glycation end product (RAGE). Elevated plasma levels of calprotectin have been reported in a variety of chronic inflammatory conditions. Elevated calprotectin levels have been reported to predict microvascular alterations in type 2 diabetes (T2DM) patients.

Objective: To make an up to date review of serum level of calprotectin as a predictor for early diagnosis of peripheral neuropathy in type 2 diabetes patients.

Patients and methods: A total number of 90 subjects were included in the study after fulfillment of the inclusion and exclusion criteria. Calprotectin was measured for all the 90 subjects.

Results: Serum calprotectin levels were significantly higher in group III "diabetics with neuropathy" compared to group II "diabetics without neuropathy "and group I "healthy controls".

Conclusion: High levels of calprotectin were detected in type 2 diabetic patients with peripheral neuropathy.

Keywords: Calprotectin, Diabetes mellitus, Neuroinflammation, Peripheral neuropathy.

INTRODUCTION

Diabetes mellitus remains as one of the most challenging healthcare problems in the 21st century, and its prevalence is higher in developing countries. While for year 2030, 170% increase is expected for developing countries, 42% increase expected for developed countries **(1)**. Diabetes mellitus is a chronic endocrine illness, which occurs when the pancreas does not secrete enough insulin, or when the body cannot efficiently use the insulin **(2)**.

The most commonly encountered microvascular complication of type 2 diabetes is diabetic neuropathy with the prevalence of 50-60%. Neuropathy may cause decreased nerve functions and nerve blood perfusion with persistent nerve damage. Diabetic peripheral neuropathy increases development of foot ulceration risk, and increases developmental risk of necrosis, which may cause lower extremity amputations. Diabetic peripheral neuropathy has significant contributions in morbidity and mortality in diabetic patients **(3, 4)**.

Although it is predicted that hyperglycemia is an important pathophysiological factor in development of diabetic neuropathy, the related mechanisms have not been much clarified. Opinions suggesting that inflammatory processes may play a role in pathogenesis of diabetic neuropathy are increasing. In previous studies, it has been shown that peripheral neuropathy was associated with increased levels of proinflammatory immune mediators in patients with type 2 diabetes **(5, 6)**.

The inflammatory Myeloid-related protein complex Calprotectin, also known as MRP8/14, is a heterodimer comprised of two intracellular calcium-binding proteins, S100A8 (MRP8) and S100A9 (MRP14), predominantly expressed in activated human neutrophils, monocytes and macrophages. Calprotectin is actively secreted during the stress response of phagocytes **(7)** and was found to be associated with inflammation more than 20 years ago**(8)**. Calprotectin was identified as an endogenous activator of Toll-like receptor 4 (TLR4) and as receptor for advanced glycation end products (RAGE) **(9)**. Calprotectin is believed to function both as an intracellular differentiation marker for phagocytes and as an extracellular protein complex (a damage-associated molecular pattern (DAMP) molecule) **(10)**. Elevated plasma levels of Calprotectin have been reported in a variety of chronic inflammatory conditions, including rheumatoid arthritis, allograft rejection, inflammatory bowel disease, and cancer lung diseases **(11)**. Elevated Calprotectin levels have been reported to predict microvascular alterations in type 2 diabetic (T2DM) patients **(12)**.

PATIENTS AND METHODS

A total number of 90 subjects were included in the study after fulfillment of the inclusion and exclusion criteria. This study was conducted from March 2019 to December 2019 in Endocrinology Clinic, Zagazig University Hospital and Endocrinology Clinic of Shbeen El Kom Teaching Hospital. This was a case-control study where the patients were classified into

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3 groups. Group I: 15 healthy control subject, group II: 15 diabetic patients without peripheral neuropathy and group III: 60 diabetic patients with early peripheral neuropathy. Calprotectin was measured for all the 90 subjects. Blood samples were collected, centrifuged and stored at -20 °C until Calprotectin levels were measured. C-reactive protein (CRP), Random blood glucose (RBG), HbA1c, High-density lipoprotein-cholesterol (HDL C), low-density lipoprotein cholesterol (LDL C), total cholesterol, triglyceride (TG) liver function tests, kidney function tests, Albumin creatinine ratio in urine and eGFR also were measured.

Inclusion criteria:

Male and female patients aged between 20-75 years with type 2 diabetes with and without peripheral neuropathy were included.

Exclusion criteria:

Patients with infectious diseases, inflammatory diseases, liver failure, malignancies, neuro-degenerative diseases, renal failure, cerebrovascular diseases, B12 vitamin deficiency, medical history of serious trauma to the limbs, use of neurotoxic medication, excessive alcohol consumption and smokers were all excluded from both study and control group.

On admission, all patients in this case-cohort study were subjected to the following: Clinical assessment including detailed history taking, full examination (general and neurological) and laboratory investigations. Serum concentration of calprotectin was expressed in ng/mL. The calibration curve was organized ranging from 1–30 ng/ mL.

Ethical approval:

Institutional Review Board (IRB) of the Faculty of Medicine, Zagazig University approved the study protocol. Informed consents was obtained from all participants or their first-degree relatives and they were informed about the aim of the study, and that the data would be used for scientific purposes only.

Statistical analysis:

All data were collected, tabulated and statistically analyzed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software bvba, Ostend, Belgium). Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) and Fisher exact were used to calculate difference between qualitative variables as indicated. Quantitative data were expressed as mean ± SD (Standard deviation) for parametric and median and range for non-parametric data. Independent T test and Mann Whitney test were used to calculate difference between quantitative variables in two groups for parametric and non-parametric variables respectively. One-way ANOVA test was used to compare between more than two dependent groups of normally distributed variables. Pearson's and Spearman's correlation coefficient were used for correlating normal and non-parametric variables respectively.

RESULTS

Demographic data and laboratory studies of the three studied groups showed that the difference between the three groups regarding age, sex & diastolic BP was non-significant. Difference between the three groups regarding BMI was highly significant. Difference between the three groups regarding systolic BP was significant**.**

Diabetes parameters of the three studied groups showed that there was highly significant difference regarding RBS, HbA1c and DM duration.

Renal parameters of the three studied groups showed that the difference between the three studied groups regarding serum creatinine, urea, ACR and eGFR was non-significant.

Lipid profile data of the three studied groups regarding TC, TG and LDL showed highly significant difference. Difference between the three groups regarding HDL was significant. Difference between the three studied groups regarding CRP was highly significant (Table 1).

Table (1): Demographic data and laboratory studies of the three studied groups

		Control (N=15)	DM (N=15)	DNP (N=60)	F / χ^2	P
Age (years) Mean ± SD		49.2 ± 9.24	51.8 ± 13.61	50.53 ± 10.52	0.206	0.814
Sex	Male	10 (66.7%)	8 (53.3%)	11 (36.7%)	3.804	0.149
	Female	5 (33.3%)	7 (46.7%)	19 (63.3%)		
BMI (kg/m^2) Mean ± SD		24.41 ± 1.35	26.88 ± 1.49	28.11 ± 1.62	21.44	< 0.001**
Systolic blood pressure Mean ± SD		111.33 ±19.13	124.64 ±13.02	123.75 ±15.04	2.165	0,048*
Diastolic blood pressure Mean ± SD		69.67 ± 13.69	74.07 ± 9.13	76.77 ± 6.47	1.241	0,297
RBS (mg/dL) Mean ± SD		132.13 ± 25.82	213.57 ± 27.82	237.04 ± 58.23	24.88	< 0.001**
HbA1c (%) Mean ± SD		5.19 ± 0.533	6.98 ± 0.307	7.49 ± 0.783	32.56	< 0.001**
DM duration (years) Mean ± SD		----	6.3 ± 3.28	10.87 ± 5.31	3.708	< 0.001**
S. Cr (mg/dL) Mean ± SD		0.910 ± 0.116	0.913 ± 0.119	0.967 ± 0.149	1.089	0.344
Urea (mg/dL) Mean ± SD		14.23 ± 3.29	18.17 ± 3.67	19.44 ± 3.39	1.477	0.237
ACR (mg/g) Mean ± SD		26.83 ± 1.49	87.1 ± 9.49	113.87 ± 9.3	2.794	0.073
eGFR (mg/24h) Mean ± SD		92.19 ± 4.42	85.91 ± 10.89	84.22 ± 13.22	2.201	0.120
TC (mg/dL) Mean ± SD		164.53 ± 13.31	185.6 ± 17.31	201.2 ± 32.15	10.561	< 0.001**
TG (mg/dL) Mean ± SD		154.67 ± 17.53	180.93 ± 7.38	192.33 ± 3.48	10.156	< 0.001**
LDL (mg/dL) Mean ± SD		81.73 ± 9.2	92.33 ± 12.07	103.37 ± 21.05	8.589	< 0,001**
HDL (mg/dL) Mean ± SD		51.86 ± 8.23	55.07 ± 9.09	63.54 ± 14.05	5.513	0,007*

****= P < 0.001 Highly significant *= P ≤ 0.05 Significant P > 0.05 Non-significant**

S. Cr: Serum Creatinine
ACR: Albumin/creatinine ratio
eGFR: estimated glomerular filtration rate

TC: Total cholesterol TG: Total triglycerides
LDL: Low-density lipoprotein HDL: High-density lipoprotein

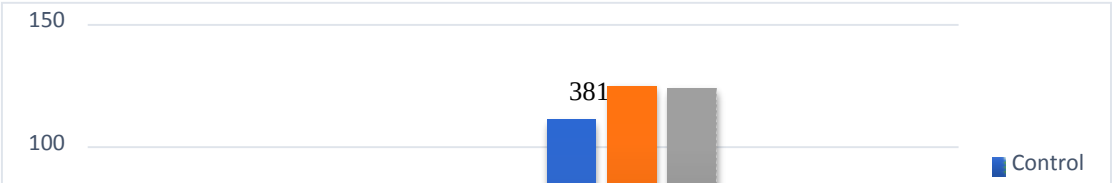

Figure (1): Patients characteristics between three studied groups.

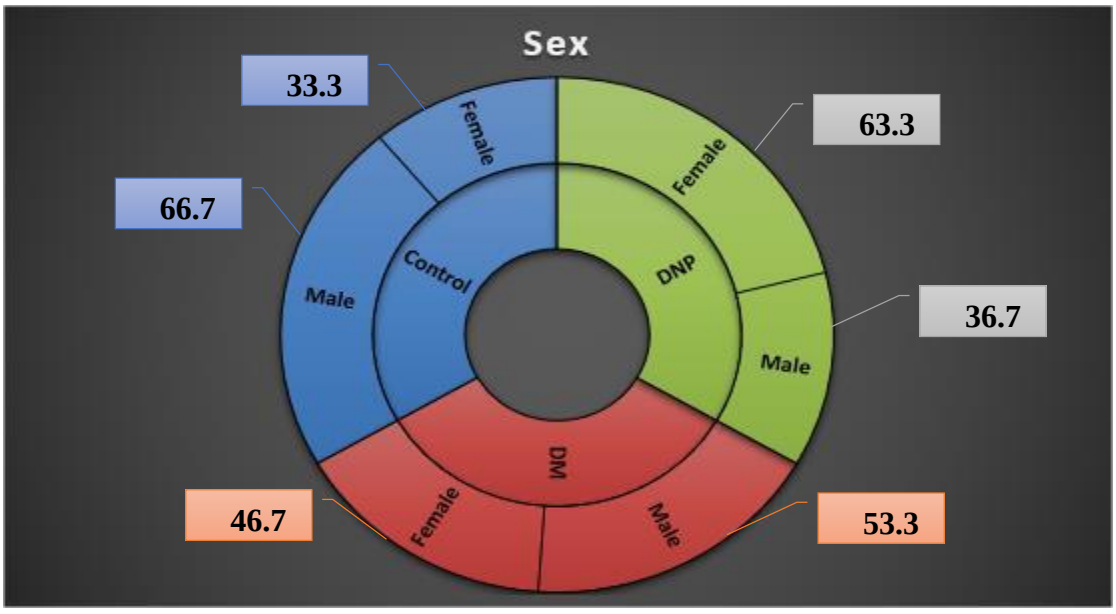

Figure (2): Sex distribution among the three studied groups.

LSD regarding CRP, RBS: between control group and diabetic group with neuropathy was highly significant, and between diabetic group without neuropathy and diabetic group with neuropathy was highly significant**.**

LSD regarding HBA1c: between control group and diabetic group with and without neuropathy was significant, and between diabetic group with and without neuropathy was significant. **LSD regarding TC:** between control group and diabetic group without neuropathy was significant, and between control group and diabetic group with neuropathy was highly significant. **LSD regarding TG:** between control group and diabetic group without neuropathy was significant, and between control group and diabetic group with neuropathy was highly significant. **LSD regarding LDL:** between control group and diabetic group with neuropathy was highly significant, and between diabetic group without neuropathy and diabetic group with neuropathy was significant. **LSD regarding HDL:** between control group and diabetic group with neuropathy was significant, and between diabetic group without neuropathy and diabetic group with neuropathy was significant **(Table 2)**.

Table (2): Post hoc (LSD) test, to indicate the difference in the three studied groups according to different parameters levels of significant difference

Variable	(I) Group	(J) Group	Mean Difference (I-J)	S.E.	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
HB	Control	DM	0.89333	0.63248	0.163	-0.3732	2.1598
		DN	-0.03333	0.54774	0.952	-1.1302	1.0635
	DM	DN	-0.92667	0.54774	0.096	-2.0235	0.1702
CRP	Control	DM	-6.88000	5.57703	0.222	-18.0478	4.2878
		DN	**-33.43333****	4.82985	**< 0.001**	-43.1049	-23.7617
	DM	DN	**-26.55333****	4.82985	**< 0.001**	-36.2249	-16.8817
RBS	Control	DM	**-104.90667****	15.96568	**< 0.001**	-136.8774	-72.9359
		DN	**-81.43333****	13.82669	**< 0.001**	-109.1208	-53.7459
	DM	DN	23.47333	13.82669	0.095	-4.2141	51.1608
HBA1C	Control	DM	**-0.50667***	0.22699	**0.030**	-0.9612	-0.0521
		DN	**-34.11333***	0.19658	**0.007**	-0.5070	0.2803
	DM	DN	0.39333*	0.19658	**0.05**	-0.0003	0.7870
TC	Control	DM	**-21.07333***	9.25810	**0.027**	-39.6124	-2.5343
		DN	**-36.67333****	8.01775	**< 0.001**	-52.7286	-20.6181
	DM	DN	-15.60000	8.01775	0.057	-31.6553	0.4553
TG	Control	DM	**-26.267***	9.656	**0.009**	-45.60	-6.93
		DN	**-37.667****	8.362	**< 0.001**	-54.41	-20.92
	DM	DN	-11.400	8.362	0.178	-28.14	5.34
LDL	Control	DM	-10.600	6.132	0.089	-22.88	1.68
		DN	**-21.633****	5.311	**< 0.001**	-32.27	-11.00
	DM	DN	**-11.033***	5.311	**0.042**	-21.67	-0.40
HDL	Control	DM	-3.210	4.341	0.463	-11.91	5.49
		DN	**-11.679***	3.823	**0.003**	-19.34	-4.01
	DM	DN	**-8.469***	3.737	**0.027**	-15.96	-0.98
Urea	Control	DM	-5.20667	3.22274	0.112	-11.6601	1.2468
		DN	-3.94000	2.79098	0.163	-9.5288	1.6488
	DM	DN	1.26667	2.79098	0.652	-4.3222	6.8555
eGFR	Control	DM	1.69000	4.86880	0.730	-8.0596	11.4396
		DN	-6.27867	4.21651	0.142	-14.7221	2.1647
	DM	DN	-7.96867	4.21651	0.064	-16.4121	0.4747
Creatinin e	Control	DM	-0.05333	0.04598	0.251	-0.1454	0.0387
		DN	0.00300	0.03982	0.940	-0.0767	0.0827
	DM	DN	0.05633	0.03982	0.163	-0.0234	0.1361
ACR	Control	DM	-45.60000	40.56854	0.266	-126.8371	35.6371
		DN	13.76667	35.13338	0.697	-56.5867	84.1201
	DM	DN	59.36667	35.13338	0.097	-10.9867	129.7201

***= P < 0.001 Highly significant *= P ≤ 0.05 Significant P > 0.05 Non-significant**

Difference between the three studied groups regarding calprotectin was highly significant (P value < 0.001) (Table 3).

Table (3): Calprotectin of the three studied groups

	Control (N=15)	**DM** (N=15)	**DNP** (N=60)	**F**	**P**
Calprotectin (ng/ mL) Mean ± SD	8.64 ± 1.91	13.71 ± 3.38	21.57 ± 4.43	**39.419**	**< 0.001****

****= P < 0.001 Highly significant *= P ≤ 0.05 Significant P > 0.05 Non-significant**

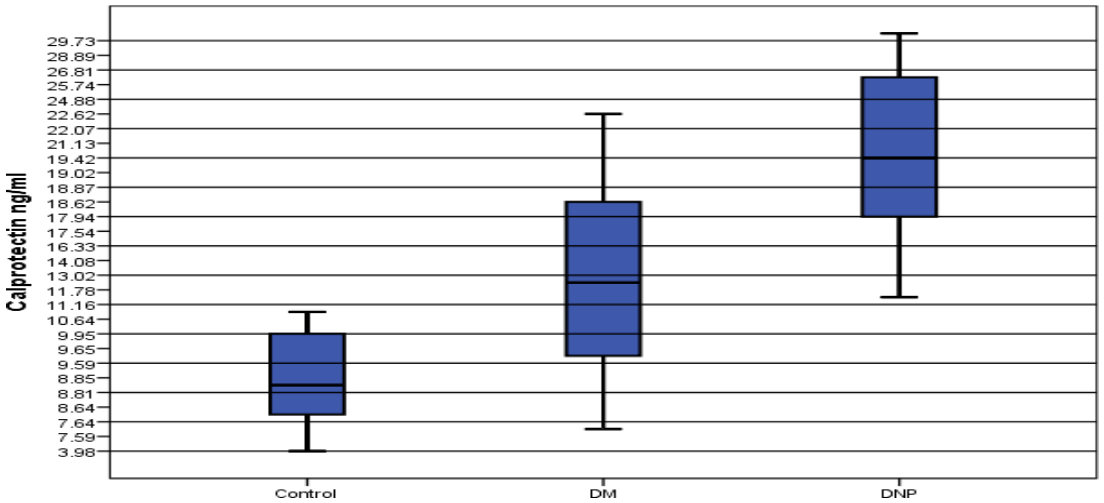

Figure (3): Calprotectin levels between three studied groups.

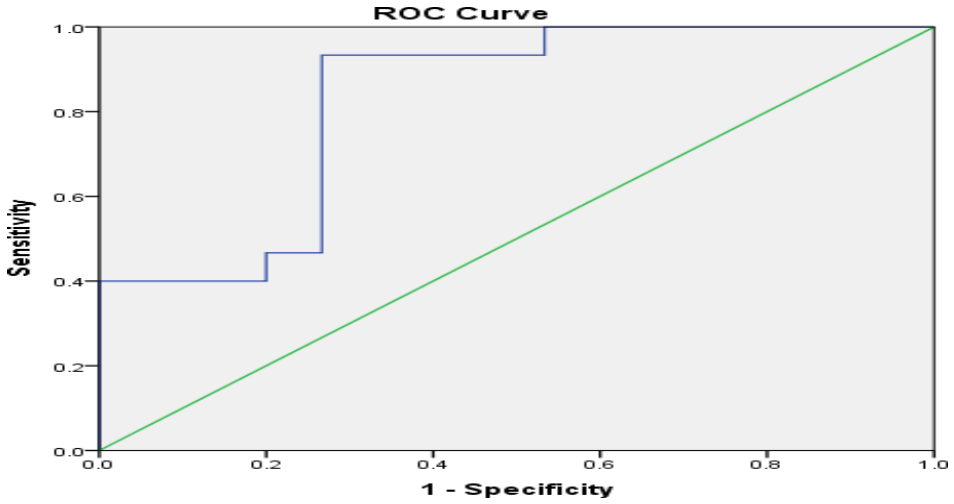

Figure (4): ROC of calprotectin as a marker of neuropathy in type 2 diabetic patients

DISCUSSION

Calprotectin is one of S100 protein. It is composed of two proteins (S100A8 and S100A9). It has high affinity to calcium and has a potent proinflammatory signaling role. It is proposed that calprotectin complex is a biomarker for inflammation, and is beneficial in monitoring disease activity. Calprotectin levels are reported to be high in chronic inflammatory diseases such as inflammatory bowel disease, atherosclerosis, rheumatoid arthritis and allograft rejection. So far, relation between high levels of calprotectin and glucose metabolism has been demonstrated in few studies. The relation between high levels of calprotectin and insulin resistance and low graded inflammation in type 2 diabetic patients has been reported. Therefore, calprotectin can be considered as an inflammatory marker and has a role in pathogenesis of diabetic peripheral neuropathy [13].

We aimed at detecting serum level of calprotectin as a marker for early diagnosis of peripheral neuropathy in type 2 diabetes patients by measuring calprotectin serum level, nerve conduction velocity studies (NCVS), modified neuropathy disability score (MNDS) and monofilament test. To achieve that we compared the absolute levels of calprotectin in between the three groups of patients.

In this study, we found no statistical differences between the three groups in age. That assures that no age related variation can affect the results. The distribution of gender in the studied groups showed no statistical difference, which prevents to get biased data due to difference in hormonal profile, variable risks, or physical activity.

In our study, patients of group I (normal healthy people) showed lower systolic blood pressure than other groups (diabetics), and that means that hypertension in addition to atherosclerosis and insulin resistance may contribute in the pathogenesis of peripheral neuropathy in type 2 diabetes patients. **Sowers *et al.*** [14, 15] reported that more than half of diabetic patients presented with coexisting hypertension and that hypertension is potent risk factor to both micro and macro vascular diseases in diabetic patients.

Our results showed that diabetic group with peripheral neuropathy had calprotectin levels higher

than diabetic group without neuropathy and healthy controls. **Tabur *et al.* [16],** supporting our study, reported also that calprotectin level in patients with diabetic peripheral neuropathy is higher than that in diabetic patients without neuropathy, and higher than that in healthy people.

Our study showed that calprotectin is an inflammatory mediator that increased in inflamed tissues and probably has a role in the neuroinflammatory conditions as it directly proportionate with CRP in patients with DPN. Like our study, positive correlation between calprotectin and CRP was described also by other studies [17, 18].

In our study, we found a positive correlation between calprotectin and HbA1c in patients with DPN. This point suggests that levels of glucose or glycation end products may affect metabolism of high calprotectin levels in diabetics**.** In agreement with our study, **Tabur *et al.* [16]** reported the same positive correlation between calprotectin and HbA1c "the marker of long-term elevation of blood sugar.

In our study, BMI was significantly higher in both diabetic groups (with and without neuropathy) compared to healthy group, while BMI was higher in diabetic neuropathy patients compared to diabetic patients without neuropathy. The high BMI in diabetic with and without neuropathy also is reported by **Avila *et al.* [19]** who showed that BMI was increased in diabetic patients with and without neuropathy than in normal healthy people.

Regarding TG, TC, HDL and LDL levels, our study showed that their levels were significantly high in both diabetic groups with and without neuropathy compared to healthy group. However, they were higher in diabetic neuropathy patients compared to diabetic patients without neuropathy. This may explain atherosclerotic changes as a risk factor of diabetes alone or if also associated with neuropathy. **Peng *et al.* [17]** reported that type 2 diabetic patients with atherosclerotic disease have increased serum level of calprotectin.

CONCLUSION

Diabetic neuropathy is associated with increased serum level of calprotectin**.**

REFERENCES

1. **Manigrasso M, Juranek J, Ramasamy R *et al.* (2014):** Unlocking the biology of RAGE in diabetic microvascular complications. Trends Endocrinol Metab., 25: 15–22.
2. **Fseha B (2017):** Glycemic Control and its Associated Factors in Type 2 Diabetic Patients in Suhul Hospital, Northwest Tigray, Ethiopia. J Diabetes Metab., 8 (3): 1-6.
3. **Vinik A, Nevoret M, Casellini C *et al.* (2013):** Diabetic neuropathy. Endocrinol Metab Clin North Am., 42: 747–87.
4. **Sandireddy R, Yerra V, Areti A *et al.* (2014):** Neuroinflammation and oxidative stress in diabetic neuropathy: Futuristic strategies based on these targets. Int J Endocrinol., 2014: 674987.
5. **Singh R, Kishore L, Kaur N (2014):** Diabetic peripheral neuropathy: currentperspective and future directions. Pharmacol Res., 80: 21–35.
6. **Herder C, Bongaerts B, Rathmann W *et al.* (2013):** Association of subclinical inflammation with polyneuropathy in the older population: KORA F4 study. Diabetes Care, 36: 3663–70.
7. **Hessian P, Edgeworth J, Hogg N (1993):** MRP-8 and MRP-14, two abundant Ca^{2+}- binding proteins of neutrophils and monocytes. J Leukoc Biol., 53: 197–204.
8. **Sorg C (1992):** The calcium binding proteins MRP8 and MRP14 in acute and chronic inflammation. Behring Inst Mitt., 91: 126–137.
9. **Leclerc E, Fritz G, Vetter S *et al.* (2009):** Binding of S100 proteins to RAGE: an update. Biochim Biophys Acta., 1793: 993–1007.
10. **Ehrchen J, Sunderkotter C, Foell D *et al.* (2009):** The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol., 86: 557–566.
11. **Bjarnason I, Sherwood R (2001):** Fecal calprotectin: a significant step in the noninvasive assessment of intestinal inflammation. J Pediatr Gastroenterol Nutr., 33: 11–13
12. **Burkhardt K, Schwarz S, Pan C *et al.* (2009):** Myeloid-related protein 8/14 complex describes microcirculatory alterations in patients with type 2 diabetes and nephropathy. Cardiovasc Diabetol., 8: 10-13.
13. **Ortega F, Sabater M, Moreno-Navarrete J *et al.* (2012):** Serum and urinary concentrations of calprotectin as markers of insulin resistance and type 2 diabetes. Eur J Endocrinol., 167: 569-578.
14. **Sowers JR, Whaley-Connell A, Hayden M (2011):** The role of overweight and obesity in the cardiorenal syndrome. Cardiorena l Med., 1: 5–12.
15. **Sowers J (2013):** Diabetes mellitus and vascular disease. Hypertension, 61 (5): 943–7.
16. **Tabur S, Korkmaz H, Ozkaya M *et al.* (2015):** Is calprotectin a novel biomarker of neuroinflammation in diabetic periferal neuropathy? Diabetol Metab Syndr., 7: 36-43.
17. **Peng W, Jian W, Li H *et al.* (2011):** Increased serum myeloid-related protein 8/14 level is associated with atherosclerosis in type 2 diabetic patients. Cardiovasc Diabetol., 10: 41-45.
18. **Pedersen L, Nybo M, Poulsen M *et al.* (2014):** Plasma calprotectin and its association with cardiovascular disease manifestations, obesity and the metabolic syndrome in type 2 diabetes mellitus patients. BMC Cardiovasc Disord., 14: 196-202.
19. **Avila-Funes J (2004):** Validity of height and weight in Mexican adults: results from the National Health and Aging Study. J Nutr Health Aging, 8: 355–67.