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High mobility group box 1 and Dickkopf-related protein 1 as new biomarkers of type 2 diabetes mellitus: associations with increased glucose toxicity and atherogenicity and lower β cell function.

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Abstract

Background. Type 2 diabetes mellitus (T2DM) is associated with increased atherogenicity and inflammatory responses, which may be related to increased levels of high mobility group box 1 (HMGB1) and Dickkopf-related protein 1 (DKK1).

Objective. The role of HMGB1 and DKK1 in T2DM is examined in association with lipid and insulin profiles.

Methods. Serum HMGB1 and DKK1 were measured in T2DM with and without hypertension and compared with controls.

Results. HMGB1 and DKK1 are significantly higher in T2DM irrespective of hypertension. T2DM was also accompanied by increased atherogenicity indices. HMGB1 and DKK1 are significantly correlated with HbA1c, glucose, indices of insulin resistance, β-cell function, and glucose toxicity, and different atherogenic indices. A large part of the variance in the β-cell index (30.5%) and glucose toxicity (34.8%) was explained by the combined effects of HMGB1 and DKK1 and hypertension. We found that 18.3% of the variance of the atherogenic index of plasma was explained by HMGB1 and DKK1 levels and that 31.2% was explained by glucose toxicity, HMGB1 and body weight.

Conclusion. The higher serum HMGB1 and DKK1 levels in T2DM patients and the associations with atherogenicity indicate that low grade inflammation and disorders in the Wnt pathways are associated with T2DM and that both HMGB1 and DKK1 may contribute to increased atherogenicity in T2DM. Moreover, both biomarkers may cause more deficits in β-cell function and increase glucose toxicity leading to the development of more inflammation and diabetic complications. HMGB1 and the Wnt pathways are new drug targets in the treatment of T2DM.

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Introduction

Diabetes mellitus (DM) is a prevalent disorder characterized by hyperglycemia resulting from multifactorial interaction between genetic, environmental, and behavioral risk factors [1]. DM is a major public health concern with a growing prevalence around the world and is estimated to contribute to 11.3% of deaths globally [2]. In 2017, it was estimated that there are 451 million people aged over 18 years with DM worldwide, which expected to reach 693 million by the year 2045 [3]. In Iraq, the prevalence of DM had risen significantly from 1.958% in the year 2000 to 4.227% in 2015 [4]. Type 2 DM (T2DM) is initiated by insulin resistance (IR) in target tissues, high circulating insulin levels, β-cell dysfunction, and subsequent β-cell failure, which develop into overt T2DM [5]. T2DM frequently coincides with hypertension and about 68.4% of T2DM patients have hypertension in the USA [6]. T2DM coupled with age and sex are the strongest predictors of uncontrolled blood pressure (BP) [7]. T2DM is usually associated with chronic inflammation due to metabolic syndrome-related immune system dysregulations [8, 9]. Chronic inflammation may be either the cause, the result or cause and result of T2DM [10]. Therefore, the delineation of inflammatory biomarkers in T2DM is important to correctly diagnose and treat the low-grade inflammation that is responsible for many its complications [11, 12]. Previous investigations reported significant relationships between high levels of C-reactive protein (CRP) and IR and T2DM progression [13, 14], while also interleukin (IL)-6 plays an important role in the development of T2DM [15]. Elevated tumor necrosis factor-alpha (TNF-α) levels may be another potential predictor of T2DM complications [15, 16].

High mobility group box 1 (HMGB1) is a nonhistone chromosomal binding protein that may be secreted by stimulated immune cells including activated monocytes, mature dendritic cells, macrophages, and natural killer cells, or passively released by necrotic and damaged cells [17, 18].

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HMGB1 is a proinflammatory mediator that is secreted in response to injury, infection, or other inflammatory stimuli [19, 20] and acts as a proinflammatory cytokine through binding the receptor for advanced glycation end products (RAGE) and Toll-Like Receptors (TLR) 2 and 4 complexes [21-23]. HMGB1 is involved in DM initiation, insulin secretion and insulin resistance by interaction with RAGE thereby promoting pancreatic islet cell apoptosis dependent on enhanced oxidative stress [24]. Moreover, HMGB1 binding with TLR-4 enhances insulin resistance via the phosphorylation of the peripheral insulin receptor substrate [24] and as such HMGB1 may sustain the chronic inflammatory state associated with diabetes [25]. Moreover, many diabetes complications are mediated by the action of HMGB1 [24, 26].

Inflammation is frequently accompanied by an upregulation of Dickkopf-related protein 1 (DKK1), a pro-inflammatory glycoprotein secreted by many cell types including endothelial cells and platelets [27]. DKK1 is a cellular inhibitor of Wnt signaling pathway after binding with its receptor, Kremen-2 [28] thereby interfering with repair mechanisms [27]. Acute infections are accompanied by a dramatic increase in systemic DKK1 levels [29] and DKK1 may contribute to inflammatory responses by inducing the secretion of proinflammatory cytokines [30]. Moreover, DKK1 directly regulates the expression of proteins involved in the pathogenesis of atherosclerosis [31] and is associated comorbid cardiovascular disease in diabetes [32, 33].

There is some evidence that DM and T2DM are accompanied by increased plasma levels of HMGB1 and DKK1 [34-36]. Nevertheless, the associations of HMGB1 and DKK1 with T2DM is relation to β-cell function, glucose toxicity and atherogenicity are still underreported. Hence, the present study aims to explore the role of DKK1 and HMGB1 as possible predictors of glucose toxicity, β cell function and atherogenicity in T2DM patients who are free of overt inflammation

 doi:10.20944/preprints202006.0011.v1

Subjects and Methods

Subjects

The current study recruited 110 T2DM patients and 51 healthy controls, age and sex-matched to the patients. Among the patients' group, 34 patients were diagnosed with hypertension in addition to diabetes (T2DM+HT) and 76 without hypertension (T2DM). The participants were recruited at the Sadr Teaching Hospital, Misan Governorate, Iraq from December 2019 to the end of January 2020. T2DM patients were diagnosed according to the World Health Organization criteria [37, 38] where they had fasting plasma glucose (FPG) ≥ 7.0 mM, and glycated hemoglobin (HbA1c) > 6.5%. HT was diagnosed according to the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) guidelines [39]. Each HT patient had a blood pressure measurement by conventional sphygmomanometry in excess of 95/140 mmHg (seated posture), with the arm in the horizontal position after five minutes of quiet sitting.

Exclusion criteria for patients were: serum FPG >25 mmol/L and fasting insulin >400pM based on HOMA calculator software requirements, and patients with evident major overt diabetic complications, such as heart diseases, liver disease, and renal diseases. We also excluded patients who are receiving metformin because the latter may have effects on IR [40] and insulin sensitivity [41] and patients with an albumin/creatinine ratio above 30 mg/g [42]. Serum CRP concentrations were < 6 mg/dL in all participants, thereby excluding subjects with overt inflammation. The subjects were considered as having sufficient physical activity when they visited a gym at least twice a week and trained for at least one hour each visit. Written informed consent was obtained from all subjects before participating in the study. Approval for the study was obtained from the IRB of the University of Kufa (444/2019), which complies with the International Guidelines for Human Research protection as required by the Declaration of Helsinki.

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Methods

Blood was sampled at 8.00 a.m. after an overnight fast. Five milliliters of venous blood was drawn from patients and controls. After complete clotting, blood was centrifuged at 3.000 rpm for 10 min, and then serum was separated to be stored at -80^{o}C until thawed for assay. Commercial ELISA sandwich kits were used to measure serum insulin (DRG® International Inc., USA), and DKK1, and HMGB1 (Elabscience®, Inc. CA, USA). The procedures were followed exactly without modifications according to the manufacturer's instructions. The sensitivities of the kits were 12.22 pM for insulin, 18.75 pg/mL for DKK1, and 18.75 pg/mL for HMGB1. All measured concentrations of HMGB1 and DKK1 were greater than the sensitivity of the assays. The intra-assay coefficient of variation (CV) (precision within an assay) were < 10.0%.

Fasting serum levels of total cholesterol (TC), triglycerides (TG), and glucose were measured by a fully automated analyzers (Cobas c 311, and Cobas e 411, Roche/Hitachi, Germany). Serum high-density lipoprotein cholesterol (HDLc) was determined after the precipitation of other lipoproteins by the reagent containing sodium phosphotungstate and $MgCl_2$, and the cholesterol contents in the supernatant were measured using the automated analyzer. Low-density lipoprotein cholesterol (LDLc) was computed from Friedewald's formula: LDLc=TC − HDLc − VLDLc. HbA1c percentage in the whole blood was determined by a fluorescence immunoassay (FIA) using the i-Chroma™ HbA1c test kit (BioLabs Diagnostics, Italy). Serum CRP was measured using a kit supplied by Spinreact®, Spain. The test is based on the principle of latex agglutination. Body mass index (BMI) was calculated by weight in kilograms divided by square of height in meters.

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We calculated three atherogenic indices, namely z score of total cholesterol – z HDLc (zTC-zHDL, reflecting the Castelli risk index 1), z LDLc -z HDLc (zLDL-zHDL, reflecting Castelli risk index 2), and z triglycerides – z HDL cholesterol (zTG-zHDL, reflecting the atherogenic index of plasma) [43, 44]. The zTC-zHDL score was significantly correlated with Castelli risk index 1 ($r=0.988$, $p<0.001$, $n=161$), zLDL-zHDL, with Castelli risk index 2 ($r=0.945$, $p<0.001$, $n=161$) and zTG-zHDL with the atherogenic index of plasma ($r=0.939$, $p<0.001$, $n=161$). Likewise, we also computed new indices reflecting IR as z glucose + z insulin (zIR), β cell function as z insulin – z glucose (zβCell), and glucose toxicity as z glucose + z HbA1C – z insulin (zGLUTOX). There were significant associations between zIR and HOMA2IR as defined with HOMA2 Calculator© (Diabetes Trials Unit, University of Oxford) ($r=0.623$, $p<0.001$, $n=161$) and between zβCell and HOMA2B ($r=0.991$, $p<0.001$, $n=161$).

Statistical analysis

Differences in the measured variables between study groups were assessed by analysis of variance (ANOVA), while the associations between nominal variables were assessed by analysis of contingency tables (χ^2-test). Associations among biomarkers were checked using correlation matrices based on Pearson's product-moment and Spearman's rank-order correlation coefficients. We employed multivariate general direct model (GLM) analysis to check the relationship among the biomarkers and the diagnosis (controls versus T2DM with and without hypertension) while controlling for background variables including age, BMI, nicotine dependence, sex, and education. Consequently, we performed tests for between-subject effects to delineate the relationships between diagnosis and each biomarker and we assessed effect sizes estimated using partial η^2 values. We also computed GLM-generated estimated marginal mean (SE) values and conducted

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protected pairwise comparisons among treatment means. Multiple regression analysis was used to define the significant biomarkers predicting the components of lipid profile and insulin resistance by using DKK1, HMGB1, and hypertension as explanatory variables using an automatic stepwise method with a p-to-enter of 0.05 and p-to-remove 0.06 while expecting R^2 changes. All results were checked for multicollinearity using VIF and tolerance values. The HMGB1 and DKK1 data distribution in the three diagnostic groups was displayed using Boxplots showing minimum, maximum, Q1, Q3, and median values as well as out-values (shown as circles) and far-out or extreme values (shown as stars). Both HMGB1 an DKK1 were processed in Ln transformation as were HbA1c, glucose, HOMA2IR, TGs, and HDLc, while TC, and LDLc were processed in square root transformation. Tests were 2-tailed and a p-value of 0.05 was used for statistical significance. All statistical analyses were performed using IBM SPSS windows version 25, 2017.

Results

Socio-demographic and clinical characteristics

The sociodemographic data of the participants are shown in **Table 1.** There were no significant differences in age, sex ratio, urban/rural residence ratio, BMI, single/married ratio, and TUD among the three study groups. The T2DM+HT group had less education years and more unemployed individuals than the control and T2DM groups. Also, there is a significant difference in the family history of disease among groups.

Biomarkers in T2DM with and without hypertension

Table 2 shows that HMGB1 and DKK1 levels were significantly different between the three study groups and that there were no significant differences in HMGB1 and DKK1 between patients

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with and without hypertension (using univariate GLM controlled for age, sed, TUD and BMI). Table 2 shows also the results of univariate GLM analysis and model-generated estimated marginal mean values of lipids and insulin status parameters in healthy controls, and T2DM and T2DM+HT patients and additionally the results of protected pairwise comparisons among groups. The results indicate that HbA1c, glucose, and the composites zIR and zGLUTOX are significantly higher in both patients groups as compared with the healthy control group. Serum insulin levels and zβCell were significantly lower in patients groups as compared with the control group. All lipid profile components (TC, TG, and LDLc) and the z-score composites zTC-zHDL, zTG-zHDLc, and zLDLc-zHDL were higher in patients as compared with controls, except HDLc, which showed a significant decrease in patients groups as compared with controls.

Correlations of HMGB1 and DKK1 with insulin/lipid biomarkers

Table 3 shows the intercorrelation matrix (obtained using partial correlation coefficients after adjusting for age, sex, BMI, and TUD) of HMGB1/DKK1 and insulin/lipid biomarkers in 161 subjects. HMGB1 and DKK1 were significantly and positively correlated with HbA1c, glucose, zGLUTOX, triglycerides, and all three atherogenic indices, and negatively with insulin, zβCELL, and HDLc.

To delineate the effects of HMGB1 and DKK1 on glycemic parameters we performed multiple regression analyses with the glycemic parameters as dependent variables while allowing for the effects of hypertension, age, BMI, sex, education, and TUD. We found that 29.3% of the variance in HbA1c (regression #1) was explained by the regression on DKK1, hypertension, and being male. **Figure 1** shows the partial regression plot of HbA1C on DKK1. A significant part of variance (21.8-34.8%) of variance in glucose (regression #2), insulin (regression #3), ZβCELL

 doi:10.20944/preprints202006.0011.v1

(regression #4), and zGLUTOX (regression #6) was explained by the combined effects of DKK1, HMGB1 and hypertension. **Figure 2** shows the partial regression plot of zGLUTOX on DKK1.

Table 5 shows the results of multiple regression analyses with atherogenic indices as dependent variables and DKK1 and HMGB1 as independent variables while allowing for the effects of age, BMI, body weight, hypertension, sex, education, and TUD. We found that (regression #1) that 13.1% of the variance in zTC-zHDL was explained the regression on HMGB1 and hypertension. We found that 18.3% of the variance in zTG-zHDL (regression #2) was explained by HMGB1 and DKK1, whereas 10.8% of the variance in zLDL-zHDL (regression #3) was explained by HMGB1 and hypertension. We have also examined whether increased GLUTOX may predict the atherogenic index and, therefore, we entered GLUTOX in the second regression. We found that 31.2% of the variance in zTRY-zHDL was explained by the cumulative effects of GLUTOX, body eight and HMGB1. **Figure 3** shows the partial regression plot of zTG-zHDL on GLUTOX levels.

Discussion

The first major finding of this study is that a) serum levels of HMGB1 and DKK1 are significantly higher in T2DM patients (irrespective of hypertension) as compared with healthy controls, and that b) increased HMGB1 and DKK1 are significantly associated with indices of glucose toxicity (computed as z HbA1c + z glucose z Insulin) and β-cells activity (computed as z Insulin – z glucose). An increase in HMGB1 in T2DM patients as compared to controls was reported previously [34-36]. HMGB1 concentrations in serum are elevated in non-diabetic hyperglycemic patients and diabetic patients [45] and higher plasma HMGB1 levels were independently associated with a higher risk of cardiovascular disease and all-cause mortality in

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patients with T1DM [46]. The increases in HMGB1 may be attributed to insulin resistance [47] or the inflammatory state in T2DM [47]. Plasma HMGB1 concentrations were also positively correlated with glucose, insulin, HOMA-IR, and HbA1c, and inversely with the HOMA-β index [47, 48]. HMGB1 was also positively related to hsCRP and associated with coronary artery disease (CAD) in nondiabetic and T2DM patients [34].

HMGB1 and RAGE are thought to play a role in the progression of DM [49] and hyperglycemia may contribute to increased expression of HMGB1 and RAGE, for example in human aortic endothelial cells [50] [51]. HMGB1 can be secreted into the extracellular milieu and may function as a proinflammatory cytokine [52]. Activation of RAGE induces an elevation in the generation of superoxide by mononuclear phagocytes in patients with DM [53]. HMGB1 may bind to TLR2 and TLR4 complexes to trigger NF-κB thereby modulating systemic immune-inflammatory responses [54] by stimulating the biosynthesis of proinflammatory mediators such as IL-1β, IL-6, and TNF-α [55]. Moreover, in T2DM, the TLR2 and TLR4 are activated and their ligands (including HMGB1) are highly expressed in the serum of newly diagnosed subjects [36]. Following binding of HMGB1 with RAGE, IL-6 may be produced thereby regulating inflammation which, in turn, modulates glucose metabolism [56] and weakens insulin sensitivity in visceral fat to promote diabetes [57] and insulin resistance [58]. As such, the HMGB1/RAGE axis could play a role in DM and constitute a potential new drug target to treat DM and related conditions such as stroke and DM [59].

Previous papers also showed that the levels of DKK1 are increased in DM [60, 61] and in T1DM children while DKK1 levels were associated with altered glycemic control [62]. The expression of DKK1 was also increased in myocytes exposed to hyperglycemia, whilst reducing DKK1 may improve glucose uptake by cells [63]. Nevertheless, in a cohort of African people at

 doi:10.20944/preprints202006.0011.v1

high risk for T2DM, DKK1 was not significantly correlated with fasting serum glucose and insulin levels and the HOMA-IR index [64]. An improvement of platelet functions in T2DM was associated with concurrent reductions in DKK1, suggesting that the latter may be involved in the inflammatory interaction between platelets and endothelial cells [61] and in the vascular disorders in T2DM [65]. Serum DKK1 levels are independently associated with the presence of cardiovascular diseases [65]. Interestingly, knocking down DKK1 prevents diabetes-induced renal dysfunction and microstructure deterioration, suggesting that inhibition of DKK1 offers therapeutic potential for diabetic nephropathy [66]. Moreover, increased levels of glucose induces the synthesis of DKK-1 and Kremen-2, which subsequently leads to initiation of diabetic nephropathy [66]. All in all, these results suggest that the combined effects of HMGB1 and DKK1 may contribute to deficits in the β-cells functions and increased glucose toxicity leading to the development of diabetic complications.

The second major finding of this study is that a) T2DM is accompanied by increased total cholesterol, triglyceride and LDLc levels and by lowered levels of HDLc irrespective of hypertension; and b) the increased levels of HMGB1 and DKK1 are also significantly and positively correlated with atherogenic indices reflecting Castelli risk indices 1 and 2 and the AIP. Atherogenic lipoprotein profiles were described in T2DM [67, 68], explaining that T2DM patients show an increased risk of atherosclerosis and cardiovascular diseases as compared with nondiabetic subjects [69, 70]. The presence of hyperglycemia with dyslipidemia may increase the risk and worsen atherosclerosis and subsequent cardiovascular diseases in diabetes patients [71, 72]. In this respect, it is interesting to note that we found that the combination of glucose toxicity and HMGB1 explained around 27.3% of the variance in the AIP index. Previous studies showed that plasma HMGB1 levels were positively correlated with triglyceride levels, BMI and the waist-

 doi:10.20944/preprints202006.0011.v1

hip ratio, and negatively with HDLc [47]. Interestingly, atorvastatin may reduce triglyceride and LDLc levels by decreasing plasma HMGB1 concentrations [73], suggesting that HMGB1 may modulate lipid metabolism. HMGB1 contributes to the pathogenesis of many disorders such as acute diabetes [74] and pancreas β cell disorders [75]. Serum RAGE levels are positively correlated with inflammatory markers in patients with T2DM [76] and, in patients with diabetic microangiopathy, mean serum RAGE levels are significantly decreased compared with those in diabetic patients without microvascular complications [77]. On the other hand, hyperlipidemic stress may increase serum HMGB1 and upregulate RAGE in tissue [23].

DKK1 levels are positively correlated with BMI, waist circumference, and adiposity, independently of age [64], suggesting that increased DKK1 may be associated with greater overall, central, and ectopic skeletal muscle adiposity [64]. DKK1 has pro-atherogenic effects for example by enhancing the endothelial–mesenchymal transition in endothelial cells [78-80]. Aberrations in the Wnt/β-catenin pathway are associated with various cardiovascular diseases [81]. Importantly, DKK1 promotes plaque formation and destabilizes plaques in part by inducing apoptosis in endothelial cells via effects on endoplasmic reticulum stress [82]. All in all, studies suggest that the HMGB1/RAGE and DKK/Wnt pathways may contribute to the pathogenesis of T2DM and the increased atherogenicity in that illness. Pharmacological interventions targeting HMGB1 and DKK1 should be trialed in T2DM to prevent the complications of T2DM.

One main limitation of the present study is that it would have been more interesting if we had measured other inflammatory mediators. Also, this is a cross-sectional correlational study, which does not allow to make firm inferences on causal associations.

Conclusions

 doi:10.20944/preprints202006.0011.v1

Serum levels of HMGB1 and DKK1 are higher in T2DM patients as compared with controls and are associated with lowered β-cell function and increased glucose toxicity and atherogenic indices. HMGB1 and DKK1 may play a role in the immune-inflammatory and atherogenic pathophysiology leading to the development of T2DM complications. HMGB1 and DKK1 are new drug targets which may be targeted to attenuate complications related to atherogenicity and inflammation.

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Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

Author’s contributions

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

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Table 1. Demographic data in healthy controls, type 2 diabetes mellitus (T2DM) patients, and diabetes with hypertension (T2DM+HT).

Variables	Control [A] n=51	T2DM [B] n=76	T2DM+HT [C] n=34	F/Ψ/χ^2	df	p
Age (years)	44.47±9.05	45.14±8.05	48.65±9.76	2.58	2/158	0.079
Sex (Female/Male)	26/25	43/33	23/11	2.33	2	0.312
Single/married	5/46	3/73	4/30	2.68	2	0.262
Residency Rural / Urban	4/47	7/69	3/31	Ψ=0.21	-	0.964
Family History of T2DM (No/Yes)	48/3[B,C]	55/21 [A, C]	13/21 [A, B]	31.64	2	<0.001
BMI (kg/m^2)	25.89±4.32	25.59±4.21	26.79±3.60	1.00	2/158	0.369
Education (years)	9.94±4.60[C]	9.86±4.34[C]	6.56±4.88[A,B]	7.24	2/155	0.001
Exercise (No/Yes)	33/18	50/26	27/7	2.47	2	0.292
Employment (No/Yes)	25/26[C]	32/44 [C]	26/8 [A,B]	11.30	2	0.004
TUD (No/Yes)	26/25	40/36	17/17	0.075	2	0.963

[A, B, C]: Pairwise comparison, BMI: Body mass index, TUD: Tobacco use disorder, T2DM: type 2 diabetes mellitus, HT: hypertension.

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Table 2. Model-generated estimated marginal mean values of lipids and insulin status parameters in healthy controls (HC), type 2 diabetes mellitus (T2DM) patients, and T2DM with hypertension (T2DM+HT).

Dependent Variables	Controls [A] n=51	T2DM [B] n=76	T2DM+HT [C] n=34	F	df	P	Partial η^2
HMGB1 (ng/mL)	6.89 (0.75) [B,C]	10.19 (0.62) [A]	11.38 (0.94) [A]	9.81	2/153	<0.001	0.114
DKK 1 (pg/mL)	677.2 (98.8) [B,C]	1012.1 (80.83) [A]	1189.4 (122.8) [A]	10.74	2/153	<0.001	0.123
HbA1c %	4.73 (0.21) [B,C]	7.93 (0.17) [A]	8.23 (0.27) [A]	86.42	2/153	<0.001	0.530
Glucose mM	5.25 (0.17) [B,C]	8.29 (0.14) [A]	8.39 (0.21) [A]	119.76	2/153	<0.001	0.610
Insulin pM	98.3 (4.115) [B,C]	81.3 (3.4) [A]	72.3 (5.2) [A]	9.12	2/153	<0.001	0.107
zIR (z scores)	-0.693 (0.126) [B,C]	0.424 (0.104) [A]	0.195 (0.160) [A]	25.23	2/153	<0.001	0.248
zβCELL (z scores)	1.074 (0.100) [B,C]	-0.383 (0.083) [A]	-0.542 (0.127) [A]	80.39	2/153	<0.001	0.512
zGLUTOX (z scores)	-1.158 (0.090) [B,C]	0.407 (0.075) [A]	0.589 (0.114) [A]	114.47	2/153	<0.001	0.599
TC mM	4.166 (0.136) [B,C]	5.130 (0.112) [A]	5.290 (0.171) [A]	20.366	2/154	<0.001	0.209
TG mM	1.626 (0.102) [B,C]	1.975 (0.084) [A]	2.243 (0.128) [A]	7.053	2/154	0.001	0.084
HDLc mM	1.135 (0.026) [B,C]	1.039 (0.021) [A]	1.035 (0.0321) [A]	5.499	2/154	0.005	0.067
LDLc mM	2.220 (0.129) [B,C]	3.066 (0.106) [A]	3.268 (0.161) [A]	18.981	2/154	<0.001	0.198
zTC-zHDL (z scores)	-0.719 (0.123) [B,C]	0.296 (0.101) [A]	0.443 (0.155) [A]	25.312	2/154	<0.001	0.247
zTG-zHDL (z scores)	-0.485 (0.132) [B,C]	0.167 (0.109) [A]	0.404 (0.166) [A]	10.814	2/154	<0.001	0.123
zLDL-zHDL (z scores)	-0.671 (0.126) [B,C]	0.267 (0.104) [A]	0.439 (0.159) [A]	21.137	2/154	<0.001	0.215

All results of univariate GLM analysis showing the model-generated marginal estimated means (SE) after controlling for age, sex, body mass index, and smoking.

[A, B, C]: Pairwise comparisons among treatment means, HDLc: high-density lipoprotein cholesterol, LDLc: low-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol, HbA1c: glycated hemoglobin; zIR: insulin resistance, zβCELL: beta-cell function, zGLUTOX: index of glucose toxicity, zTC-zHDL: z total cholesterol – z HDL cholesterol (reflects the Castelli risk index 1), zLDL-zHDL: z LDL cholesterol – z HDL cholesterol (reflects the Castelli risk index 2), zTG-zHDL: z triglycerides – z HDL cholesterol (reflects the atherogenic index of plasma).

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Table 3. Correlation matrix showing the partial correlations adjusted for age, sex, BMI, smoking, and exercise.

Parameters	HMGB1	DKK1
HMGB1	1	0.396(<0.001)
HbA1c	0.321(<0.001)	0.433(<0.001)
Glucose	0.338(<0.001)	0.369(<0.001)
Insulin	-0.347(<0.001)	-0.382(<0.001)
zIR	-0.005(0.951)	-0.009(0.913)
zβCell	-0.386(<0.001)	-0.440(<0.001)
zGLUTOX	0.399(<0.001)	0.469(<0.001)
Total cholesterol	0.168(0.036)	0.106(0.186)
Triglycerides	0.333(<0.001)	0.221(0.006)
HDL cholesterol	-0.276(<0.001)	-0.268(0.001)
LDL cholesterol	0.135(0.093)	0.084(0.296)
zTC-zHDL	0.311(<0.001)	0.262(0.001)
zTG-zHDL	0.395(<0.001)	0.317(<0.001)
zLDL-zHDL	0.272(0.001)	0.233(0.003)

HDLc: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol, HbA1c: glycated hemoglobin, Glu: glucose, HMGB1: high mobility group box 1, DKK1: Dickkopf-related protein-1, zIR: index of insulin resistance, zβCELL: index of beta-cell function, zGLUTOX: index of glucose toxicity, zTC-zHDL: z total cholesterol – z HDL cholesterol (index of Castelli risk index 1), zLDL-zHDL: z LDL cholesterol – z HDL cholesterol (index of Castelli risk index 2), zTG-zHDL: z triglycerides – z HDL cholesterol (reflects the atherogenic index of plasma).

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Table 4: Results of multiple regression analysis with glucose/insulin data as dependent variables.

Dependent variables	Explanatory variables	β	t	p	F_{model}	df	p	R^2
#1. HbA1c	**Model**				21.740	3/157	<0.001	0.293
	DKK1	0.391	5.682	<0.001				
	Hypertension	0.251	3.627	<0.001				
	Sex	-0.144	-2.126	0.035				
#2. Glucose	**Model**				16.772	3/157	<0.001	0.243
	DKK1	0.261	3.425	0.001				
	Hypertension	0.238	3.340	0.001				
	HMGB1	0.197	2.621	0.010				
#3. Insulin	**Model**				14.558	3/157	<0.001	0.218
	DKK1	-0.285	-3.672	<0.001				
	HMGB1	-0.207	-2.715	0.007				
	Hypertension	-0.148	-2.047	0.042				
#4 zIR	**Not significant**							
#5. zβCELL	**Model**				22.98	3/157	<0.001	0.305
	DKK1	-0.327	-4.476	<0.001				
	Hypertension	-0.226	-3.305	0.001				
	HMGB1	-0.218	-3.028	0.003				
#6. zGLUTOX	**Model**				27.948	3/157	<0.001	0.348
	DKK1	0.351	4.966	<0.001				
	Hypertension	0.254	3.845	<0.001				
	HMGB1	0.218	3.129	0.002				

HbA1c: glycated hemoglobin, HMGB1: high mobility group box 1, DKK1: Dickkopf-related protein-1, zIR: z score of insulin resistance, zβCELL: z score of beta-cell function, zGLUTOX: z score of glucose toxicity.

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Table 5. Results of multiple regression analysis with atherogenic indices as dependent variables.

Dependent variables	Explanatory variables	β	t	p	F_{model}	df	p	R^2
#1. zTC–zHDL (reflects Castelli risk index 1)	**Model**				11.927	2/158	<0.001	0.131
	HMGB1	0.275	3.668	<0.001				
	Hypertension	0.201	2.689	0.008				
#2. zTG–zHDL (reflects the atherogenic index of plasma)	**Model**				17.685	2/158	<0.001	0.183
	HMGB1	0.288	3.703	<0.001				
	DKK1	0.226	2.906	0.004				
#3. zLDL– zHDL (reflects the Castelli risk index 2)	**Model**				9.601	2/158	<0.001	0.108
	HMGB1	0.240	3.168	0.002				
	Hypertension	0.194	2.563	0.011				
#4. zTG-zHDL	**Model**				16.64	3/157	<0.001	0.312
	GLUTOX	0.389	5.41	<0.001				
	Body weight	0.198	2.98	0.003				
	HMGB1	0.212	2.95	0.004				

HDLc: high-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol, HMGB1: high mobility group box B1, DKK1: Dickkopf-related protein-1.

GLUTOX: Glucose toxicity index computed as the z score of glucose + z score glycated hemoglobin – z score insulin.

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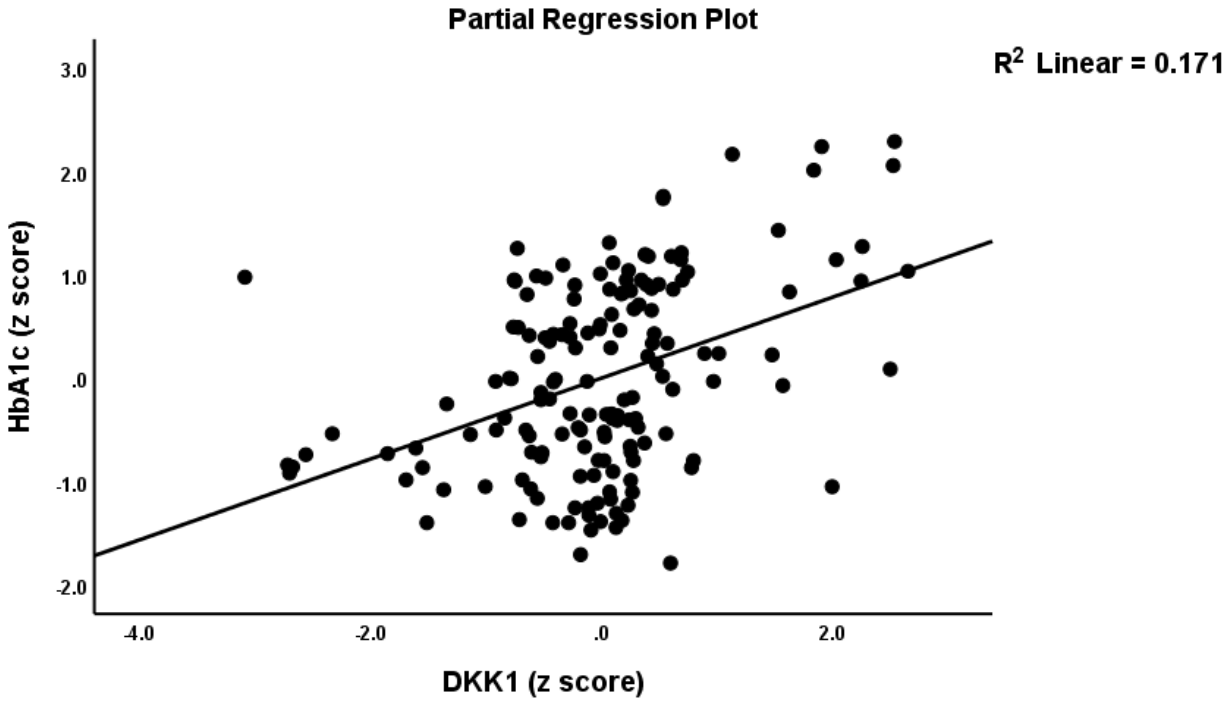

Figure 1 Partial regression plot of glycated hemoglobin (HbA1C) on Dickkopf-related protein 1 (DKK1).

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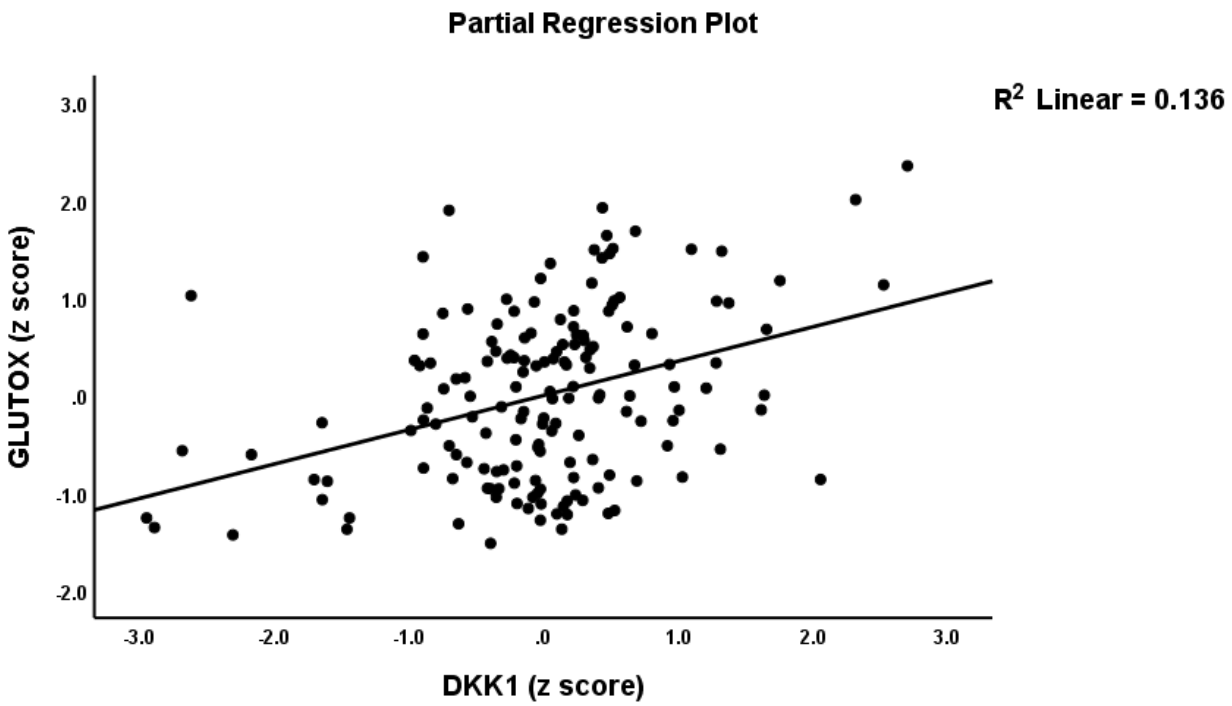

Figure 2 Partial regression plot of an index of glucose toxicity (GLUTOX) on Dickkopf-related protein 1 (DKK1).

The glucose toxicity index is computed as the z score of glucose + z score glycated hemoglobin – z score insulin.

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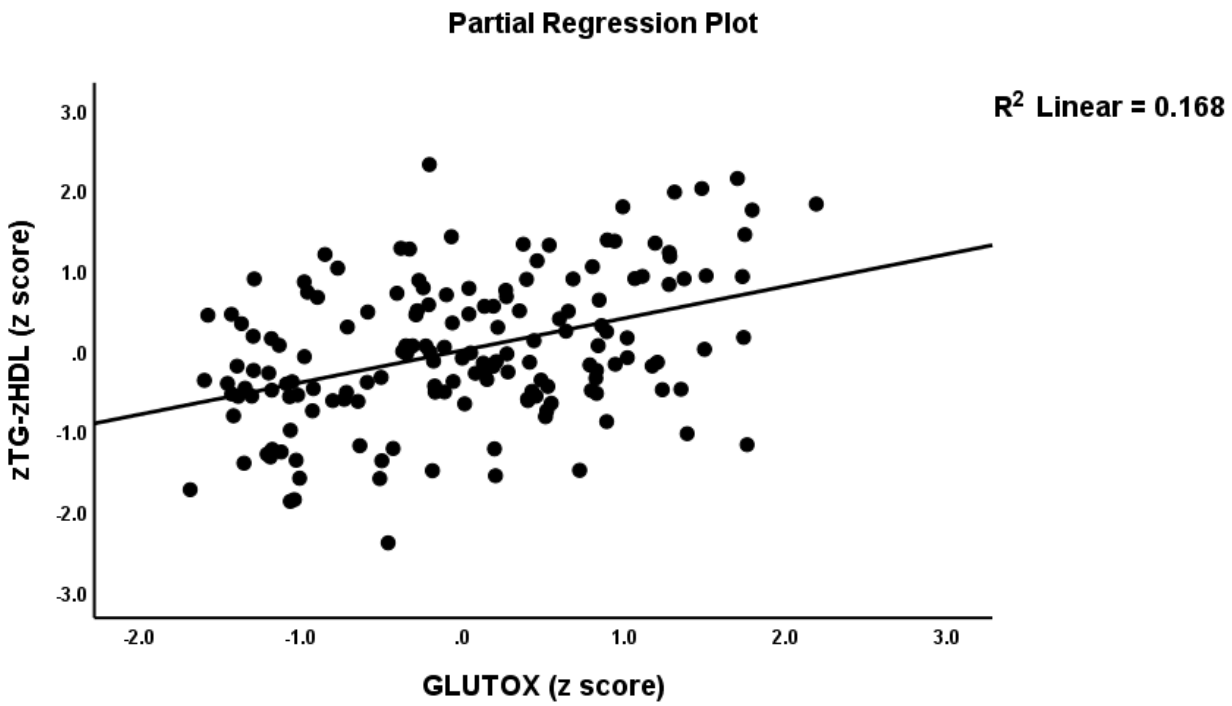

Figure 3 Partial regression plot of the triglyceride / high density lipoprotein cholesterol (zTG-zHDL) ratio on an index of glucose toxicity (GLUTOX).

zTG-zHDL is computed as z score of triglycerides – z score high density lipoprotein cholesterol, and reflects the atherogenic index of plasma

The glucose toxicity index is computed as the z score of glucose + z score glycated hemoglobin – z score insulin.