Association of Endothelin-I and A symmetric Dimethylarginine Levels with Insulin Resistance in Type-2 Diabetes Mellitus Patients

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Abstract:
Endothelin-I (ET-I) is one of the potent vasoconstrictors secreted from endothelial cells when needed. Many studies revealed the elevation of serum ET-I with human diabetes and microangiopathies. Since insulin resistance is a case of mixed diabetic and pre-diabetic cases, many risk factors beyond obesity and inflammation are proposed. The current study aims to demonstrate the association between serum ET-I and asymmetric dimethylarginine (ADMA) and insulin resistance in type 2 diabetes mellitus (T2DM). Sera of 73 subjects were enrolled currently (control= 35 subjects, and 38 with T2DM for more than 7 years), aged (40-60) years old, with distinct body mass index (BMI) ≤ 25 for control volunteers and (BMI) ≥ 25 for obesity and diabetes patients. Peripheral serum ET-I and ADMA levels were significantly (P≤ 0.0001) higher in T2DM than the control subjects. Receiver operating characteristic curve analysis regarded ET-I and ADMA as good markers for T2DM disease and insulin resistance, correlations between ET-I and anthropometrics revealed a strong increase of urotensin-II (UII), ADMA, homeostatic model assessment for insulin resistance (HOMA-IR) and hemoglobin A1C (HbA1C) with an increase of ET-I. These results are supported by the data of multiple regression analysis, showing that HOMA-IR, HbA1C, UII, BMI, and mean arterial pressure (MAP) are related to ET-I independently. The endothelin-I and ADMA had a positive relationship with increase insulin resistance and may serve as prognostic and diagnostic clinical biomarkers of insulin resistance. Collectively, Therefore, these measurements could evaluate the incidence of DM, and help to better rise up the knowledge about the progression of DM complications.

Keywords: ADMA, Endothelin-I, HOMA-IR, Insulin Sensitivity, Urotensin-II.

Introduction:
Endothelin-I (ET-I) is one of the potent vasoconstrictors synthesized and secreted from endothelial cells of vasculatures. This argumentative vasoconstrictor is still under debate through its vast effects and diverse pathways of action, ET-I has been implicated in the progression of diabetes mellitus and cardiovascular diseases (CVD), through endothelial dysfunction. Diabetes mellitus is the disability of tissues to use glucose, initiating a cascade of unwanted events, passing from hyperglycemia, insulin resistance toward cardiovascular events, and endothelial dysfunction. The molecular basis of hyperglycemia is the key role in mediating oxidative stress through damaging of cellular deoxyribonucleic acid (DNA) and production of adenosine di-phosphate ADP-ribose with derangement of transcription levels; the high glucotoxicity in the blood activates nuclear factor-κB (NF-κB) increased inflammation events. According to recent studies, the vasculature remodeling and profound vasoconstriction is the priority mechanism of DM through the implication of nitric oxide (NO) production and increasing the secretion of vasoactive substances as ET-I and UII.

Asymmetric dimethylarginine has pleiotropic cardiomyopathy effects associated with DM, NO resistance-associated aging, and an increase in ADMA levels have been reported with the overproduction of reactive oxygen species (ROS). According to Muniyappa et al. insulin...
has a pivotal role in regulating vascular tone through increasing endothelial NO synthase production (eNOS) and secretion of ET-1 through the myosin-activated protein kinase (MAPK) pathway. In insulin resistance subjects, the deterioration of NO leads to an increase of ET-1, thus unbalancing the entire system and mitigate the NO

There is also an established relationship between increased ADMA levels and insulin resistance regarding obesity in patients with DM. To date, no studies have investigated the relationship between ET-1 and ADMA with insulin resistance, accordingly, this case-control study aims to establish the relation between ET-1 and ADMA in insulin-resistant subjects.

Materials and Methods:

Ethics statement: The study has been approved by the local ethics committee of Hawler Medical University, Erbil/Iraq (protocol code 4).

Subject features and study design: This case-control study was executed at the Department of Biology/Salahaddin University-Erbil/Iraq. Sera samples were obtained from 73 individuals aged 40-60 (35 volunteers without DM with BMI ≤ 25, and 38 patients with DM and BMI ≥ 25). Detection of insulin resistance was examined by HOMA-IR test. Subjects with systemic diseases, thyroids, insulin resistance was examined by HOMA-IR test.

Measurement of nitric oxide (NO) and malondialdehyde (MDA): Nitric oxide levels were evaluated indirectly using Griess’s reaction according to conversion to nitrate concentration in the cadmium filled medium, then converted to nitric acid, serum levels were measured by spectrophotometry at 543 nm (UNICO spectrophotometer SN SQU10111012002) MDA, measured through a reaction of thiobarbituric acid TBA with lipid peroxidation end products (UNICO spectrophotometer SN SQU10111012002).

Statistical analysis: The data are expressed as mean value ± standard error of the mean (SEM) unless otherwise detected. The t-test was used to compare between all subjects by using GraphPad Prism 8 software, a p-value less than 0.05 is statistically significant. Unpaired. Mann-Whitney t-test was used for non-parametric variables, receiver operating characteristic curve was applied to compare the sensitivity of variables in all subjects. Statistical package for social science (SPSS) version 25.0 (IBM Corp, released 2017, IBM SPSS statistics for windows, Version 25.0, Armonk, NY) was applied to analyze the correlation coefficient of ET-1 and all other anthropometric and clinical parameters, Spearman and Pearson (r) correlation was used, stepwise multiple regressions were performed to predict the relationship of ET-1 as a dependent variable with other independent variables.

Results:

The anthropometric measurements and clinical characteristics of this case-control study are summarized in Table 1. ET-1 increased significantly in DM patients compared to the control group, however, non-significant differences were noticed.
for insulin, LDL, and urea levels among the groups, (0.5240), (0.6737), and (0.8623) are p-values arranged respectively for each item individually. Insulin in hyperglycaemic group 0.7432(0.4920-1.540) is slightly different from the control group 0.7466(0.5734-2.978) but still, they are not significant. The same result is observed in data of comparing LDL in control group 99.70(57.30-139.8) with diabetic group 100.6(52.80-199.2) and comparing urea levels in control group 29.00(15.08-49.80) with diabetic group 28.50(3.30-67.00). Pearson’s correlation analysis shown in Table 2 was used to evaluate the profound correlation between ET-I and other metabolic parameters. In this study, ET-I exhibited a positive correlation with most of the parameters except with insulin, LDL, and Urea. ET-I correlated strongly to most of the parameters mentioned before with p<0.0001 significant value especially waist circumference, BMI, weight, SBP, glucose, HbA1c, HOMA-IR, QUICKI, NO, ADMA, and UII, the other parameters are correlated with less significant levels but they still strongly correlated to ET-I. According to data illustrated in Fig. 1, ROC curves, serum ET-I concentrations are significantly higher in DM patients and hence, both diabetes and non-diabetes groups were compared using t-test, the area under the curve in females was slightly higher than males but the total AUC was 0.9117 with a 0.0001 significant level. Correlations between ET-I and other parameters in Fig. 2. The results revealed strong relations between ET-I and insulin-resistant indices as waist circumference, BMI, MAP, FBS, HbA1C, HOMA-IR, NO, ADMA, DDAH. The results of the ROC curve revealed high values of AUC for male (85%) and female (97%) subjects. There was a positive association between ET-I and other parameters, this showed using a multivariate regression model by adding parameters one by one (Table 3).

### Table 1. Anthropometric and clinical characteristics for diabetic and non-diabetic participants (Mean±SEM).

| Parameters | Non-diabetic (n=35) | DM (n=38) | p-values |
|------------|---------------------|-----------|----------|
| ET-I       | 6.62±0.4042         | 11.20±0.4167 | 0.0001   |
| FBS        | 90.88±2.467         | 248.3±9.476  | 0.0001   |
| HbA1C      | 5.42±(4.000-6.080)  | 8.26(6.330-11.20) | 0.0001   |
| Insulin    | 0.7466(0.5734-2.978)| 0.7432(0.4920-1.540) | 0.5240   |
| HOMA-IR    | 0.1880(0.112-1.217) | 0.4142(0.2199-1.407) | 0.0001   |
| QUICKI     | 0.5328(0.3491-0.6280)| 0.4415(0.3651-0.5390) | 0.0001   |
| ADMA       | 1.755(0.1739-6.546) | 3.998(0.8808-11.73) | 0.0001   |
| DDAH       | 47.84±4.759         | 98.28±7.343  | 0.0001   |
| MDA        | 2.898(0.2940-8.820) | 10.37(7.266-16.04) | 0.0001   |
| NO         | 6.988(0.1469-16.08) | 20.33(8.103-104.0) | 0.0001   |
| Cholesterol| 188.7(146.0-210.0) | 210.0(163.0-367.0) | 0.0003   |
| HDL-C      | 58.76(40.80-83.00)  | 47.00(31.00-97.00) | 0.0057   |
| LDL-C      | 99.70(57.30-139.8)  | 100.6(52.80-199.2) | 0.6737   |
| VLDL-C     | 28.97(11.00-41.40)  | 38.00(14.80-151.4) | 0.0004   |
| TG         | 141.8(55.00-207.0)  | 200.5(107.0-757.0) | 0.0001   |
| AST        | 21.00(7.000-31.00)  | 27.23(20.00-47.00) | 0.0001   |
| ALT        | 19.10(10.00-31.00)  | 29.00(20.00-58.00) | 0.0001   |
| ALP        | 60.50(41.00-113.0)  | 72.00(33.00-210.0) | 0.0426   |
| Urea       | 29.00(15.08-49.80)  | 28.50(3.300-67.00) | 0.8623   |
| Creatinine | 0.7442(0.4000-0.8900)| 0.9150(0.7000-1.890) | 0.0001   |
| Uric acid  | 4.568±0.2971        | 6.530±8.580  | 0.0022   |

DM: diabetes mellitus; BMI: body mass index; HOMA-IR - homeostasis model assessment of insulin resistance; QUICKI - quantitative insulin sensitivity check index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein; VLDL-C: Very low-density lipoprotein cholesterol; ET-I: endothelin-I; DDAH: dimethyl diethyl amino hydrolase; ADMA: asymmetric dimethylarginine; MDA: malondialdehyde; NO: nitric oxide; HbA1c: hemoglobin A1C; AST: aspartate aminotransferase; ALT: alanine transaminase; ALP: alkaline phosphatase.
Table 2. Correlation coefficient of Endothelin-I with anthropometrics and metabolic parameters of the study parameters:

| parameters                  | Endothelin-I | (n=73) |
|-----------------------------|--------------|--------|
| Age                         | 0.213        | 0.056  |
| Waist circumference         | 0.547        | 0.0001 |
| Weight                      | 0.463        | 0.0001 |
| Height                      | -0.296       | 0.013  |
| BMI                         | 0.592        | 0.0001 |
| SBP                         | 0.567        | 0.0001 |
| DBP                         | 0.419        | 0.001  |
| MAP                         | 0.387        | 0.001  |
| Glucose                     | 0.722        | 0.0001 |
| HbA1c                       | 0.595        | 0.0001 |
| Insulin                     | 0.108        | 0.212  |
| HOMA-IR                     | 0.527        | 0.0001 |
| QUICKI                      | -0.541       | 0.0001 |
| Cholesterol                 | 0.212        | 0.056  |
| Triglyceride                | 0.370        | 0.002  |
| HDL-C                       | -0.273       | 0.020  |
| LDL-C                       | 0.004        | 0.489  |
| VLDL-C                      | 0.323        | 0.007  |
| Urea                        | 0.047        | 0.363  |
| Creatinine                  | 0.377        | 0.002  |
| Uric acid                   | 0.329        | 0.006  |
| eGFR                        | 0.339        | 0.005  |
| ALP                         | 0.318        | 0.008  |
| AST                         | 0.413        | 0.001  |
| ALT                         | 0.369        | 0.002  |
| NO                          | 0.501        | 0.0001 |
| MDA                         | 0.397        | 0.001  |
| ADMA                        | 0.466        | 0.0001 |
| DDAH                        | 0.324        | 0.007  |
| UII                         | 0.567        | 0.0001 |

DM: diabetes mellitus; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; HOMA-IR - homeostasis model assessment of insulin resistance; QUICKI - quantitative insulin sensitivity check index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low density lipoprotein cholesterol; ET-I: endothelin-I; DDAH: dimethyldiethyl amino hydrolase; ADMA: asymmetric dimethyl arginine; MDA: malondialdehyde; NO: nitric oxide; eGFR: estimated glomerular filtration rate; HbA1c: hemoglobin A1C; AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase; UII: urotensin-II.

Figure 1. Receiver operating characteristics curves for Endothelin-I in both males and females, AUC=area under the curve.
Discussion:

Vascular endothelium is responsible for the vessel's health and disease, once the integrity of this fundamental layer overexposed to potential risk factors, the endothelial dysfunction (ED) develops to atherosclerosis, the impairment of endothelium up-regulates reactive oxygen species ROS via decreasing the production of NO. Generally,
Hyperglycemia has been well known for its corrosive effect on the endothelium of the vessels leading to ED through the deterioration of NO production and vasoconstrictors of deep impact on the vessels as ET-I. This unconventional risk factor is responsible for developing coronary syndromes by the generation of pro-oxidants to vasoconstrictor events.

ET-I is a hormone discovered first from endothelial cells of the porcine aorta with 21 amino acids, different authors have hypothesized the mechanisms of action of ET-I as decreases in NOS and insulin-stimulated blood flow to small capillaries. Although extensive studies have been carried out on ET-I and diabetes, no previous study was undertaken to investigate the correlation between ET-I and both insulin resistance markers and ADMA which were done currently. On average, parameters were shown to have a significant correlation with ET in both non-diabetic and diabetic patients. Overall, ET-I did not affect non-diabetics and diabetics differently in serum insulin, LDL, and urea measurements. One paradoxical result has already drawn attention to the stability of serum insulin in diabetic’s patient despite increasing serum glucose and HOMA-IR in blood, the only explanation, is due to proinsulin, is a marker of insulin resistance to diagnose T2DM, secreted by dysfunctional pancreatic β-cells. It activates the MAP-Kinase pathway via binding to the insulin receptor, initiating inflammations, and secreting ET-I.

The results of the correlational analysis showed a strong correlation of serum ET-I with serum glucose, HbA1c, HOMA-IR, and HOMA-IS. These indices are increasing with further exposure to metabolic or insulin resistance risk factors, stress, obesity, improper lifestyle, and high fat and carb diets, which are all increasing the risk of insulin resistance to T2DM.

Compared to normoglycemic subjects, ET-I, glucose, HbA1c, HOMA-IR, and HOMA-IS were significantly higher in glycemic subjects indicating a strong relation of insulin resistance and increased circulating levels of ET-I in diabetic patients.

Recently, have found that there is a strong and strict relationship between increasing ET-I and ADMA levels in vivo. It’s well-known that ADMA is one of the highlighted cardiovascular risk factors, since it’s an endogenous NOS inhibitor, ET-I further inhibits NO production by inhibiting NOS, thus ED is a multifactorial disorder affected by both ET-I and ADMA as well. Moreover, NO regulation of production is not limited to L-arginine only, but on other cellular cofactors and substrates, thus when ADMA (an endogenous competitive antagonist of L-arginine at the active site of NOS) increased in the blood it inhibits NOS. Following the present results, ADMA is hydrolyzed and degraded by the enzyme DDAH, which was significantly increased in diabetic subjects. It can be seen from the data in Table (1) that ADMA and ET-I are increased both significantly, the findings observed recently mirror those of the previous studies but in vitro designs, suggesting increasing oxidative stress and free radicals of metabolic origin increasing the incidence of endothelial dysfunction leading to DM and insulin resistance. These effects can be antagonized by using ETA receptor antagonists. ADMA is one of the potent guanidino-substituted analogs of L-arginine inhibits NOS and has a role in the implication of cardiovascular diseases. Many hypotheses suggest lipid hydroperoxides as the main cause of increasing ADMA, but the detailed mechanism is still under debate.

The empirical findings in this study provide a new understanding of the relations between ET, ADMA, and insulin resistance. Both impair NO production through the deterioration of enzyme NOS, but the turning point is that not only L-arginine regulates the production of NO, thus it’s not necessarily ADMA responsible for inhibiting NO production. A corporation of ET-I is served in this phenomenon by further inhibiting NO production and increasing oxidative and glycostress through decreasing insulin availability for blood flow. Accordingly, our study suggests a strong correlation between ET, ADMA, and insulin resistance.

It is also worthy to mention that ET-I is significantly more frequently increased in glycemic subjects with increased indices of cardiometabolic risk factors, waist circumferences, BMI, elevated SBP, DBP, decreased levels of HDL-C, and increased LDL-C. Increasing BMI is further associated with the initiation of insulin resistance, these findings are confirmed by other previous studies. Waist circumferences are additionally related to abdominal obesity or intra-abdominal fat, increasing waist circumferences increase the incidence of insulin resistance. Subsequent complications of increased ET-I has increased another vasoconstrictor of interest, UII. Results from stepwise multiple regressions in Table (3) represent UII as an independently associated indicator of ET-I, upon this result we can hypothesize UII as a trigger of insulin resistance cascade through increasing oxidative damage of endothelial layer and modifying c-Jun N-terminal kinase phosphorylation, which is a common pathway of insulin underlying transduction.
mechanism. NADPH oxidase is another trigger of UII to phosphorylate kinases and production of ROS of different origins, this action could be attenuated by apocynin, an inhibitor of NADPH. UII can stimulate ET-I since it is 10 times potent than ET, and increases the production of mitochondrial ROS. The most striking result to emerge from the data is that integrating ET-I as a dependent parameter, and UII, HOMA-IR, and HbA1C as independent parameters offer some important insights into understanding the role of ET-I in deteriorating and increasing incidences of insulin resistance.

Conclusions:
One of the most significant findings of this study is that determination of serum ET-I level permits early detection of insulin resistance cases and this relation is supported by our study findings. From data of HOMA-IR and HbA1C, we can conclude that an increase in ET-I levels may indicate irreversible and sustained insulin resistance. Moreover, strong pieces of evidence of the contribution of independent variables as ADMA and UII are found when correlated to ET-I levels, interestingly, there is a significant positive correlation for those subjects with ET-I as a dependent variable. Generally, we can suggest that the idea of the contribution of ADMA and UII with ET-I in diabetic patients is highly adopted by our case study. Independently, BMI, blood pressure, and waist circumferences are significantly correlated with ET-I and have a great enrollment in the development of endothelial dysfunction and releasing ET-I through increasing incidences of insulin resistance.

It would be interesting to assess the effects of proinsulin markers to further investigate the relation of ET-I and UII with insulin resistance; further work needs to be done to establish whether ET is secreted before UII or not toward the exact pathway of ET-I and UII independently through insulin resistance. More investigation and experimentation into ET-I, ADMA, and UII are strongly recommended besides their role as triggers of endothelial dysfunction.

Authors' declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for republication attached with the manuscript.
- The author has signed an animal welfare statement.

Authors' contributions statement:
The authorship of the title above certify that they have participated in different roles as follows:
*is collecting the entire sample and enrollment the research results, discussion and writing (Ismail M. Maulood) implements the idea, experimental design and data analysis with interpretations, (Almas MR Mahmud) contributes in proof reading, interpretation and writing.

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