

Evaluation of Ischemia-Modified Albumin, Malondialdehyde, and Advanced Oxidative Protein Products as Markers of Vascular Injury in Diabetic Nephropathy

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ABSTRACT

AIM: This study aimed at evaluation of ischemia-modified albumin (IMA), malondialdehyde (MDA), and advanced oxidative protein products (AOPP) as markers of vascular injury in diabetic nephropathy (DN) with derivation of cutoff values for the same.

MATERIALS AND METHODS: Study population comprised 60 diabetes patients and 30 controls, with diabetes patients further categorized into three groups based on urine albumin/creatinine ratio (UACR) of <30 mg/g (diabetes without microalbuminuria), 30–300 mg/g (early DN), and >300 mg/g of creatinine (overt DN). Serum IMA, MDA, and AOPP were estimated by enzyme-linked immunosorbent assay; HbA1c, serum creatinine, urine albumin, and urine creatinine were estimated using automated analyzers. Statistical analysis was done using analysis of variance, Pearson's correlation coefficient, and receiver-operating characteristic curve.

RESULTS: A statistically significant difference was found in the levels of IMA among patients with early DN (154 ng/mL), diabetes without nephropathy (109.4 ng/mL), and healthy controls (45.7 ng/mL), with highest levels in early DN cases. Similar increase was seen in AOPP as well. A significant correlation was observed between IMA and UACR in diabetes without nephropathy ($r = 0.448$).

CONCLUSION: The present study postulates serum IMA as a novel biomarker for the assessment of disease progression in diabetes even before microalbuminuria, and a cutoff point ≥ 99 ng/mL can be used for detection of early DN.

KEYWORDS: advanced oxidative protein products, diabetic nephropathy, ischemia-modified albumin, malondialdehyde, vascular injury

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Introduction

Diabetic complications are associated with increased incidence, severity, and delayed recovery from the stress-induced ischemia participating in endothelial dysfunction, which may lead to the development of angiopathy, especially diabetic nephropathy (DN).¹ The pathogenesis of DN is a complex process and it is not yet fully understood. The cause of increased urinary albumin excretion, which is considered to be an early sign of DN, can be attributed to a defect in the glomerular membrane filtration, resulting from renal endothelial damage due to hyperglycemia, oxidative stress, ischemia, and inflammation.^{2–5} However, several studies have reported development of DN in 29%–61% of type 2 diabetes patients having normal urine albumin levels, as microalbuminuria develops only when significant damage to the glomerular function has already occurred.^{4,6–8} This significant observation impedes the use of microalbuminuria as a good predictive marker^{6,9} and necessitates the need for a

better biomarker with high sensitivity and specificity for early detection of DN.^{2,10}

Free radical generation coupled with decreased antioxidant defense mechanism and associated ischemia in diabetes leads to an alteration in the tertiary structure of human serum albumin within minutes of arterial occlusion; hence, it is termed ischemia-modified albumin (IMA). As a result of increased generation of reactive oxygen species (ROS), structural modifications of the albumin molecule can occur. IMA is now being used as a sensitive biomarker for detecting cardiac ischemia as an indicator of oxidative stress.¹¹ Recently, some studies were published regarding the role and diagnostic significance of IMA in noncardiac disorders. For the first time, Piwowar et al reported the association of IMA in diabetes and reported 75% increased value of IMA in diabetes compared with control after estimating it by manual spectroscopic method.¹² Elevated IMA levels were also detected in diabetic ketoacidosis, pulmonary embolism, peripheral vascular disease, brain ischemia, infection,



liver disease, trauma, some neoplasms, and complicated and uncomplicated pregnancies.^{11,13–16}

Complications of diabetes are associated with increased activity of free radicals generated as a result of malignant exposure of endothelial cells to glucose, leading to oxidation of lipid molecules.^{17,18} Malondialdehyde (MDA), one of the highly reactive three carbon dialdehyde byproducts, is produced from peroxidation of polyunsaturated fatty acids and also during arachidonic acid metabolism for the synthesis of prostaglandins.^{19,20} This stress marker has been identified to reflect the complications of diabetes.²¹ The protein molecules that are susceptible to oxidation and glycation are another set of biomolecules that cause damage to vascular endothelium in DN. Advanced oxidative protein product (AOPP), an advanced glycation end product, is progressively related to the increase in albuminuria.²²

Despite the associative studies on IMA, MDA, and AOPP with DN, the clinical application with regard to reference ranges and diagnostic criteria is limited and not homogenous across populations. To find the effect of hyperglycemia-induced stress on albumin and lipid macromolecules in vascular endothelial injury of diabetes, this study was aimed at estimating the levels of IMA, MDA, and AOPP in diabetes cases as markers of vascular injury with derivation of cutoff value to evaluate their utility in early DN.

Materials and Methods

Study population. A cross-sectional study was conducted including 60 type 2 diabetes mellitus (T2DM) cases aged between 30 and 65 years and 30 age- and sex-matched controls from Medical College Hospital between December 2014 and June 2015. Based on urine albumin/creatinine ratio (UACR), all diabetes cases were further divided into three groups of 20 each.

The diagnosis of diabetes was made on the basis of fasting plasma glucose (FPG) ≥ 126 mg/dL, 2-hour postprandial plasma glucose (2-hour PPPG) ≥ 200 mg/dL, and glycated hemoglobin (HbA1c) $\geq 6.5\%$, and that of nephropathy was made based on UACR. Group I (diabetes without microalbuminuria) included diabetes with UACR < 30 mg/g; group II included diabetes with microalbuminuria (early DN) with UACR between 30 and 300 mg/g; and diabetes with macroalbuminuria (overt DN) having UACR > 300 mg/g of creatinine constituted group III.

This research complied with the principles of the Declaration of Helsinki. The study was approved by the Institutional Ethics Committee, and informed consent was obtained from all the individuals before enrolment into the study. Patients having serum albumin level < 3 g/dL and > 5.5 g/dL, those suffering from acute infections, inflammatory diseases, known cases of hepatic, renal, cardiovascular, or endocrine diseases, pregnancy, cancer/HIV patients, and patients on steroid medications or hormonal therapy were excluded from the study. Patients' relevant data were recorded about routine biochemical parameters such

as FPG and two-hour PPPG (glucose oxidase method), HbA1c (ion-exchange high-performance liquid chromatography in Bio-Rad Turbo II Variant analyzer), serum and urine creatinine (Jaffe's method), and serum and urine albumin (Turbidimetric method), which were estimated using Roche commercial kits on automated clinical chemistry analyzers in Central Biochemistry Laboratory of the hospital. UACR was calculated for each subject.

Blood sample was collected in plain vacutainer for the estimation of IMA, MDA, and AOPP. After separation, serum was stored at -20 °C until further analysis. IMA, MDA, and AOPP were estimated by solid-phase enzyme-linked immunosorbent assay (ELISA) with kits from Shanghai Yehua Biological Technology Co., Ltd, based on double-sandwich principle on ELx 800 by BioTek® Instruments, Inc. The sample was added to the precoated monoclonal antibody wells. After incubation, the antibodies labeled with biotin were added to form immune complex with streptavidin–horseradish peroxidase. Unbound enzymes were removed by washing, and then, the substrate was added to give color, which was measured at 450 nm by a colorimeter. The intensity of color was proportional to the concentration of the analyte and was positively correlated.²³ Assay range of the ELSIA kits used was 2–600 ng/mL (sensitivity 1.08 ng/mL) for IMA, 0.2–60 nmol/mL (sensitivity 0.14 nmol/mL) for MDA, and 0.1–40 ng/mL (sensitivity 0.05 ng/mL) for AOPP, with coefficient of variation for intraassay being $< 10\%$ and that for interassay being $< 12\%$.

Statistical analysis. Data were analyzed using IBM SPSS Statistics version 20 (SPSS). Intergroup comparison of means was done using analysis of variance and Tukey's test as the post hoc test. Correlation analysis was done using Pearson's correlation coefficient. Receiver-operating characteristic (ROC) curve was used to distinguish the positive results from negative results. The area under the curve (AUC) ranges from 0.6 to 1, and the test parameter that approaches 1 was considered as a perfect discriminator. All data are expressed as mean \pm standard deviation. $P < 0.05$ was considered significant.

Results

The baseline characteristics of each group are presented as mean \pm standard deviation (Table 1). Patients in groups II and III were having higher blood pressure and a longer duration of diabetes than group I. Significant difference was observed with regard to two-hour PPPG and HbA1c in group III as compared with groups I, II, and IV. Mean levels of FPG, two-hour PPPG, and HbA1c were found in the order of group III $>$ group II $>$ group I. Serum creatinine, UACR, systolic blood pressure, and diastolic blood pressure were also found to be significantly raised in group III compared with groups I, II, and IV.

IMA, MDA, and AOPP levels. Statistically significant difference was present in the mean of IMA, MDA, and AOPP in all three groups when compared with the control group



Table 1. Comparison of baseline characteristics among the groups.

| | GROUP I (N = 20) | GROUP II (N = 20) | GROUP III (N = 20) | GROUP IV (N = 30) |
|-----------------------------|------------------------------|------------------------------|--------------------------------|-------------------------------|
| Age (Year) | 48.1 ± 17.1 (31–65) | 50.2 ± 14.1 (36–64) | 49.8 ± 16.9 (33–65) | 47.3 ± 17.2 (30–64) |
| Sex (M/F) | 12/8 | 12/8 | 9/11 | 16/14 |
| FBG (mg/dL) | 164.25 ± 61.7 ^{c,d} | 180.5 ± 34.6 ^d | 193.8 ± 16.5 ^{a*,d} | 80 ± 7.9 ^{a,b,c} |
| PPPG (mg/dL) | 249.35 ± 43.3 ^{c,d} | 274.3 ± 32.7 ^{c*,d} | 320.3 ± 79.0 ^{a,b*,d} | 129.3 ± 10.8 ^{a,b,c} |
| HbA1c (%) | 7.6 ± 0.84 ^{b*,c,d} | 8.9 ± 0.7 ^{a*,c,d} | 10.8 ± 2.5 ^{a,b,d} | 4.8 ± 0.5 ^{a,b,c} |
| UACR (mg/g) | 18.9 ± 7.1 ^{b,c} | 126 ± 62.3 ^{a,c,d} | 535.1 ± 144 ^{a,b,d} | 16.9 ± 6.1 ^{b,c} |
| Serum creatinine (mg/dL) | 1.12 ± 0.25 ^{b,c} | 1.5 ± 0.2 ^{a,d} | 1.7 ± 0.38 ^{a,d} | 0.98 ± 0.2 ^{b,c} |
| SBP (mm/Hg) | 145 ± 8 ^{c*,d} | 150 ± 7 ^d | 155 ± 16 ^{a*,d} | 121 ± 3 ^{a,b,c} |
| DBP (mm/Hg) | 88 ± 4 ^d | 88 ± 4 ^d | 90 ± 4 ^d | 80 ± 3 ^{a,b,c} |
| Duration of diabetes (Year) | 4.4 ± 0.9 | 9.6 ± 1.7 | 14.8 ± 2.1 | |

Notes: *P* < 0.001 is denoted when ^agroup I, ^bgroup II, ^cgroup III, ^dgroup IV, **P* < 0.05.

Abbreviations: N, number of subjects; SD, standard deviation; M/F, male/female; FBG, fasting blood glucose; two-hour PPPG, two-hour postprandial plasma glucose; TC, total cholesterol; TG, triglyceride; SBP, systolic blood pressure; DBP, diastolic blood pressure.

(Table 2). IMA and AOPP levels in group II was significantly high when compared with groups I and IV, but no significant difference was found when compared with group III. Among the diabetes groups, group I had the lowest levels. Diabetic patients with early nephropathy (group II) were having highest MDA levels (18.6 ± 9.2 nmol/mL) as compared with groups I, III, and IV. However, the difference in the values of groups II and III was minimal and not statistically significant.

Correlation of IMA, MDA, and AOPP. Pearson’s correlation was used to determine the influence of these markers in pathogenesis of nephropathy; serum levels of IMA, MDA, and AOPP were correlated with HbA1c, UACR, and serum creatinine (Table 3). Among the three analytes, only IMA had significant correlation with UACR in groups I, II, and III. IMA was also positively associated with serum creatinine in group III and with HbA1c in groups II and III. MDA had no association in group I, but was found to have positive association only with UACR in group II and with HbA1c in group III. AOPP was found to have significant correlation with HbA1C and UACR in groups II and III.

The ROC curves were generated to evaluate and compare the diagnostic performance of the serum levels of IMA, MDA, and AOPP for differentiating between subjects with normal kidney function (UACR <30 mg/g) and those with UACR >30 mg/g (both with micro- and macroalbuminuria;

Figure 1). Cutoff value for IMA to distinguish early DN in our study population was found to be ≥99 ng/mL at maximum sensitivity of 97.5% and specificity of 78%. We observed similar AUC for IMA (0.93) and AOPP (0.933) in comparison with MDA (0.746; Table 4).

Discussion

The present study aimed to estimate the IMA, MDA, and AOPP levels in the serum of T2DM patients and find their correlation with the stage of DN, suggested by their HbA1c, UACR, and serum creatinine levels. In this study, higher mean levels of serum IMA were found in diabetes cases in comparison with controls. IMA increased progressively with the degree of albuminuria, supporting other studies.^{1,24} Among those with diabetes, lowest levels were observed in patients without microalbuminuria, which was significantly different from those in the micro- and macroalbuminuria groups, but there were no significant differences between micro- and macroalbuminuria patients.¹

These results suggest that albumin modification occurs in the early stage of the disease provoked probably by hyperglycemia-induced stress and takes part in the pathogenesis of diabetic complications. IMA as an ischemic marker indicates underlying subclinical disease or vascular dysfunction in diabetic kidney, as suggested by Dash et al and

Table 2. Comparison of serum IMA, MDA, AOPP, and albumin levels between groups stratified according to UACR status.

| | GROUP I | GROUP II | GROUP III | GROUP IV |
|----------------|--------------------------------|------------------------------|-----------------------------|------------------------------|
| IMA (ng/mL) | 109.4 ± 50.3 ^{b*,c,d} | 154.5 ± 43.1 ^{a*,d} | 178.1 ± 67.9 ^{a,d} | 45.7 ± 23.9 ^{a,b,c} |
| MDA (nmol/mL) | 13.9 ± 6.1 | 18.6 ± 9.2 ^{d*} | 18.3 ± 6.5 ^{d*} | 12.5 ± 5.2 ^{b*,c*} |
| AOPP (ng/mL) | 7.5 ± 3.4 ^{b,c,d*} | 12.4 ± 3.0 ^{a,d} | 13.6 ± 4.6 ^{a,d} | 4.89 ± 1.4 ^{a*,b,c} |
| Albumin (g/dL) | 3.9 ± 0.4 ^{d*} | 3.8 ± 0.2 ^{d*} | 3.7 ± 0.25 ^d | 4.2 ± 0.5 ^{a*,b*,c} |

Notes: Data are expressed as mean ± standard deviation. *P* < 0.001 is denoted when ^agroup I, ^bgroup II, ^cgroup III, ^dgroup IV, **P* < 0.05.



Table 3. Correlation between serum IMA, MDA, AOPP with HbA1c, UACR, and serum creatinine in each group.

| | | | HBA1c | UACR | CREATININE |
|------|-----------|---|--------|--------|------------|
| IMA | Group I | r | 0.051 | 0.448* | 0.381 |
| | | p | 0.801 | 0.034 | 0.091 |
| | Group II | r | 0.473* | 0.46* | 0.41 |
| | | p | 0.029 | 0.03 | 0.06 |
| | Group III | r | 0.509* | 0.502* | 0.445* |
| | | p | 0.022 | 0.023 | 0.035 |
| MDA | Group I | r | 0.07 | 0.11 | 0.15 |
| | | p | 0.71 | 0.61 | 0.51 |
| | Group II | r | 0.42 | 0.467* | 0.38 |
| | | p | 0.06 | 0.04 | 0.09 |
| | Group III | r | 0.463* | 0.39 | 0.22 |
| | | p | 0.04 | 0.08 | 0.39 |
| AOPP | Group I | r | 0.17 | 0.33 | 0.03 |
| | | p | 0.46 | 0.15 | 0.80 |
| | Group II | r | 0.452* | 0.453* | 0.36 |
| | | p | 0.03 | 0.03 | 0.11 |
| | Group III | r | 0.493* | 0.454* | 0.17 |
| | | p | 0.03 | 0.03 | 0.46 |

Notes: Group I is DM with UACR < 30 mg/g, group II is early DN with UACR 30–300 mg/g, and group III is overt DN with UACR >300 mg/g of creatinine (20 in each group). UACR, urinary albumin/creatinine ratio. *P < 0.05.

Borderie et al.^{24,25} Analyzing the relationships between IMA and parameters applied in the routine diagnosis of renal dysfunction, significant correlation was found with the UACR in all the three groups. With serum creatinine, IMA correlated only in macroalbuminuria groups, which proves that IMA is a helpful marker for detecting early renal dysfunction.¹

Recent observations have shown that increased ROS generation is directly proportional to hyperglycemia and oxidative protein damage.^{26,27} These changes profoundly influence cellular function and lead to abnormal renal vascular remodeling that lead to the development of DN.²⁸ Hyperglycemia-induced ischemia, inflammation, and oxidative stress might increase the levels of IMA in the serum and also in the kidney, resulting in podocyte malfunction. Their excess accumulation along the extracellular matrix in the glomerulus and tubulointerstitium leads to vascular endothelial damage in diabetes.^{3,29–32}

An increase in protein damage manifests as increased serum levels of AOPP and IMA. There are a fewer studies concerning AOPP in diabetes and hardly any of them correlating with the stages of DN.^{33–35} The levels of AOPP rose progressively and significantly with the degree of albuminuria. These findings were in support of the previous study by Piwowar et al.³³ In agreement with the study by Fathy et al.³⁶, our study found a significant correlation between AOPP, HbA1c, and UACR in T2DM patients with microalbuminuria as compared with diabetes patients without microalbuminuria.³⁷ Increased serum AOPP promotes kidney inflammation in diabetes

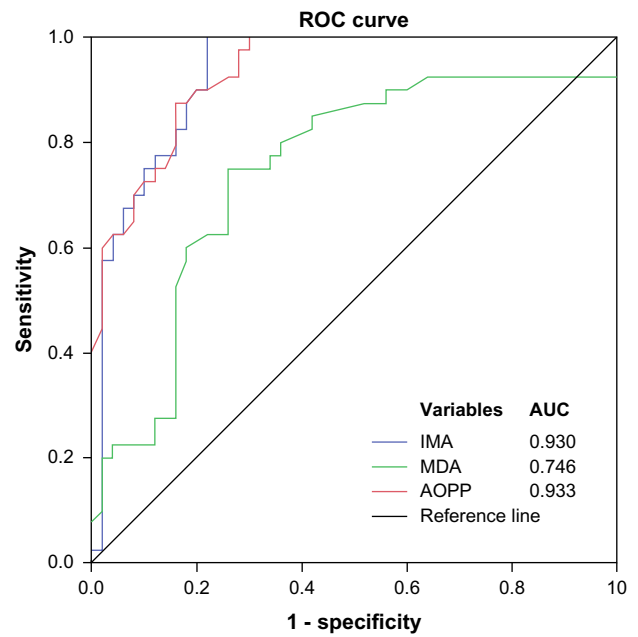


Figure 1. ROC curves (N = 90) for serum levels of IMA, MDA, and AOPP for differentiating individuals with normal renal function (normalalbuminuria) and those with upper normal albuminuria (both micro- and macroalbuminuria).

patients through the action of renal NADPH oxidase.³⁸ Telci et al explained that damage to proteins may be more or occur earlier than damage to lipids during oxidative stress *in vivo*.³⁹ Supporting this statement, the current study also reports significant correlation of IMA and AOPP with HbA1c, UACR, and serum creatinine in DN cases (micro- and macroalbuminuria patients) as compared with MDA.^{23,39} Our study also recorded increased MDA levels in diabetes cases compared with controls, and its correlation with HbA1c, UACR, and serum creatinine was found to be significant. These findings are

Table 4. Comparison of the diagnostic performance of IMA, MDA, and AOPP serum levels for distinguishing between renal function (normalalbuminuria) and those with upper normal albuminuria (both micro- and macroalbuminuria).

| | IMA (ng/mL) | MDA (nmol/mL) | AOPP (ng/mL) |
|-------------|-------------|---------------|--------------|
| AUC | 0.930 | 0.746 | 0.933 |
| Cut off | 99 | 12.3 | 7.4 |
| Sensitivity | 0.975 | 0.80 | 0.975 |
| Specificity | 0.78 | 0.64 | 0.70 |
| PPV | 0.78 | 0.64 | 0.72 |
| NPV | 0.025 | 0.2 | 0.027 |
| +LR | 4.43 | 2.2 | 3.25 |
| -LR | 0.03 | 0.31 | 0.036 |
| DOR | 141.8 | 7.1 | 91 |

Notes: AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; +LR, positive likelihood ratio; -LR, negative likelihood ratio; DOR, diagnostic odd ratio.



indicative of hyperglycemia-induced oxidative stress, causing lipid peroxidation that leads to further exacerbation of renal injury in diabetes.

To evaluate the discriminatory power of the tests and their diagnostic utility, ROC analysis was used for IMA, MDA, and AOPP. Evaluation was done by their ability to distinguish individuals without any kidney disturbances (normoalbuminuria) from those with *upper normal* albuminuria, manifested by both micro- and macroalbuminuria. According to these criteria, the result showed highest and similar mean AUC for IMA and AOPP (0.930 and 0.933, respectively) in comparison with MDA (0.746). This indicates that after a certain period, oxidative stress-induced protein modification and inflammatory component are more strongly associated with endothelial damage and ischemia to cause DN than the oxidatively modified lipids.²³ In an attempt to select appropriate cutoff values for the best marker of the three parameters from the ROC plot, it was found that IMA and AOPP were having the same sensitivity of 97.5%, but differed in specificity (78% and 70%, respectively). This indicates that IMA can be used with higher specificity to reveal individuals with upper normal albuminuria or early DN. In contrast to our finding, Piwowar et al.³³ reported AOPP as a better marker than IMA, but the current study found that serum IMA levels of ≥ 99 ng/mL are ideal for differentiating these patients because of its high value of specificity, positive predictive value, and positive likelihood ratio.³²

Strengths and Limitations

IMA, MDA, and AOPP were estimated by ELISA technique, which is a more sensitive method as compared with several previous spectrophotometric techniques. To nullify the effect of serum albumin on IMA and AOPP levels, the current study included only those subjects having serum albumin level between 3 and 5.5 g/dL. A potential limitation could be the cross-sectional design from which the cause and effect relationship cannot be established. Large-scale longitudinal prospective studies are required to add on to the existing knowledge and to establish the significance of IMA as an early marker of oxidative stress-induced vascular injury in the pathogenesis of DN.

Conclusion

The current study supports the essential relationship between the serum levels of IMA in type 2 diabetes patients and its utility in predicting vascular damage in DN. Increase in serum IMA level was more marked in diabetes without microalbuminuria and early DN cases compared with normal individuals, reinforcing the hypothesis that estimation of serum IMA may be used as a diagnostic marker to assess early detection and progression of nephropathy in diabetes. The clinical significance of IMA lies in its high negative predictive value, which may help in excluding the presence of early DN in a population. A cutoff value ≥ 99 ng/mL of serum IMA,

as determined in this study, may help clinicians to diagnose early DN among normoalbuminuria cases or diabetes without microalbuminuria, which may help in the prognosis of the disease as well. Hence, along with the established markers such as serum creatinine and UACR, serum IMA estimation can be used as an auxiliary marker for early detection of DN.

Author Contributions

Conceived and designed the experiments: AfA, PM. Analyzed the data: AfA, AsA. Contributed to the writing of the manuscript: PM, CY, AfA, AsA. Jointly developed the structure and arguments for the paper: PM, AfA, RMS, AH. Made critical revisions and approved the final version: PM, AfA, CY. All the authors reviewed and approved the final manuscript.

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