RESEARCH ARTICLE

The lost correlation between leptin and CRP in type 2 diabetes

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To cite this article: Morteza A, Nakhjavani M, Asgarani F, Ghaneei A, Esteghamati A, Mirmiranpour H. The lost correlation between leptin and CRP in type 2 diabetes. *Eur. Cytokine Netw.* 2013; 24(1): 53-9 doi:10.1684/ecn.2013.0329

ABSTRACT. C reactive protein (CRP) is an inflammatory marker believed to be of value in the early prediction of type 2 diabetes (T2DM). Recent studies have shown a positive correlation between leptin and CRP levels. Here, we aimed to study the correlation between leptin and CRP in patients with T2DM. We also studied the effect of metformin therapy on the CRP-leptin correlation in patients with newly diagnosed diabetes. We performed a follow-up study on three groups of participants defined as 1: patients with newly diagnosed T2DM, 2: patients with long-standing T2DM, and 3: healthy controls. Patients with newly diagnosed diabetes were followed for three months after the initiation of metformin therapy. The homeostatic model assessment of insulin resistance (HOMA-IR) decreased, while leptin levels (15.9 ± 1.6 *versus* 21.4 ± 2.5, $p<0.01$) increased after metformin therapy. Leptin levels correlated significantly with CRP in healthy controls ($r = 0.48$; $p<0.01$); patients with newly diagnosed diabetes before ($r = 0.35$; $p<0.05$), and after ($r = 0.55$, $p<0.001$) metformin therapy, while there was no significant correlation between leptin and CRP in patients with long-standing diabetes ($r = 0.15$; $p = 0.55$). After multiple adjustments for potential confounders, leptin was the best predictor of CRP in controls (β coefficient = 0.433, $p<0.01$), and patients with newly diagnosed T2DM who received metformin (β coefficient = 0.584, $p<0.01$). Statin treatment did not have any significant effect on the results. This is the first report demonstrating the restorative role of metformin on the leptin-CRP correlation in patients with newly diagnosed diabetes.

Key words: leptin, C reactive protein (CRP), type 2 diabetes, inflammation

Leptin is a protein hormone secreted by stored fat that was believed to regulate food intake and energy expenditure. Recent studies suggest that leptin increases in acute inflammation and is hence an inflammatory marker [1]. Leptin can stimulate the production of C Reactive Protein (CRP) by cultured human hepatocytes [2, 3]. This suggests that leptin may play a role in the physiological regulation of CRP. The association between leptin and CRP has been demonstrated in healthy individuals [4, 5], and in patients with type 2 diabetes (T2DM) [6, 7].

On the other hand, it has long been known that null mutations in genes encoding leptin and leptin receptor cause hyperphagia, severe obesity and a rise in inflammatory markers such as CRP [8]. A similar situation occurs in T2DM, with decreased leptin and increased CRP levels [9]. So we questioned why there is a positive correlation between leptin and CRP in healthy individuals [10], when they do not respond in a similar manner to chronic inflammation. Healthy subjects have lower serum CRP and higher serum leptin levels compared to patients with T2DM [11-13]. These seemingly contradictory observations imply a more complex situation controlling the cause and effect relationship between serum leptin and CRP levels. Here, we aimed to study the correlation between serum leptin and CRP levels, in two groups of patients with T2DM, defined as patients with newly diagnosed diabetes and patients with long standing diabetes, plus healthy controls. We also wondered if three months metformin therapy affected this correlation.

Abbreviations

T2DM	Type 2 diabetes
CRP	C reactive protein
HOMA-IR	Homeostasis model assessment of insulin resistance
TNF-α	Tumor necrosis factor α
IL-6	Interleukin 6
IL-1	Interleukin 1
BMI	Body mass index
FBS	Fasting blood sugar
HDL-C	High-density lipoprotein-cholesterol
LDL-C	Low-density lipoprotein-cholesterol
SEM	Standard error of mean

DONORS AND METHODS

We studied the established groups of patients with T2DM defined as 1: patients with long-standing diabetes for more than three years; and 2: patients with newly diagnosed diabetes within the previous six months who were not on any glucose-lowering treatment other than dietary means alone and 3: healthy controls. Patients were recruited from

doi: 10.1684/ecn.2013.0329

the diabetes clinic of the Vali-Asr Hospital, affiliated with the Tehran University of Medical Sciences. Controls were healthy volunteers from the patients' associates or from hospital staff.

Healthy controls were selected to include those without any known disease including T2DM, hyperlipidemia, ischemic heart disease, or malignancy. Patients and controls were matched according to age, sex and body mass index (BMI).

On the basis of the results of an exploratory analysis, we designed a follow-up study of patients with newly diagnosed diabetes, before and after three months of metformin treatment. The patients commenced treatment immediately following diagnosis. All of these patients had poor glycemic control, sufficient to merit an oral hypoglycemic agent. Metformin was the first choice of glucose-lowering therapy in these patients. Treatment was started with metformin only, and the patients had a three-month period of therapy with three phases of initiation, titration and maintenance. The average dose of metformin was 1,000-1,500 mg per day. Diabetes was diagnosed according to the criteria of the American Diabetes Association [14]. Exclusion criteria included smoking, pregnancy, creatinine >1.5 mg/dL or GFR $<$ 70 cc/min, glomerulonephritis, congestive heart failure, use of antioxidants and hospital admission in the previous six months. None of the participants had overt diabetes complications. None of the participants were on hormone replacement therapy. Demographic and anthropometric data including age, sex, duration of diabetes, height, weight in light clothing, and blood pressure in a sitting position were recorded.

Blood pressure was re-measured twice after five minutes, and averaged. The BMI (Kg/m^2) was calculated according to the Quetelet formula. The research was carried out according to the principles of the Declaration of Helsinki. All the patients received and signed a written informed consent. The local ethics review committee of the Tehran University of Medical Science approved the study protocol. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to; fasting insulin ($\mu U/mL$) $\times$ fasting blood sugar (FBS) (mg/dL)/405, as described by Matthews *et al*. [15].

Blood samples

Blood samples were collected after 12 hours of fasting. They were centrifuged and kept at -70 °C until analysis. Serum creatinine, FBS, total cholesterol, triglycerides, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and HbA1c were measured for all participants. Glucose measurements (intra-assay coefficient of variants [CV] 2.1%, inter-assay CV 2.6%) were carried out using the glucose oxidase method. Cholesterol, HDL-C, LDL-C and triglycerides were determined using direct enzymatic methods (Parsazmun, Karaj, Iran). Serum leptin concentration was determined using an enzyme-linked immunosorbent assay (DRG Instruments GmbH, Germany), with an intra-assay coefficient of variation of 5.9-6.9% and an inter-assay coefficient of variation of 8.6-11.5%. Hs-CRP was assessed using a two-site, enzyme-linked immunosorbent assay (ELISA) (Diagnostic Biochem, London, Ontario, Canada). Sensitivity of the assay was 10 ng/L. Intra- and inter-assay coefficients of variation (CV) were 8% and 10% respectively.

Statistical analysis

The statistical package SPSS 17 for windows (Chicago, Illinois, USA), was used for the analysis. Variables distributed normally are presented as mean and standard error of mean (SEM). The independent sample t test was employed to compare variables between groups. The paired sample t test was used to compare variables, before and after treatment. The independent sample t test was employed to compare men and women. Pearson's correlation test was employed to study the correlation between leptin and CRP. As statin effects are pleomorphic and include an LDL- and CRP-lowering effect, we hence repeated the analysis, stratifying the patients according to the presence or absence of statin therapy. Multiple linear regression analyses were undertaken to determine contributors to serum CRP concentration. Potential predictor variables included HbA1c, leptin, HOMA-IR, cholesterol, LDL, HDL and FBS.

RESULTS

The main characteristics of the groups of patients and controls are presented in *table 1*. The drop- out rate due to side effects following commencement of metformin therapy was (0%) in patients with newly diagnosed diabetes. The frequency of insulin therapy was (9/44; 20%) in patients with long-standing diabetes. Forty six per cent (21/44) of the patients with long-standing diabetes were on alternative night 10 mg atorvastatin therapy. None of the patients with newly diagnosed diabetes were on any other treatment apart from metformin.

Patients with T2DM had a higher serum CRP level, larger waist circumference, FBS, HbA1C, triglyceride, HOMA-IR, and a lower serum leptin level than controls (*table 1*). HOMA-IR (3.26 ± 0.23 *versus* 2.93 ± 0.32; $p<0.01$) decreased when leptin levels (15.9 ± 1.6 *versus* 21.4 ± 2.5, $p<0.01$) increased after three months of metformin therapy (*table 2*). Leptin correlated significantly with CRP in healthy controls ($r = 0.48$; $p<0.01$), and in patients with newly diagnosed diabetes before ($r = 0.35$; $p<0.05$) and after metformin therapy ($r = 0.55$, $p<0.001$), while there was no significant correlation between serum leptin and CRP levels in patients with long-standing diabetes ($r = 0.15$; $p = 0.55$).

We repeated the analysis after stratifying the patients in the long-standing diabetes group, according to statin and insulin therapy. There were no significant correlations between leptin and CRP in patients with ($r = 0.064$, $p = 0.822$) or without statin treatment ($r = 0.145$; $p = 0.565$). When stratifying the patients according to insulin therapy, the correlation coefficient was significantly increased in patients who were not on insulin therapy ($r = 0.39$; $p = 0.057$), compared to those who were on insulin treatment ($r = -0.12$, $p = 0.75$).

Predictors of serum CRP levels

Multiple linear regression modeling was employed to study the correlation between leptin and CRP after multiple adjustments for HbA1c, FBS, HDL, LDL, HOMA-IR and cholesterol (*table 3*). Leptin was the best predictor of serum CRP in controls (β coefficient = 0.433, $p<0.01$), and patients with newly diagnosed diabetes after

Table 1
Comparing demographic and biochemical variables among patients with long-standing diabetes, patients with newly diagnosed diabetes and healthy controls.

	Healthy controls (n = 41)	**Patients with type 2 diabetes**		P value
		Newly diagnosed diabetes (N = 44)	**Diabetes for more than three years (n = 44)**	
Age (years)	52.73 ± 1.72	52.09 ± 1.77	55.18 ± 1.45	NS
BMI (kg/m^2)	26.8 ± 0.50	27.84 ± 0.72	27.46 ± 0.58	NS
Waist circumference (cm)	99.00 ± 1.23	97.28 ± 1.58	91.20 ± 1.49	NS
Diastolic blood pressure (mmHg)	78.44 ± 1.16	87.51 ± 2.39	78.66 ± 1.48	NS
Systolic blood pressure (mmHg)	125.22 ± 1.55	127.94 ± 3.54	125.77 ± 2.03	NS
FBS (mg/dL)	85.92 ± 1.26	181.90 ± 8.48	187.27 ± 10.143	<0.001
HbA1c (%)	4.88 ± 0.06***	8.37 ± 0.36	7.88 ± 0.28	<0.001
HOMA-IR	1.40 ± 0.13***	3.26 ± 0.23	4.42 ± 0.58*	<0.001
Creatinine (mg/dL)	0.93 ± 0.024	0.89 ± 0.033	0.89 ± 0.031	NS
Triglyceride (mg/dL)	135.00 ± 9.85**	201.97 ± 14.86	185.11 ± 15.50*	<0.001
Cholesterol (mg/dL)	183.14 ± 5.0**	206.4 ± 6.3	215.84 ± 5.56	<0.001
LDL (mg/dL)	92.78 ± 2.73**	123.96 ± 6.03	113.97 ± 2.91	<0.01
HDL (mg/dL)	43.24 ± 2.22	44.71 ± 1.85	43.54 ± 1.054	NS
CRP (mg/mL)	1.84 ± 0.21**	3.61 ± 0.35	3.41 ± 0.27	<0.01
Leptin (ng/mL)	23.6 ± 2.9*	15.9 ± 1.6	14.5 ± 1.4	<0.05

Normally distributed variables are expressed as mean ± standard error of mean (SEM). Variables with skewed distribution are presented as median [interquantile range]. *: P<0.05, **: P<0.01, ***: P<0.001 when comparing patients with long-standing diabetes or controls *versus* patients with newly diagnosed diabetes. The column of "P value" is presented when comparing patients with long-standing diabetes *versus* controls.

Table 2
Characteristics of patients with newly diagnosed diabetes before, and after three months of metformin therapy.

	Before metformin treatment	**After metformin treatment**	**P valve**
BMI (kg/m^2)	27.84 ± 0.72	27.6 ± 0.64	NS
FBS (mg/dL)	188.90 ± 8.48	144.76 ± 6.25	<0.001
HbA1c (%)	8.37 ± 0.36	7.36 ± 0.59	<0.001
Creatinine (mg/dL)	0.80 ± 0.033	0.84 ± 0.03	NS
Cholesterol (mg/dL)	206.77 ± 6.161	182.97 ± 5.57	<0.01
HDL-C (mg/dL)	44.71 ± 1.85	46.06 ± 1.92	NS
LDL-C (mg/dl)	123.96 ± 6.03	102.171 ± 4.19	<0.01
Triglyceride (mg/dL)	201.97 ± 14.86	189.55 ± 12.86	NS
Leptin (ng/mL)	15.9 ± 1.6	21.4 ± 2.5	<0.01
HOMA-IR	3.2 ± 0.23	2.9 ± 0.32	<0.01
CRP (mg/mL)	3.61 ± 0.35	3.62 ± 0.36	NS

Variables are presented as mean ± standard error of mean (SEM). The "P value" column is presented when comparing the variables studied between patients with newly diagnosed diabetes, before, and after three months of metformin therapy.

treatment (β coefficient = 0.584, p<0.01) (*table 3*). Before starting treatment, no correlation was observed between leptin and CRP in patients with long-standing diabetes and patients with newly diagnosed diabetes. We then studied serum CRP levels in different quartiles of leptin (*figure 1*). Quartiles are a choice for presenting the quantitative data, especially while the variable has a skewed distribution, such as leptin and CRP (Shamsuzzaman *et al.* 2004). In controls and patients with newly diagnosed diabetes after treatment, CRP was higher in upper leptin quartiles, and lower in lower leptin quartiles. This did not happen in patients with long-standing diabetes or patients with newly diagnosed diabetes before treatment (*figure 1*).

DISCUSSION

Our findings clearly demonstrated that while CRP correlated significantly with leptin in controls; there was no such correlation in patients with long-standing diabetes. Serum leptin and CRP levels correlated in patients with newly

Table 3
Linear regression modeling demonstrating the predictors of serum CRP levels in the groups studied.

		Control	Patient with newly diagnosed diabetes before treatment	Patient with newly diagnosed diabetes after treatment	Patients with long-standing diabetes
Model R		0.69	0.60	0.66	0.48
Independent variables	FBS (mg/dL)	0.11	-0.04	-0.14	0.19
	Cholesterol (mg/dL)	0.51	0.75*	-0.25	-0.08
	Leptin (ng/mL)	0.43**	0.29	0.58**	0.11
	HOMA-IR	0.43**	0.17	0.05	0.09
	HDL (mg/dL)	-0.09	-0.17	-0.02	-0.01
	LDL (mg/dL)	-0.28	-0.84*	-0.15	0.40
	HbA1c (%)	0.08	0.10	0.16	0.13

*: P<0.05, **: P<0.01, ***: P<0.001, CRP as the dependent variable

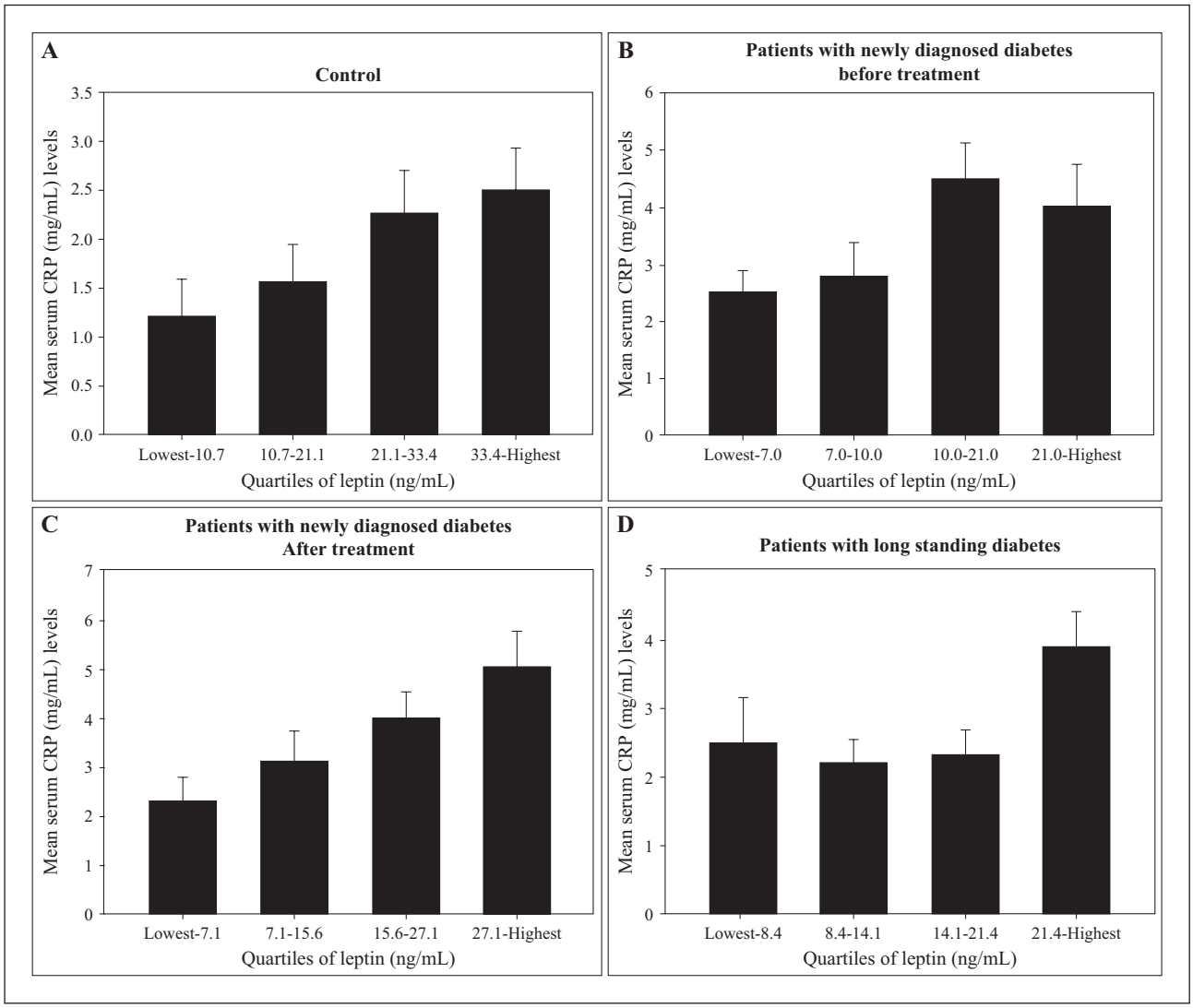

Figure 1
CRP levels in quartiles of leptin A controls, B patients with newly diagnosed diabetes before treatment, C patients with newly diagnosed diabetes after treatment D patients with long-standing diabetes.

diagnosed diabetes, both before and after metformin therapy; however, while leptin and CRP correlation weakly in newly diagnosed patients, this correlation increased significantly after treatment. Multiple linear regression analysis demonstrated that leptin is the best predictor of CRP, only in controls and patients with newly diagnosed diabetes. This is the first report demonstrating the restorative role of metformin on the leptin-CRP correlation.

The mechanisms linking leptin and CRP are not clear. It is suggested that adipocytes, by serving as a common source for leptin and inflammatory cytokines such as IL-6, IL-1 and TNFα, which contribute to the hepatic synthesis of

CRP, link the correlation between leptin and CRP levels [16-18]. However, while we observe higher serum leptin and CRP levels in women with T2DM, there are no gender differences as regards serum IL-6, IL-1 and TNFα levels [19]. Moreover serum CRP, IL-6, IL-1 and TNFα are increased in diabetes while serum leptin levels are decreased [12, 13].

Leptin is the key molecule regulating energy intake and expenditure, including appetite and metabolism [20, 21]. Serum leptin's entry into the central nervous system is proportional to its plasma concentration [21]. Body fat, under normal conditions, is responsible for the constant production of leptin [21]. Insulin linearly stimulates leptin secretion from incubated adipocytes [22].

Starvation decreases both plasma insulin and leptin levels while obesity is strongly associated with hyperinsulinemia and hyperleptinemia [22]. Consistently, serum leptin levels are lower in patients with type 1 diabetes and will increase after intensive insulin therapy [23, 24]. In line with our previous studies, we showed lower serum leptin levels in patients with T2DM compared to BMI-matched, healthy controls [9]. This explains the carbohydrate craving and increased appetite of patients with uncontrolled diabetes. Consistently, leptin increased after metformin therapy. The effects of metformin could be related to AMP-activated protein kinase (AMPK) activation [25], which consequently enhances insulin action. This results in a rise in serum leptin levels in patients with T2DM.

Leptin administration, in addition to enhanced leptin expression in rodents and its reinstatement in the hypothalamus of leptin-deficient ob/ob mice, confers a broad range of health benefits [26]. These benefits include maintenance of reduced adiposity, abolition of dyslipidemia, normalization of the pancreatic-glucose axis, and suppression of TNF-α and insulin growth factor-1 [26-30]. Our findings show that suppression of insulin resistance and hence inflammation, as is shown by decreased HOMA-IR and improved glycemic index, increases serum leptin levels in T2DM. So, while leptin has all these beneficial effects, why does it correlate positively with CRP in healthy individuals, and why does this correlation fade out in patients with long-standing diabetes? Moreover, despite the increase in serum leptin levels, the leptin-CRP correlation is restored after treatment in patients with newly diagnosed diabetes. The study controlled for lipids, but not for statin therapy. When we stratified the patients with long-standing diabetes according to insulin therapy, the leptin-CRP correlation was significantly increased in patients who were not on insulin therapy.

The beneficial effect of leptin is neutralized by serum CRP levels [2]. This has been suggested by the paradoxical observation that the majority of obese individuals have elevated rather than depressed levels of leptin [31]. Increased serum leptin levels do not provide beneficial effects in these patients, which is possibly due to high serum CRP levels [2]. CRP blocks the effects of exogenous leptin on food intake and body weight in leptin-deficient ob/ob mice [2]. Furthermore, since CRP can avidly bind leptin in the blood: restriction of leptin entry across the blood brain barrier by the CRP-leptin complex may engender leptin insufficiency in the hypothalamus, which, in turn, promotes fat accrual [2]. Since CRP is increased in obesity, diabetes and cardiovascular disorders [32], by interacting with leptin and interfering with leptin-mediated signaling, it is, in turn, involved in the regulation of adiposity by stimulating appetite.

Hence, in normal physiology, we observed a positive correlation between leptin-CRP levels. In physiological concentrations, leptin induces CRP secretion from hepatocytes [10]. On the other hand, in pathological concentrations, when both CRP and leptin are influenced by different pathways, the correlation reverses. Selective, increased leptin expression in the hypothalamus, suppresses inflammatory markers such as CRP and IL-6 in leptin-deficient diabetic obese mice [33-35]. It is also reasonable to propose that the high blood CRP levels found in ob/ob mice results from a lack of leptin-induced restraint from hypothalamus on hepatocyte CRP efflux, as has been shown by many studies [26, 29, 33, 36]. T2DM is an inflammatory condition with a cascade of reactive oxygen species. CRP, like other
inflammatory markers, is induced by different pathways [37]. So the positive correlation that is observed between leptin and CRP fades out in T2DM and pre-diabetes situations. This also explains the restoring role of metformin on the CRP-leptin correlation in patients with newly diagnosed diabetes. Metformin has anti-inflammatory and anti-oxidative properties, which improve serum leptin levels and hence any negative feedback between leptin and CRP [38]. Together, these observations imply a more complex role for inflammatory markers such as CRP and leptin in body weight regulation and fat accrual. Future prospective studies may elucidate other biochemical pathways linking leptin and CRP.

The principal limitation of the present study is its short-term follow-up. Furthermore, we did not have placebo group, as it is considered unethical not to start patients on treatment immediately following a diagnosis of diabetes. Moreover, we did not control for the use of statins in patients with long-standing diabetes. Hhowever our patients were indeed sensitive to the side effects of statins [39]. Hence, statin therapy was not started concomitantly with metformin in patients with newly diagnosed diabetes. None of the patients in the "newly diagnosed diabetes" or "control" groups were on statin therapy (or any other drug) before entering the study. Forty six percent of the patients with long-standing diabetes were on 10 mg alternate night atorvastatin treatment. As is also presented in the results, patient LDL levels are not within the recommended range of $\leq$100 mg/dL. However, we took advantage of a relatively large sample size and close similarity between groups for most of the potentially confounding variables. In conclusion, we showed the value of leptin in the prediction of CRP levels in patients with T2DM. This is the first report demonstrating the restorative role of metformin on the leptin-CRP correlation in patients with newly diagnosed diabetes. These observations pave the way for future experimental studies.

AUTHOR CONTRIBUTION

MN designed the study and assembled the patients. AM designed the study, performed the statistical analysis, and

wrote the primary draft of the manuscript. FA approved the ethics and assembled the patients. AE, AG, HM assembled the patients and prepared the study. All the authors read the final draft and contributed to the discussion.

Disclosure. *Financial support:* none. *Conflict of interest:* none.

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