Original article:

Insulin Resistance and markers of Endothelial dysfunction in Metabolic Syndrome, Delhi; a case-control study

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Abstract:
Background: Insulin resistance is strongly associated with components of the metabolic syndrome (MS) including endothelial dysfunction. Our present work aims to study markers of endothelial dysfunction and calculate insulin resistance in patients fulfilling the definition of MS and to compare the results with healthy controls.

Methodology: A hospital based observational, case-control study was conducted. It included 46 cases of MS (according to International Diabetes Federation-2006 criteria) attending medicine OPD of a tertiary care hospital of New Delhi and 46 healthy volunteers. The study was ethically cleared by hospitals’ ethical committee and written consent was taken from the study population. Routine chemistries, plasma insulin levels, Endothelin-1 (ET-1) and Nitric oxide (NO) were estimated. Insulin resistance was assessed using HOMA-IR. Appropriate statistical tests were applied using SPSS.

Results: The results of the study are expressed as Mean± Standard error of Mean. The mean fasting plasma Insulin level was significantly high among cases [11.7 ±1.7 mU/L vs 6.93±0.6mU/L, (p<0.05)]. The mean value of HOMA-IR was significantly high among cases [5.3 ±1.2 vs 1.3 ±0.1, (p<0.001)]. The mean plasma NO level was significantly high among cases [22.5 ±2.9 µmole/L vs 14.1±2.5 µmole/L, (p<0.05)]. The mean plasma Endothelin-1 (ET-1) level was significantly high among cases [(8.6 ± 0.6 pg/mL vs. 5.0 ± 0.3pg/dL, (p<0.001)]. Significant positive correlation was seen in Insulin and NO levels (p value <0.05, r=0.348) whereas no significant correlation was seen in between Insulin and ET-1. On regression analysis, association of Insulin and NO was seen (Standardized coefficient beta=0.348, t=2.4, p value<0.05).

Conclusion: In present study, patients with metabolic syndrome had significantly higher Insulin, Insulin resistance, higher levels of Endothelin-1 and higher Nitric oxide levels. The characteristic finding of endothelial dysfunction of reduced Nitric oxide is absent. It points to other mechanisms leading progression of the disease process. An understanding of the primary mechanisms resulting in these phenotypes may reveal new therapeutic targets in metabolic and cardiovascular disease.

Keywords: Cardio-metabolic Syndrome, Insulin Resistance, Endothelial Dysfunction, Nitric oxide, Endothelin-1, HOMA-IR.

Introduction
Metabolic syndrome is a serious health concern. The global burden of diabetes mellitus has been estimated at 382 million and is going to rise to 592 million by the year 2035 [1]. Number of Type 2 diabetes mellitus (T2DM) patients is increasing in both developed and developing countries but 80% contribution is from low and middle income countries.
Asian Indians are a high risk population with respect to Diabetes, CAD and the numbers are consistently on a rise. Prevalence of MS (study done in 2010) in urban Indians according to IDF criteria is 51.8% [3]. Insulin resistance (IR) is postulated to be the common underlying pathogenic link between the various components of the MS and may explain the presence of the MS even in non-obese subjects [4, 5]. Diabetes is a metabolic disorder characterised by chronic hyperglycaemia. The long-term effects of diabetes mellitus include cellular injury, inflammation and failure of various organs [6]. The complications of diabetes are divided into macrovascular complications i.e., coronary artery diseases, peripheral vascular disease and stroke and, microvascular complications i.e., diabetic nephropathy, retinopathy and neuropathy [7]. Among all complications, endothelial dysfunction is a common problem in all diabetic patients. Endothelial cells secrete different mediators such as vasodilators i.e., nitric oxide, and vasoconstrictors i.e., endothelin-1. Hyperglycaemia and other metabolic changes may lead to impairment of nitric oxide (NO) production [6]. Impairment of endothelial function in T2DM patients ultimately leads to cardiovascular diseases. Nitric oxide is a gaseous molecule secreted by the endothelium and a major modulator of endothelial function [9]. NO is synthesized from L-arginine by the family of enzymes called nitric oxide synthases (NOSs) viz. neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) [10]. NO is a key regulatory molecule with extensive metabolic, vascular, and cellular effects [11]. While low levels of NO is beneficial for several physiological and cellular functions, high levels of NO may cause detrimental effects in the cells. High levels of NO may react with superoxide anion to generate peroxynitrite radical, which binds to proteins and thus affects their function [12]. Altered serum NO levels in T2DM were reported by different investigators previously [13–15]. The serum NO data in T2DM patients that reported by different scientific literature is controversial. Some research articles reported increased NO levels in diabetes patients [14] whereas others reported the opposite [15]. The presence of endothelial dysfunction can be regarded as a clinical syndrome that per se is associated with and predicts an increased rate of adverse cardiovascular events [16]. Endothelial dysfunction, the initial step of atherosclerotic process is induced by abnormal insulin signalling in the vasculature. It is however unclear whether the impact of the MS variables on various outcome parameters, including endothelial function, is uniform across ethnicity. Not much data is available on the development of endothelial dysfunction in population at risk of developing Diabetes or CAD later in life. The observation that certain individuals do not develop atherosclerotic manifestations despite the presence of several cardiovascular risk factors suggests the existence of a “threshold switch” that, only when activated, translates the risk factor into an unfavourable vascular effect [17].

The present study was undertaken to determine association between insulin resistance and endothelial dysfunction markers: Endothelin-1 (ET-1) and Nitric oxide (NO) in newly diagnosed cases of Cardio-metabolic syndrome and to compare the findings with healthy controls.

Material and methods

This observational, analytical, cross sectional study was conducted in the Department of the Biochemistry in collaboration with the Department of Medicine for

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a period of one year in a tertiary care hospital in New Delhi.

Patients attending the Diabetes clinic with MS [according to criteria of International Diabetes Federation (IDF)] were recruited in a non-randomized manner after a bilingual written informed consent. They were newly diagnosed cases of Diabetes, duration less than five years. As the IDF criteria does not have waist circumference cut-off for Indian populations, we used the cut offs for South Asian population. A total of 46 patients both males and females, meeting the inclusion criteria and 46 healthy matched controls were enrolled into this study. Patients having any ongoing acute or chronic inflammatory conditions or with any type of debilitating illness were excluded from the study. Ethical approval was obtained from scientific review board and ethics committee of institution.

Upon enrolment, a structured and standardized questionnaire was applied concerning the parameters of interest. Data regarding demographic profile, family history of cardiovascular disease (CVD), diabetes mellitus, systemic hypertension, cigarette smoking, use of medications that might affect the lipid profile and physical inactivity was obtained. Measurement of adiposity (waist circumference, body mass index), systolic blood pressure (SBP), and diastolic blood pressure (DBP) was done.

Samples were drawn in appropriate vaccutainers for estimation of blood glucose, serum lipid profile (total cholesterol, LDL, HDL and triglycerides), LFT, KFT, fasting insulin, plasma Nitric oxide levels and plasma Endothelin levels.

The routine biochemistry (blood glucose, lipid profile, LFT, KFT) was analyzed immediately. Plasma was separated and stored at -20 C and batch analysis was done for Insulin, NO and Endothelin-1 levels.

**Assays**

Blood glucose levels were estimated using glucose-oxidase (GOD-POD) method, Serum urea was estimated using urease-GLDH kinetic method and serum creatinine levels were measured using modified jaffe’s kinetic method. Liver enzymes ALT, AST and ALP were measured using IFCC recommended enzymatic methods. Serum Total Cholesterol was estimated by a timed end point method by Richmond L (1973). Serum Triglyceride was estimated by a timed end point method Jacobs N et al. HDL Cholesterol kit was used for estimation of HDL cholesterol by PEG precipitation and enzymatic method. All the mentioned biochemical tests were done using automated analyzer (AU480, Beckman Coulter). LDL- Cholesterol was estimated by Friedwald’s formula (LDL-C = Total Cholesterol – (HDL + VLDL) where VLDL = TG/5).

Serum Insulin (fasting) were estimated using chemiluminescence based immunoassay; with coefficient of variation 5.10% (Beckman Access II, Beckman Coulter, Inc., Fullerton, CA). Insulin resistance was assessed using HOMA-IR calculated with the formula; [HOMA-IR = fasting plasma glucose (mmol/L) × fasting plasma insulin (mIU/L)/22.5]. Plasma endothelin was estimated by ELISA using DRG’s human Endothelin-1 Enzyme Immunometric Assay kit. Determination of NO in plasma was performed indirectly by the measurement of stable decomposition product nitrite (NO2), employing the Griess reaction (Mathew et al. (1996) . Nitrite can be directly detected by observing the magenta colored azo dye that is formed from nitrite (NO2) and the Griess reagent, the absorbance of which was determined at 543 nm using
semiautoanalyser. The detection limit of the assay is approximately 1.0 nmole nitrite/well, or 10 µM.

Statistical analysis

Continuous variables were expressed as mean ± standard error of mean (SEM), and categorical variables as percentages (%) and number of observations. Means of continuous variables were compared using unpaired 2-tailed Student’s t-test. Pearson’s correlation coefficient was used for measurement of association. P< 0.05 was considered as significant. The statistical package for the social sciences programme (SPSS) version 19.0 was used in all analysis.

Results:

Demographic profile and anthropometric characteristics is depicted in Table 1. The study population both cases and controls, consisted of 74% Females and 26% Males. More than 40% of the study group was in age group of 41-50 years.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Cases</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>50.3 ± 1.4</td>
<td>49.0 ± 0.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex</td>
<td>12 M, 34F</td>
<td>12M, 34F</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Waist circumference in cm</td>
<td>98.8 ± 1.0</td>
<td>86.8± 1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hip circumference in cm</td>
<td>99.8 ±1.3</td>
<td>89.4 ± 1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist-Hip ratio</td>
<td>0.99 ± 0.0</td>
<td>0.97± 0.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Body Mass Index kg/m²</td>
<td>27.2 ± 0.6</td>
<td>23.2 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic Blood Pressure mm Hg</td>
<td>137.8 ±12.9</td>
<td>129.6 ± 7.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic Blood Pressure mm Hg</td>
<td>85.2 ± 9.3</td>
<td>74.8 ±4.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Mean waist circumference, mean hip circumference, mean waist/hip ratio and BMI were significantly higher in cases than in controls. BMI of both groups is in the upper part of normal range (18-25 kg/m²) indicating susceptibility of control group also for Cardio-metabolic syndrome. Metabolic profile in study group shows significantly higher Systolic BP and Diastolic BP in cases as compared to controls. The distribution of risk factors of cardio-metabolic syndrome in cases is depicted in Table 2. It shows all cases in present study group had central obesity, deranged blood sugar fasting and hypertension.
Table 2: Distribution of risk factors defining cardio-metabolic syndrome in cases (n=46)

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>% of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference (Male&gt;90cm,Female&gt;80cm)</td>
<td>100</td>
</tr>
<tr>
<td>BMI (&gt;30kg/m²)</td>
<td>19.6</td>
</tr>
<tr>
<td>Fasting blood glucose &gt;100 mg/dl</td>
<td>96</td>
</tr>
<tr>
<td>Hypertension (SBP&gt;130 mm/Hg or DBP&gt;85 mm/Hg or antihypertensive treatment)</td>
<td>100</td>
</tr>
<tr>
<td>Serum triglycerides&gt;150 mg/dl</td>
<td>63</td>
</tr>
<tr>
<td>HDL levels (Males&lt;40 mg/dl, Females&lt;50 mg/dl)</td>
<td>70</td>
</tr>
<tr>
<td>Family history of Diabetes, Hypertension, CAD</td>
<td>35</td>
</tr>
</tbody>
</table>

SBP-systolic blood pressure  
DBP-diastolic blood pressure  
BMI- body mass index  
HDL- high density lipoprotein  
CAD-coronary artery disease

Table 3 and 4 depicts biochemical parameters in cases and controls. Significant difference was observed in lipid profile, kidney function tests, and blood glucose profile. The difference in Uric acid levels among cardio-metabolic cases and normal controls was highly significant, being<0.001. Similarly we observe that the difference in serum Triglyceride levels were also highly significant (<0.001) compared to other lipid parameter like VLDL and HDL. No significant difference was observed in Serum Cholesterol levels.
Table 3: Routine biochemical parameters in study group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cases (n=46)</th>
<th>Controls (n=46)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mg/dL)</td>
<td>154.3 ± 12.1</td>
<td>83.5 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Triglyceride mg/dl</td>
<td>165.7 ± 10.2</td>
<td>117.9 ± 7.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum VLDL mg/dl</td>
<td>36.6 ± 2.8</td>
<td>23.6 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum HDL mg/dl</td>
<td>44.6 ± 1.5</td>
<td>51.2 ± 2.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urea mg/dl</td>
<td>26.9 ± 1.2</td>
<td>23.6 ± 0.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>1.0 ± 0.0</td>
<td>0.9 ± 0.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Uric acid mg/dl</td>
<td>5.0 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4: Special Tests results in study group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cases Mean ± SEM</th>
<th>Controls Mean ± SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Insulin (mU/L)</td>
<td>11.7 ± 1.7</td>
<td>6.9 ± 0.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.33 ± 1.25</td>
<td>1.36 ± 0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nitric oxide µmole/L</td>
<td>22.5 ± 2.9</td>
<td>14.1 ± 2.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Endothelin -1 pg/mL</td>
<td>8.6 ± 0.6</td>
<td>5.0 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Endothelial dysfunction markers: The mean plasma NO level was significantly higher in cases than in controls (p<0.05). The mean plasma Endothelin-1 (ET-1) level among cases was also higher in cases than in controls (p<0.001).

Association of insulin resistance and endothelial dysfunction in Cardio-metabolic syndrome: After applying Pearson’s correlation coefficient, association between Insulin resistance and ET-1 is found to be statistically non-significant. Whereas, significant positive correlation was seen in Insulin and NO levels (p value <0.05, r=0.348). The regression analysis was applied in cases group; Insulin as the constant and NO levels as dependent variable confirmed association of the two parameters (Standardized coefficient beta=0.348, t=2.4, p value<0.05).

![Correlation between Insulin resistance and Nitric oxide.](image)

**Fig 1: Correlation between Insulin resistance and Nitric oxide.**

**Discussion:**

There are several limitations inherent in the study design. First, this was a non-randomized observational, analytical, case control study with small sample size. Although the gold standard as measure of insulin resistance is the hyperinsulinemic glycemic clamp method [18], because of its simplicity, the HOMA index has been used as a surrogate of insulin resistance and of beta-cell function in this study as in present day large clinical or population-based studies[19]. Assessment of endothelial dysfunction remains a challenge as currently available techniques are expensive, technically difficult and time consuming. Yet, for practical reasons, it is the current standard to measure endothelial function by the study of its vasomotor regulation function. It is also customary to use the generic term ‘endothelial function’ as a synecdoche for endothelium-dependent vascular reactivity.

In the present study, endothelial function markers; plasma NO level and plasma Endothelin-1 (ET-1) level among cases was higher than in controls. We have hypothesised that increased glucose levels in blood may enhance the nitric oxide levels in blood as significant positive correlation was seen in Insulin and NO levels by correlation and regression analysis.
It has been noted in previous literature, elevated levels of glucose may enhance NO production through increased expression of eNOS and iNOS gene and protein levels [20–23]. Although several works showed decreased levels of nitric oxide and increased endothelin1 in T2DM patients [23], increased levels of NO were also reported by other investigators [13]. Some studies have shown that NO levels were increased in T2DM patients with coronary artery disease but not with hypertension. The conflict among the results of previous studies might be due to geographical location and genetic background of the population. Smoking and food habit may also affect serum NO levels as reported previously [24]. It is usual to expect that endothelial dysfunction is characterized by reduced production of nitric oxide (NO) and increased synthesis and secretion of endothelin-1[25]. The vasoconstrictor response to various stimuli is increased by endothelin-1 concentrations, which are elevated in the plasma of patients with early and advanced atherosclerosis as well as in culprit lesions [26]. Decreased NO production promotes platelet aggregation and release of growth factors in the vessel wall and reduced vasodilator effect [27]. Studies in past have reported significantly lower NO levels in subjects having diabetes since more than 5 years compared to the subjects having a history of diabetes for less than 5 years. Their cell culture data with HUVEC cells also confirmed that high glucose exposure enhanced NO production at early time point but reduced subsequently after exposure for a longer duration [28]. In our study the cases were newly diagnosed cases of Diabetes, duration less than five years. Increase in NO might be a step towards pathogenesis of endothelial dysfunction which finally results in reduced vasodilators and increased vasoconstrictors.

**Conclusion:**
Asymptomatic subjects with Cardio-metabolic syndrome had greater endothelial dysfunction in comparison to subjects without syndrome. Both insulin resistance & endothelial dysfunction are associated independently with MS. Nitric oxide, along with endothelin-1, markers of endothelial dysfunction is seen to rise in early cases of metabolic syndrome. Rise in NO could be due to hyperglycemia or due to stimulation of iNOS gene. A follow up of cases will throw light on the sequence of events leading to complication of MS and can help in development of new therapeutic targets.  

**Clinical implications and public health perspective:**
Insulin resistance and endothelial dysfunction could potentially be used as markers for the detection of early atherosclerotic process. Multiple invasive and non-invasive techniques have been developed to assess endothelial function and insulin resistance. However, assessment of endothelial function remains primarily a research area, as currently available techniques are expensive, technically difficult and time consuming. There is requirement of validated screening tools for assessing endothelial dysfunction in the clinical setting.

**Conflict of interest:** Conflict of interest declared none.
References:


