Title Page

Title: The immune-opioid axis in prediabetes: prediction of prediabetes with insulin resistance by plasma interleukin-10 and Endomorphin-2 to kappa-opioid receptors ratio.

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The Novel Findings: There is an upregulation in opioid peptides and their receptors in prediabetes depending on the significance of IR. These changes in opioid system are dependent on the immune cytokines. Both systems need to be normalized as a mean to prevent further development into diabetes mellitus.

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All procedures performed in a study involving human participants were in accordance with the ethical standards of the relevant national and institutional committees on human experimentation

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Title: Immune–opioid axis in prediabetes: prediction of prediabetes with insulin resistance by plasma interleukin-10 and endomorphin-2/kappa-opioid receptor ratio

Abstract

Background: Prediabetes is characterized by a hemoglobin A1c of 5.7%–6.4% and fasting blood glucose of 100–125 mg/dl. A high percentage of prediabetes subjects develops into type 2 diabetes mellitus in the following years. The effect of opioid peptides and their receptors, in addition to immunological cytokines on prediabetes, is not well understood.

Objective: We hypothesize that opioid peptides and their receptors affect the insulin and the insulin resistance (IR) in patients with prediabetes and that the immune cytokines, IL-6 (inflammatory factor) and IL-10 (anti-inflammatory factor), influence the opioid system.

Methods: A total of 60 patients with prediabetes and IR (prediabetes+IR), 60 patients with prediabetes without IR (prediabetes-IR), and 60 controls participated in the study. The IR state was HOMAIR > 2.5. The enzyme linked immunosorbent assay was used to measure interleukin (IL)-6, IL-10, μ - and κ -opioid receptors (MOR and KOR), endomorphin-2 (EM2), and β -endorphin (β EP).

Results: The subjects with prediabetes had dyslipidemia, and not all of them underwent the IR state. The IL-6, IL-10, β -endorphin, MOR, and endomorphin-2 were higher in the prediabetes subgroups compared with the control group. MOR was correlated with IL-10 and KOR. Prediabetes+IR can be predicted by the increased levels of the combination of IL-10, β EP, and EM2 and by the combination of IL-10 and endomorphin-2/KOR with good sensitivity and specificity.

Conclusion: Opioid peptides and their receptors were upregulated in patients with prediabetes depending on the significance of IR. These changes in the opioid system depend on the immune

cytokines. Both systems need to be normalized to prevent further development into diabetes mellitus.

Keywords: prediabetes, insulin resistance, interleukin, endorphin, endogenous opioid receptor

Abbreviations: fasting blood glucose (FBG), insulin resistance (IR), Enzyme linked immunosorbent assay (ELISA), interleukin (IL), μ - and κ -opioid receptors (MOR and KOR), endomorphin-2 (EM2) and β -endorphin (β EP).

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Introduction

Impaired fasting blood glucose (FBG) or glucose tolerance occurs years before developing into a clear type 2 diabetes mellitus (T2DM), and this state is known as a prediabetes, which is a considerable risk factor for developing diabetes [1]. More than one-third of American adults above the age of 20 years develop prediabetes, a condition in which the blood glucose or the hemoglobin A1c (HbA1c) and FBG levels are higher than normal but not sufficiently elevated to be grouped as diabetes [2,3]. In the most recent US guidelines, prediabetes is defined as HbA_{1c} of 5.7%–6.4% and FBG of 100–125 mg/dl [4]. Approximately 87 million American adults have prediabetes, and approximately 30%–50% develop T2DM [5]. One of the dangerous factors of prediabetes is insulin resistance (IR), which refers to the reduced sensitivity or reactivity of tissues to insulin-mediated biologic activity [6]. IR leads to high blood sugar

levels, prediabetes, and T2DM. IR, impaired insulin action, and insulin hypersecretion are central to the pathophysiology of prediabetes [7]. However, not all patients with prediabetes experience a sufficient increase in IR to be classified as an insulin-resistant patient. The clear condition of IR is when the HOMAIR value is more than the cutoff value (> 2.5) [8,9]. As the blood glucose increases, it joins the hemoglobin and elevates the level of HbA1c. This glycated hemoglobin is associated with the roles of IR [10].

Many evidence associates insulin with neuron functions and structure. Insulin signaling is important for neuronal survival, learning, and memorization. In animal models, impaired insulin signaling leads to a collection or gathering of A β oligomers [11,12]. Upon formation, A β oligomers exhaust insulin receptors from the surface membrane of neurons, thereby contributing to IR [13]. and generating an abnormal phosphorylation of insulin receptor substrate (IRS). This condition reduces the normal prosurvival signaling in neurons and promotes their death by apoptosis [14]. Notably, most of these studies focus on brain IR rather than systemic IR. These structural network abnormalities are related to the delay of the information processing speed [15]. IR occurring during midlife may elevate the danger of cognitive decline later in life. Elevated IR is associated with increased risk of cognitive decline, including impaired verbal fluency and slow simple reaction time [16]. Therefore, the relationship between IR roles and the molecules that have a function in the brain duties in some insulin-related disorders, including prediabetes, remains to be elucidated. These groups of molecules include endogenous peptides (e.g., β -endorphin and endomorphin-2) and their receptors, μ - (MOR) and κ -opioid (KOR) receptors. Another significant subject is the immune system molecules especially interleukin (IL)-6 and IL-10. In addition to yielding analgesia, glucose homeostasis is regulated by opioids through changing the insulin secretion [17]. The demonstration of β -endorphins in the human

endocrine pancreas [18], and the possibility of causing insulin and glucagon liberation in animals and humans [19], suggest that opioids may affect glycoregulation [17]. Opioid receptors are expressed in the pancreas and on the pancreatic alpha and beta cells [20], thereby affecting endogenous opioid-mediated glucose and insulin homeostasis [21, 22]. An interaction occurs between the MOR and the IRS 1 in muscle tissues and cells [23]. Moreover, disorders related to the IR line polycystic ovary syndrome are accompanied with increased plasma immunoreactive β -endorphins [24]. On the basis of the linear regression analysis, the strongest proteins related to beta-cell function/HOMA-IR is the beta-endorphin [25]. The beta-endorphin is an influential variable in the ROC curve analysis for discrimination between high and low beta-cell functions and has a strong relationship with the beta-cell function, as observed in regression analysis [25]. Therefore, endogenous opiates are partially responsible for hyperinsulinemia and IR in prediabetes. The analysis of the isolated islets indicates that islet insulin hypersecretion is mediated directly by MOR, which is expressed on islet cells via a mechanism downstream of the ATP-sensitive K channel activation by glucose. Therefore, MOR manages the body weight by insulin release [26]. Opioids influence the beta cell function, as observed in individuals using heroin (MOR agonist) with elevated blood glucose levels [27]. Increased glucose utilization and decreased hepatic gluconeogenesis following the enhancement of peripheral MOR and the variation of genes in glucose metabolism [28] are among the proposed mechanisms. The enhancement of the chronic activation of opioid receptors stimulates hyperglycemia [29]. Puerarin enhances MOR expression and phosphorylation and elevates insulin-stimulated the glucose transporter 4 (GLUT4) translocation to the plasma membrane in the skeletal muscle of diabetic rats [23]. This study first supports the interaction between the MOR and the IRS 1 in the muscle tissues and cells [23]. The mechanism of enhancing IR may be associated with rising

cell-surface level of GLUT4 by decreased FBG and fasting serum insulin levels and increased GLUT4 trafficking and intracellular insulin signaling [30]. The EM-2-induced inhibition of the gastrointestinal transit in diabetic mice is significantly weakened compared with that in nondiabetic mice. The inhibitory effects of EM-2 are mediated by MOR, which exerts a reduced role in diabetes. Moreover, poor blood glucose control may result in the attenuated effects of EM-2 [31].

IL-6 was studied because the impairment of the insulin-degrading enzyme (IDE) is associated with obesity and T2DM. IL-6 and IDE concentrations are significantly increased in the plasma of humans after an acute exercise compared with the pre-exercise values. Positive correlations between IL-6 and IDE activity and between IL-6 and IDE protein expression are observed. Our outcomes denote the novel role of IL-6 on insulin metabolism, suggesting new potential therapeutic strategies and focusing on insulin degradation, for the treatment and/or prevention of diseases linked to hyperinsulinemia, such as obesity and T2DM [32].

Interleukin-6 (IL-6) is a proinflammatory cytokine that conclusively motivates the progression of IR and the pathogenesis of T2DM through the generation of inflammation by controlling differentiation, migration, proliferation, and cell apoptosis [33]. The presence of IL-6 in tissues is a normal consequence, but its irregular generation and long-term exposure leads to the development of inflammation, which stimulates IR and overt T2DM [33]. A mechanistic association occurs between the promotion of IL-6 and IR. IL-6 is the purpose of IR by impairing the phosphorylation of insulin receptor and IRS-1 through the stimulation of the expression of SOCS-3, a potential inhibitor of insulin signaling [33]. IL-10 is a cytokine with anti-inflammatory characteristics that can modulate inflammatory responses by repressing the

generation of proinflammatory cytokines [34]. Serum IL-10 has a converse association with hyperinsulinemia and IR as IL-10 decreases with an increase in HOMA-IR [35].

This study was performed because the direct link between opioid and opioid receptors in patients with prediabetes is still weak, and molecular, physiological, and clinical studies are needed to determine the role of the opioid system in the etiology and pathogenesis of prediabetes.

This clinical study was designed to add clinical information on the diagnosis, prevention, and identification of the risk factors related to the initial occurrence of prediabetes to improve the quality of life by enhancing the health care and education of individuals. The results of the current study may make a difference in the care of future individuals. The association of these factors with prediabetes remains to be elucidated. We hypothesized that opioid peptides and their receptors affect the insulin and IR functions of patients with prediabetes. Moreover, we explored the effects of immune cytokines IL-6 (inflammatory factor) and IL-10 (anti-inflammatory factor) on the opioid system and IR parameters in patients with prediabetes.

The MOR is hypothesized to have an important role in IR. Several reports have investigated the role of MOR in insulin sensitivity and IR, but the results are somehow controversial. Thus, this study was conducted. This research is essential for the pharmacological intervention for the potential targeting of MOR for IR therapy.

Materials and Methods

Subjects

Patients: More than 500 subjects, who check FPG routinely in the laboratories, were examined to select our study group with restricted criteria. A total of 120 subjects with prediabetes were chosen to participate in the study. These subjects were diagnosed by a physician in accordance

with the American Diabetes Association's criteria (FPG = 5.55–6.94 mM, HbA1c = 5.7%–6.4%). The samples were collected from private clinics and laboratories and the Rizgari Teaching Hospital in Erbil City, Kurdistan Region, Iraq from October 2019 to December 2019. The patients fasted for 8–12 h. All procedures were conducted in accordance with the established ethical standards. All study subjects provided written informed consent prior to participation in the study. The study was carried out in accordance with the international and Iraq ethics and privacy laws and approved by the Ethics Committee of Medical Research at College of Pharmacy/Hawler Medical University. The study was performed in accordance with the ethical standards in the Helsinki Declaration of 1975, as revised in 2008, for experiments involving humans.

The subjects with prediabetes were further divided into two subgroups in accordance with the results of HOMA2IR. The first group, prediabetes+IR, comprised subjects with high IR state ($\text{HOMA2IR} > 2.5$). The second group, prediabetes–IR, comprised subjects with low IR state ($\text{HOMA2IR} < 2.5$). This classification occurred deliberately in the same number of subjects in the two groups to remove the possible bias of the number of cases. A total of 55 healthy adult subjects were selected as the control group. Age ranges and sex ratios were comparable to that of the prediabetes groups. None of these subjects manifested an evident systemic disease or took drugs. Furthermore, in all subjects, the C-reactive protein (CRP) was lower than 6 mg/l, which excluded overt inflammation. Tobacco use disorder was examined in accordance with the DSM-IV-TR criteria. Body mass index (BMI) was calculated using the formula: body weight (kg)/squared length (m^2). Subjects performing more than 30 min of moderate activity for 2–3 times/week and never or less than one time per week were considered to do physical activity [4].

Exclusion criteria: The present study excluded patients who satisfied the following criteria: patients with serum TG ≥ 5.32 mM based on the Friedewald's formula, FPG > 25 mM, and fasting insulin > 400 pM based on the HOMA calculator software requirements. Patients with evident major diabetic complications, such as heart diseases, and those receiving lipid-lowering medications (e.g., simvastatin or atorvastatin) and metformin were also excluded. Subject with abnormal blood pressure were also excluded. No subject had a urinary albumin/creatinine ratio of more than 30 mg/g to exclude microalbuminuria, which indicated microvessel damage.

Measurements:

All blood samples were collected in the morning after 12 h of fasting. After collection and separation, the serum samples were separated into three aliquots and stored in a refrigerator until use. The serum glucose, total cholesterol, and TG were determined spectrophotometrically by observing the enzymatic reactions by using commercially available kits (Spinreact[®], Spain). Serum HDLc was determined after the other lipoproteins were precipitated using a reagent containing sodium phosphotungstate and magnesium chloride. The cholesterol contents in the supernatant were determined using a cholesterol kit. VLDLc was calculated on the basis of TG/2.19 and LDLc by using Friedewald's equation ($LDLc = Tc - HDLc - VLDLc$). The HbA1c percentage in the whole blood and the urinary albumin/creatinine ratio were determined using the immunofluorescence analyzer *Finecare*[™] II *FIA* Meter (Guangzhou Wondfo Biotech Co., Ltd, China). The normal range of the HbA1c kit was 4%–6% and that of microalbumin ratio was ≤ 30 mg/g. The IR parameters were calculated on the basis of fasting glucose and insulin concentrations by using the HOMA calculator software (<http://www.dtu.ox.ac.uk/homa-calculator/download.php>). This software was used to generate the IR (HOMA2IR), insulin sensitivity (HOMA%S), and beta-cell function (HOMA%B) indices. An ideal normal-weight

individual aged < 35 years had HOMA2IR of 1 and HOMA%B cell function of 100%. The serum insulin level was assayed using the insulin enzyme-linked immunosorbent assay (ELISA) kit, which is a solid-phase ELISA based on the sandwich principle, supplied by Calbiotech®, China. The serum levels of IL-10 (Elabscience®, Inc. CA, USA), MOR and KOR, endomorphin-2 (Mybiosource®, Inc. CA, USA), and IL-6 and β -endorphin (Melsin Medical Co, Jilin, China) were assayed using commercial ELISA kits. The sensitivities of β -endorphins, MOR, KOR, endomorphin-2, and IL-6 ELISA kits were 0.1, 7.18, 1.0, 0.33, and 0.1 pg/ml, respectively. All measured concentrations were greater than their assay sensitivities. All intra-assay coefficients of variation were < 10.0%. Serum CRP was measured using the kit supplied by Spinreact®, Spain through a test based on the latex agglutination principle.

Statistical Analysis

Analysis of variance (ANOVA) was employed to assess the differences in all measured biomarkers between diagnostic categories, and the χ^2 test was used to compare the proportions and nominal variables. The associations among variables were computed using the Pearson's product-moment and the Spearman's rank-order correlation coefficient. The multivariate general linear model (GLM) analysis was used to delineate the effects of diagnosis for the prediabetes+IR, prediabetes-IR, and control groups while controlling the background variables, including age and sex. Protected LSD tests were used to check pairwise comparisons among treatment means. The model-generated estimated marginal mean (SE) values were computed after adjusting for covariates. Multiple regression analysis was used to delineate the significant biomarkers associated with the prediabetes+IR, and the results were checked for multicollinearity (tolerance and VIF values) and homoscedasticity (White and Breusch-Pagan tests). The binary logistic regression analysis was used to delineate the important explanatory

variables that predict prediabetes (versus control as reference group). The data were subjected to ln transformation to normalize the data distribution of the measured biomarkers (tested using the Kolmogorov–Smirnov test). However, the nonlinearity of the mean and variance of any biomarker is a predictable source of variability that is eliminated using z scores. The natural logarithm of the relevant z unit scores was computed to transform the nonparametric variables into normally distributed components and apply the statistical analysis as a linear group. All tests were two-tailed, and a p value < 0.05 was used for statistical significance. All statistical analyses were performed using the IBM SPSS windows version 25, 2017. Odds ratios (OR) and 95% confidence intervals (CI) for unfavorable glycemic status by study factors were calculated.

Results

Sociodemographic data

Table 1 shows the sociodemographic data of the prediabetes–IR, prediabetes+IR, and healthy control groups. No significant difference was observed among the groups in terms of age, BMI, sex ratio, employment, and marital status. The patients in the prediabetes–IR group were slightly more educated than those in the prediabetes+IR group.

A significant increase ($p < 0.05$) in TC, TG, VLDLc, and LDLc and a significant decrease in HDLc were observed in the prediabetes+IR group compared with the control group. No such difference was observed between the prediabetes–IR and the prediabetes+IR groups. The atherogenic indices, TC/HDLc and LDLc/HDLc, were significantly different among the three study groups, and the scores followed the order: controls $<$ prediabetes–IR $<$ prediabetes+IR. The TG/HDLc showed a significant increase in prediabetes+IR group compared with the control group.

No significant difference was observed in the levels of the ratios of opioids to their receptors zLn (β EP/MOR) and zLn (EM2/KOR) among the study groups. The level of other opioids in the prediabetes groups was higher than that in the control group: zLn β EP ($F = 6.834$, $df = 2/174$, $p = 0.002$), zLnMOR ($F = 4.087$, $df = 2/174$, $p = 0.024$), zLnEM2 ($F = 6.615$, $df = 2/174$, $p = 0.002$), and zLnKOR ($F = 3.964$, $df = 2/174$, $p = 0.031$).

Serum IL-10 was significantly different among the three study groups ($F = 9.362$, $df = 2/174$, $p < 0.001$), and the score followed the order: controls < prediabetes-IR < prediabetes+IR. zLnIL-6 was significantly increased in the prediabetes groups compared with the control group, whereas no such difference was observed between the prediabetes subgroups.

The IR parameters, namely, glucose ($F = 49.734$, $df = 2/174$, $p < 0.001$), insulin ($F = 256.628$, $df = 2/174$, $p < 0.001$), I/G ($F = 52.921$, $df = 2/174$, $p < 0.001$), HOMA2%S ($F = 174.254$, $df = 2/174$, $p < 0.001$), and HOMA2IR ($F = 303.865$, $df = 2/174$, $p < 0.001$), followed the order: controls < prediabetes-IR < prediabetes+IR. The HbA1c% in the prediabetes-IR and the prediabetes+IR groups was higher than that in the control group ($F = 132.746$, $df = 2/174$, $p < 0.001$). zLnHOMA2%B showed no significant difference among the study groups ($F = 0.764$, $df = 2/174$, $p = 0.469$).

Differences in the biomarkers between the study groups

In the entire study group, significant correlations were observed between the zLnMOR and the following parameters: IL-10 ($r = 0.306$, $p = 0.017$), zLnKOR ($r = 0.311$, $p = 0.016$), and zLnIL-6 ($r = 0.368$, $p = 0.004$). zLnKOR was correlated with IL-6 ($r = 0.451$, $p < 0.001$) and zLnEM2 with ($r = 0.377$, $p = 0.003$).

Table 1: Demographic and clinical data of healthy controls (HC) and Prediabetes-IR versus Prediabetes+IR groups.

Parameter	Control ^A N=58	Prediabetes-IR ^B N=60	Prediabetes+IR ^C N=60	F/ χ^2	df	p
Sex (F/M)	28/30	28/32	27/33	0.364	2	0.552
Age year	36.67±10.03	35.03±11.44	37.9±11.88	0.499	2/174	0.609
BMI kg/m ²	26.39±3.72	27.76±4.01	27.63±5.14	0.909	2/174	0.407
Smoking (Y/N)	17/41	18/42	19/41	0.321	2	0.714
Marital status (M/S)	33/25	31/29	36/24	1.308	2	0.067
Education years	10.2 (4.7)	10.3 (5.2) ^C	9.9 (4.3) ^B	2.107	2/174	0.054
Employment (Y/N)	36/22	40/20	38/22	1.163	2	0.088
Physical activity (Y/N)	21/37	20/40	18/42	1.412	2	0.056
TC mM	4.07±0.59 ^C	4.42±0.56	4.72±0.70 ^A	8.288	2/174	0.001
TG mM	1.40±0.14 ^C	1.49±0.17	1.57±0.36 ^A	3.701	2/174	0.029
VLDLc mM	0.64±0.07 ^C	0.68±0.08	0.72±0.16 ^A	3.701	2/174	0.029
HDLc mM	1.12±0.11 ^C	1.06±0.14	0.99±0.13 ^A	6.789	2/174	0.002
LDLc mM	2.32±0.60 ^C	2.69±0.58	3.01±0.74 ^A	8.725	2/174	<0.001
TC/HDLc	3.69±0.64 ^{B,C}	4.27±0.81 ^{A,C}	4.81±0.80 ^{A,B}	16.512	2/174	<0.001
TG/HDLc	1.27±0.19 ^C	1.44±0.26	1.61±0.42 ^A	9.298	2/174	<0.001
LDLc/HDLc	2.11±0.60 ^{B,C}	2.61±0.75 ^{A,C}	3.07±0.81 ^{A,B}	13.214	2/174	<0.001
zLn IL-6	-0.35±1.15 ^{B,C}	0.16±0.90 ^A	0.19±0.86 ^A	4.562	2/174	0.019
IL-10 pg/ml	7.9±2.27 ^{B,C}	11.46±4.52 ^{A,C}	14.08±8.19 ^{A,B}	9.362	2/174	<0.001
zLn β EP	-0.50±0.39 ^{B,C}	0.37±1.39 ^A	0.13±0.76 ^A	6.834	2/174	0.002
zLn MOR	-0.34±1.15 ^{B,C}	0.19±0.96 ^A	0.15±0.81 ^A	4.087	2/174	0.024
zLn (β EP/MOR)	-0.09±1.11	0.10±1.02	0.01±0.87	0.279	2/174	0.757
zLn EM2	-0.50±0.89 ^{B,C}	0.16±0.98 ^A	0.34±0.96 ^A	6.615	2/174	0.002
zLn KOR	-0.35±1.20 ^{B,C}	-0.21±1.14 ^A	0.14±0.64 ^A	3.964	2/174	0.031
zLn (EM2/KOR)	-0.15±1.14	-0.038±0.94	0.19±0.90	0.888	2/174	0.415
HbA1c %	5.01±0.43 ^{B,C}	6.02±0.22 ^A	6.17±0.2 ^A	132.746	2/174	<0.001
Glucose mM	5±0.71 ^{B,C}	5.92±0.59 ^{A,C}	6.49±0.39 ^{A,B}	49.734	2/174	<0.001
Insulin pM	68.54±10.79 ^{B,C}	100.74±13.64 ^{A,C}	140.48±12.38 ^{A,B}	256.628	2/174	<0.001
I/G mM	14.07±3.40 ^{B,C}	17.12±2.46 ^{A,C}	21.79±2.84 ^{A,B}	52.921	2/174	<0.001
zLnHOMA2%B	0.13±1.39	0.18±0.84	0.05±0.63	0.764	2/174	0.469
HOMA2%S	80.72±13.82 ^{B,C}	52.63±7.51 ^{A,C}	36.85±2.80 ^{A,B}	174.254	2/174	<0.001
HOMA2IR	1.27±0.19 ^{B,C}	1.94±0.27 ^{A,C}	2.73±0.22 ^{A,B}	303.865	2/174	<0.001

^{A,B,C}: pairwise comparisons between group means.

zLn: z-score of the natural logarithm, FPG: fasting plasma glucose, TC: serum total cholesterol, TG: serum triglycerides, VLDLc: very low density lipoproteins, HDLc: high density lipoproteins, LDLc: Low density lipoproteins, IL: interleukin; KOR: κ -opioid receptor; MOR: μ opioid receptor; EM2: Endomorphin-2; β EP: β -endorphin; BMI: Body mass index, HOMA2IR: homeostasis model assessment 2 of insulin resistance, HOMA2%S: homeostasis model assessment 2 of insulin sensitivity percentage:

HOMA2%B: homeostasis model assessment 2 of beta-cell function percentage, IR: insulin resistance (HOMA2IR>2.5),

Multivariate GLM analysis

Table 2 displays the outcomes of the multivariate GLM analysis comparing the differences in the measured biomarkers among the three study groups while adjusting for age, BMI, sex, physical activity, education, IR parameters, and smoking. Significant differences ($p = 0.040$) were observed in the biomarkers among the groups with an effect size of 0.198, whereas the other covariates had no significant effects ($p > 0.05$). The tests for between-subject effects in Table 2 and the results in Table 3 showed the SE values and indicated that all eight biomarkers of the patients with prediabetes were significantly higher compared with those of the control group. Furthermore, the IL-0, zLn β EP, zLnMOR, and zLnEM2 in the prediabetes subgroups were significantly higher compared with those in the control group. Among all the examined biomarkers, zLn β EP had the highest effect on the diagnosis of prediabetes ($F = 5.128$, $df = 2/174$, $p = 0.027$, partial $\eta^2 = 0.067$).

Table 2: Results of multivariate GLM analysis showing the associations between biomarkers and diagnosis while adjusting for background variables

Type	Dependent variable	Explanatory variable	F	df	p	Partial η^2
Multivariate	IL-10, zLn β EP, zLn (β EP/MOR), zLn IL-6, zLnMOR, zLnEM2, zLnKOR, zLn (EM2/KOR)	Diagnosis	2.203	16/320	0.045	0.192
		Sex	0.450	8/168	0.867	0.046
		Age	1.138	8/168	0.351	0.109
		BMI	1.803	8/168	0.102	0.163
		Smoking	0.604	8/168	0.750	0.061
		Marital status	1.426	8/168	0.210	0.133
		Physical activity	0.798	8/168	0.612	0.084
		HbA1c	1.076	8/168	0.389	0.104
		Glucose	0.801	8/168	0.590	0.079
		Insulin	0.851	8/168	0.550	0.084
		I/G	0.968	8/168	0.462	0.094
		HOMA2%S	0.495	8/168	0.835	0.051
		HOMA2IR	0.841	8/168	0.558	0.083
		zLnHOMA2%B	1.177	8/168	0.328	0.112
		TC, TG, VLDL, HDL, TC/HDL, TG/HDL, and LDL/HDL	0.002	8/168	0.966	0.001
Tests of Between-Subjects Effects	IL-10	Diagnosis	2.102	2/174	0.152	0.029
	zLn β EP	Diagnosis	5.128	2/174	0.027	0.067
	zLn (β EP/MOR)	Diagnosis	0.884	2/174	0.350	0.012
	zLn IL-6	Diagnosis	0.202	2/174	0.654	0.003
	zLnMOR	Diagnosis	1.131	2/174	0.291	0.016
	zLnEM2	Diagnosis	0.003	2/174	0.957	0.001
	zLnKOR	Diagnosis	3.745	2/174	0.057	0.050
	zLn (EM2/KOR)	Diagnosis	2.213	2/174	0.141	0.030

zLn: z-score of the natural logarithm, FPG: fasting plasma glucose, IL: interleukin; KOR: κ -opioid receptor; MOR: μ opioid receptor; EM2: Endomorphin-2; β EP: β -endorphin.

Table 3. Model-generated estimated marginal means values (SE) of the biomarkers in Prediabetes (versus healthy controls) and Prediabetes-IR versus Prediabetes+IR and healthy controls

Biomarkers	Control ^A	Prediabetes-IR ^B	Prediabetes+IR ^C
IL-10	7.981(1.062) ^{B,C}	11.461(1.026) ^A	14.082(1.026) ^A
zLn β EP	12.146(2.308) ^{B,C}	23.652(2.230) ^A	20.638(2.230) ^A
zLn (β EP/MOR)	5.362(0.677)	5.976(0.654)	5.572(0.654)
zLn IL-6	0.314(-0.187)	0.157(0.180)	0.194(0.180)
zLnMOR	0.431(-0.180) ^{B,C}	0.194(0.174) ^A	0.149(0.174) ^A
zLnEM2	0.518(-0.180) ^{B,C}	0.163(0.174) ^A	0.338(0.174) ^A
zLnKOR	0.362(-0.186)	0.213(0.180)	0.140(0.180)
zLn EM2/KOR	0.158(-0.191)	0.038(-0.185)	0.188(0.185)

^{A,B,C}: pairwise comparisons between group means

zLn: z-score of the natural logarithm, IL: interleukin; KOR: κ -opioid receptor; MOR: μ opioid receptor; EM2: Endomorphin-2; β EP: β -endorphin.

Table 4 shows the results of two binary logistic regression analyses examining the best predictors of prediabetes (versus controls) and prediabetes-IR (versus prediabetes+IR) by using an automatic stepwise method with biomarkers as explanatory variables while allowing the effects of other cofounders (age, sex, smoking, employment, and education). The first regression analysis showed that prediabetes was best predicted by increased levels of IL-10, zLn β EP, and zLnEM2 ($\chi^2 = 38.122$, $df = 7$, $p < 0.001$, Nagelkerke = 0.480) with an accuracy of 76.7%, sensitivity of 85.0%, and specificity of 75.6%. The second regression analysis showed that the

combination of IL-10 and zLnEM2/KOR were the best predictors of prediabetes+IR versus prediabetes-IR ($\chi^2 = 14.780$, $df = 7$, $p = 0.031$, Nagelkerke = 0.364) with an accuracy of 71.2%, sensitivity of 74.4%, and specificity of 72.7%.

Correlation:

Table 4: Results of two different binary logistic regression analyses with Prediabetes (versus healthy controls) and Prediabetes-IR versus Prediabetes+IR as dependent variables and the biomarkers as explanatory variables.

Dichotomies	Explanatory variables	B	SE	Wald	df	p	OR	95% CI
Prediabetes / Controls	IL-10	0.359	0.128	7.876	1	0.005	1.431	1.114-1.839
	zLnβEP	0.316	0.137	5.311	1	0.021	1.372	1.048-1.795
	zLnEM2	0.846	0.396	4.569	1	0.033	2.331	1.073-5.063
Prediabetes-IR / Prediabetes+IR	IL-10	0.618	0.05	2.505	1	0.043	1.083	0.981-1.195
	zLnEM2/KOR	0.555	0.433	4.846	1	0.024	2.611	1.187-3.947

zLn: z-score of the natural logarithm, IL: interleukin; KOR: κ-opioid receptor; MOR: μopioid receptor; EM2: Endomorphin-2; βEP: β-endorphin; IR: insulin resistance (HOMA2IR>2.5)

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Table 5 shows different stepwise multiple regression analyses with the IR parameters as dependent variables and the eight biomarkers as explanatory variables while allowing the effects of education, age, and sex. Regression #1 showed that 21.1% of the variance in the total FPG can be explained by the regression on IL-10, zLnKOR, and zLn (EM2/KOR). Regressions #2, #4, #5, and #6 showed that the same variables explained a considerable part of the variance in insulin (22.4%), HbA1c (29.0%), HOMA2IR (29.3%), and HOMA2%S (29.7%). Regression #3 showed

that 13.0% of the variance in *I/G* ratio was explained by IL-10. Regression #7 showed that zLnHOMA%B cannot be explained by any measured biomarker ($p = 0.742$).

Table 5: Results of multiple regression analysis with IR parameters as dependent variables and biomarkers as explanatory variables.

Dependent variables	Explanatory variables	β	t	p	F _{model}	df	p	R ²
#1. FPG	Model				3.142	7/82	0.005	0.211
	IL-10	0.279	2.661	0.009				
	zLnKOR	0.374	2.625	0.010				
	zLn(EM2/KOR)	0.302	2.537	0.130				
#2. Insulin	Model				4.669	7/82	<0.001	0.224
	IL-10	0.397	3.973	<0.001				
	zLnKOR	0.339	2.498	0.015				
	zLn(EM2/KOR)	0.271	2.393	0.019				
#3. I/G	Model				2.907	7/82	0.009	0.13
#4. HbA1c	IL-10	0.322	3.041	0.003	6.200	7/82	<0.001	0.290
	Model							
	IL-10	0.318	3.333	0.001				
	zLnKOR	0.386	2.977	0.004				
#5. HOMA2IR	zLn(EM2/KOR)	0.356	3.284	0.002	4.809	7/82	<0.001	0.293
	Model							
	IL-10	0.401	4.029	<0.001				
	zLnKOR	0.355	2.624	0.010				
#6. HOMA2%S	zLn(EM2/KOR)	0.280	2.485	0.015	4.836	7/82	<0.001	0.297
	Model							
	IL-10	-0.348	-3.497	0.001				
	zLnKOR	-0.371	-2.749	0.007				
#7. zLnHOMA %B	zLn(EM2/KOR)	-0.290	-2.577	0.012	0.615	7/82	0.742	-
	Model							
								0.031

zLn: z-score of the natural logarithm, FPG: fasting plasma glucose, IL: interleukin; KOR: κ -opioid receptor; MOR: μ opioid receptor; EM2: Endomorphin-2; β EP: β -endorphin; HOMA2IR: homeostasis model assessment 2 of insulin resistance, HOMA2%S: homeostasis model assessment 2 of insulin sensitivity percentage: HOMA2%B: homeostasis model assessment 2 of beta-cell function percentage.

Discussion

This case-control observational study was designed to investigate the possible role of opioid system in the pathogenesis of prediabetes. Accordingly, this problem was solved in this study. Dyslipidemia and increased atherogenic indices were observed in the prediabetes+IR group compared with the controls, indicating the negative effect of elevated IR on the lipid metabolism (Table 1). IR can also alter systemic lipid metabolism, leading to the generation of dyslipidemia. Along with endothelial dysfunction, which can also be created by an abnormality of insulin signaling, IR contributes to the atherosclerotic plaque production [36]. IR in the myocardium produces damage by signal transduction modification, impaired regulation of substrate metabolism, and variation in the delivery of substrates to the myocardium [36]. These variations are engaged in the presence of increased IR in patients with prediabetes, indicating the huge effect of the elevated IR on the lipid metabolism.

The first main finding is the elevation in the levels of the endogenous opioid and their receptors in the prediabetes groups compared with the controls. These results signify the dependence of these parameters on the glucose metabolism state more than its dependence on the response to insulin hormone. To our knowledge, this study was the first to deal with the endogenous opioid in prediabetes subjects. Some previous studies have dealt with the opioid peptides and receptors in the IR state in animals [28, 37] and humans [38, 39]. MOR is enhanced on insulin-sensitive tissues, such as skeletal muscle, originating in an inversion of the impairment of insulin-stimulated glucose disposal in genetically obese Zucker rats via exercise training [37]. This enhancement of IR is associated with the increased circulation of β -endorphin, thus enhancing the post receptor and insulin signaling cascade, including the downstream effectors of the phosphatidylinositol 3-kinase (PI3-kinase) signaling pathway implicated in the translocation of the glucose transporter [28, 37]. The increase in β EP release from the adrenal

gland may stimulate peripheral opioid MOR to boost the expression of muscle glucose transporters and/or reduce hepatic gluconeogenesis at the gene level, thereby directing to enhance glucose utilization in peripheral tissues for the improvement of severe hyperglycemia [28].

Insulin sensitivity is improved through the peripheral MOR stimulation, overcoming defects in insulin signal transduction associated with the post receptor in IRS-1 associated with the PI3-kinase step. Insulin sensitivity is enhanced through the peripheral MOR activation. Opioid receptor stimulation, especially that of the μ -subtype, may supply merits in the improvement of defective insulin function. The opioid system manages the endocrine function of β -cells [40]. Within the pancreas, EOPs result in insulin secretion via the paracrine and the intracrine mechanisms [41]. Opioid activity can perform an important role in the physiopathology of hyperinsulinemia in hyperandrogenic women [38]. Hence, locally increased opioid activity results in insulin release from the pancreas. IR is a consequence of the downregulation of hepatic insulin receptors, thereby increasing the insulin levels in peripheral blood [6, 42] Sinaiko and Caprio 2012). Interestingly, brain damage and white matter disruption occur in the IR stage without overt diabetes, and these structural abnormalities may be involved in early cognitive impairment [43] and secrete endogenous peptides to the circulation and increase their levels. Patients with insulin-resistant conditions (like polycystic ovarian syndrome) have greater limbic MOR availability than the controls. Moreover, receptor availability is associated with the severity of IR, and MOR availability is normalized after insulin-regulating therapy [39].

The typical brain areas for opioid neurotransmission are the amygdala and nucleus accumbens, which affect the management of appetite and mood.

The elevation of EM2 in prediabetes subjects may be due to the increase in their release in response to any possible hurt of the beta cells. In animal studies, EM2 can maintain the beta-cell islets from the injury caused by streptozotocin, alloxan, and hydrogen peroxide [44]. EM2 enhances the viability of islet and elevates the insulin accumulation of the cell supernatant after streptozotocin and alloxan stimulation. Our observations imply that endomorphins may have protective effects on islet cell oxidative injury [44]. When the μ and κ opioid antagonists are microinjected into the hypothalamic paraventricular nucleus or the nucleus accumbens of rats, they decrease food intake under deprivation states. MOR and KOR seem accountable for the inclusion of various food intake by the nucleus [45]. The outcomes also propose that MOR and KOR may be altered by blood glucose levels, possibly participating cellular energetics-mediated variation in potassium channels in female rats [46]. Moreover, elevated insulin levels lead to the variation in the sensitivity of KOR and MOR to nociception by agonists [47]. The blood glucose of diabetic mice decreases significantly behind the therapy with a selective KOR agonist. This phenomenon is time-dependent and related to the corresponding variation of GLUT4 translocation in the skeleton muscles. Hence, the enhancement of KOR decreases the hyperglycemia in streptozotocin-induced diabetic mice [48]. Insulin elevates the efficacy of KOR motivation by phosphorylating two tyrosine residues in the first and second intracellular loops of the receptor. Thus, tyrosine phosphorylation may supply an influential mechanism for the variation of G protein-coupled receptor signaling [49]. Together, these studies support that the insulin in the CNS may reduce the effect of CNS KOR system(s) that mediate palatable feeding [50]. Our results are in accordance with the mutual effect of IR and hyperinsulinemia with the endogenous opioid receptors. The presence of these changes in the prediabetic groups in

comparison with controls should be considered when seeking for a new treatment target to prevent the development of prediabetes into T2DM.

Serum IL-10 was significantly different among the three study groups, and the score followed the order: controls < prediabetes-IR < prediabetes+IR. In a previous study, the serum IL-10 level is elevated in T2DM but not in prediabetes compared with the healthy controls [51]. However, this study is not choosing a homogenized group in number, and patients with positive CRP were excluded. The decreased levels of the proinflammatory cytokine IL-12 in the prediabetes stage and the increased levels of the anti-inflammatory cytokine IL-10 in the T2DM stage suggest a continuous organismal effort for halting the activation of the Th1 subpopulation that acts as a main generator of proinflammatory $\text{INF}\gamma$, possibly as a mechanism to maintain homeostasis in relevant tissues. Notably, several studies have reported the addition of the Th1 cell subset and causal participation of this phenomenon in inflammation and IR in the mouse models of diabetes [52]. IL-6 was significantly elevated in the prediabetes groups compared with the control group, whereas no such difference was observed between the prediabetes subgroups. IL-6 has exhibited no significant variation between the prediabetes groups and controls [53]. However, increased IL-6, another proinflammatory marker, is observed in hyperglycemic/hyperinsulinemic conditions [54, 55]. The studies on inflammatory markers have indicated that participants with a combined increase of IL-6 and IL-1 β have approximately threefold elevated risk of developing diabetes, whereas low levels of IL-1 β alone exhibit no substantial elevation in risk [56].

Results exhibited significant associations between IL-10 and MOR and between IL-6 and KOR. The connection among endogenous opioids and immunosuppression has been explored in vitro and in vivo. However, results are conflicting. Exogenous narcotics hinder the capacity of

macrophages, natural killer cells, and T-cells to weaken the gut barrier in vitro and in animal models [57]. High dosage and the commencement of opioid therapy for pain correspond with a high danger of infectious diseases, such as pneumonia. However, immune cells secrete endogenous opioid peptides, which are linked to peripheral opioid receptors to relieve inflammatory pain. In general, the immune framework and endogenous opioids have an equal collaboration (57). IL-10 and MOR has a relationship in depression [58], indicating that the mood causes on such association in addition to the suggested correlation between immune and opioid systems.

The second major finding was obtained from the multivariate GLM analysis, which removed the effects of the covariates (age, BMI, sex, education, IR parameters, physical activity, and smoking) to compare the parameters in accordance with the type of diagnosis (Table 2). Approximately 20% (0.198) of the values of immune–opioid biomarkers can be explained by the presence of prediabetes and IR. Based on the results on Tables 2 and 3 (SE), all eight biomarkers were significantly higher in patients with prediabetes compared with the controls. Among all the examined biomarkers, zLnβEP had the highest effect on the diagnosis of prediabetes. βEP cells have been demonstrated in areas surrounding the pancreatic β-cells, and opioids enhance insulin secretion [42]. The improvement of IR is associated with the increased circulating β-endorphin to ameliorate the postreceptor insulin signaling cascade and the improvement in insulin sensitivity through the peripheral MOR motivation [59]. MOR manages body weight by sharing insulin release, exhibiting a novel target for new diabetes treatments [60].

The endogenous opioid system negatively manages insulin secretion from isolated Langerhans islets in vitro [40]. In vivo, the β-endorphin also weakens insulin secretion when administered through intravenous infusion [61]. In addition, some groups have reported the dual

stimulatory/inhibitory action of β -endorphin on insulin secretion depending on dose, obesity condition, or circulating glucose level [62]. MORs act as a part of the complex opioid system and mediate the effects of endogenous opioids, such as endomorphin-2 and various exogenous opioid agonists [63]. The determination of the endogenous opioid peptide expression in the Langerhans islet and peptide secretion after glucose motivation [64]. transform the endocrine pancreas as a potential candidate for MOR management. MOR participates in glucose homeostasis by negatively regulating glucose tolerance by inhibiting insulin release from the β -cell and decreasing β -cell mass [60].

The binary logistic regression analyses in Table 4 indicated that the increased levels of the combination of IL-10, zLn β EP, and zLnEM2 can be used to differentiate prediabetes+IR from other groups with good accuracy (76.7%). Another compilation of increased IL-10 and zLnEM2/KOR can be used to predict prediabetes+IR with good accuracy (71.2%). These results further indicate the importance of these parameters in prediabetes+IR and the strong correlation with the immune–opioid system. Endogenous opioid activity increases by acting on the pancreatic B cells, an increase in insulin secretion, resulting in elevated insulin levels in the hepatic circulation. Elevated insulin levels lead to a downregulation of hepatic insulin receptors, thereby causing and maintaining hyperinsulinemia [65]. Morbid obesity is related to reduced MOR availability in the brain, and the endogenous opioid system is a key component underlying human obesity [66]. The increase in body mass is associated negatively with MOR availability [66]. The central stimulation of the MOR impairs glucose tolerance and insulin sensitivity and motivates hepatic gluconeogenesis [67]. The stimulation of peripheral MOR can enhance IR in animals and provide a novel target for the treatment of IR [68]. MOR activation may ameliorate IL-6-induced IR through various insulin signals opposite the responsiveness of insulin [69].

Mice lacking MOR in the whole body exhibit increased glucose tolerance and insulin sensitivity and elevated fatty acid oxidation when fed with a high-fat diet [67]. The stimulation of KOR results in the reduction in IL-6 levels [70]. Table 5 shows an important result on the effect of the measured biomarkers on the IR parameters. Approximately one-fifth (21.1%) of the variance in FPG can be explained by the regression on IL-10, zLnKOR, and zLn(EM2/KOR). The elevated endogenous opiates may affect the insulin response to glucose in subjects with obesity through impaired or normal oral glucose tolerance test [71]. Same changes explain an important part of the variations in insulin, HbA1c, HOMA2IR, and HOMA2%S. Opioid activity can play a critical role in the physiopathology of hyperinsulinemia in hyperandrogenic women [38]. The β EP level is not affected during the oral glucose tolerance test. Furthermore, reducing the IR condition after the reduction in body weight has not affected the β EP level [72]. In healthy persons, the independent parameters (FBG, insulin, and HbA1c) associated well because the beta cells worked properly. Elevated blood glucose stimulates the elevation in insulin concentrations as released from the healthy beta cells to keep normal blood glucose concentrations over long periods [73]. The hyperproduction of endogenous opioid peptides in obesity increases insulin release motivated by food intake but does not appreciably affect the insulin release stimulated by circulating glucose or amino acids [74].

Conclusion

The subjects with prediabetes had dyslipidemia, but all of them underwent the IR state. IL-10, β EP, MOR, and EM2 were significantly higher in the prediabetes subgroups than in the control group. Among all the examined biomarkers, β EP had the highest effect on the diagnosis of prediabetes. Serum IL-10 increased among the three study groups, and the score followed the order: controls < prediabetes-IR < prediabetes+IR. Serum IL-6 was significantly increased in the

prediabetes groups compared with the control group, and the increase was independent of the IR. MOR was correlated with IL-10 and KOR, and KOR was correlated with IL-6 and EM2. Prediabetes+IR can be predicted using the increased levels of the combination of IL-10, β EP, and EM2 and by a combination of IL-10 and EM2/KOR with good sensitivity and specificity. A significant percentage of FPG, insulin, HbA1c, HOMA2IR, and HOMA2%S can be explained by the regression on IL-10, KOR, and EM2/KOR in the prediabetes group. Overall, the opioid peptides and their receptors were upregulated in prediabetes depending on the significance of IR. These changes in the opioid system are dependent on the immune cytokines.

Limitations of the Study

The results of this study should be discussed with respect to its limitations. First, the delta opioid receptor, T regulatory phenotypes, and some other cytokines, including those produced by M1 macrophage, T helper cells 1, 2, and 17 should be measured to examine the associations of opioids with the full spectrum of cytokines. Second, the effects of dynorphin and β -endorphin on the production of cytokines and the effects of immune activation (by mitogen or LPS stimulation) on KOR–MOR protein production should be examined by stimulated and unstimulated whole blood cultures of patients and controls.

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