ORIGINAL RESEARCH

Comparison of the usefulness of plasma levels of oxidatively modified forms of albumin in estimating kidney dysfunction in diabetic patients

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Manuscript submitted 23rd November, 2009

Manuscript accepted 2nd February, 2010

Clin Invest Med 2010; 33 (2): E109-E116.

Abstract

Purpose: Advanced oxidation protein products (AOPP) and ischemia-modified albumin (IMA) are forms of oxidatively modified albumin and have recently been investigated as indicators of oxidative stress. They are increased in different disorders, including diabetes mellitus, as a result of hyperglycaemia, oxidative stress and hypoxia. The usefulness of the plasma levels of these two parameters in estimating kidney dysfunction in type 2 diabetic patients (T2DM) was compared in this study.

Methods: Plasma levels of AOPP and IMA were determined spectrophotometrically in 218 individuals, 153 patients with T2DM and 65 healthy people. The urinary albumin/creatinine ratio (UACR) was used as the reference to define the stage of kidney dysfunction by the assessment of the degree of albuminuria.

Results: Receiver Operating Characteristic (ROC) curve analysis, likelihood ratio (LR), and Youden's index (J) revealed that AOPP and IMA had acceptable sensitivities and specificities in individuals with different degrees of albuminuria; however, AOPP had higher values of the area under the curve (AUC: 0.934) than IMA, as well as 100% sensitivity and 77.01% specificity for distinguishing patients with micro- and macroalbuminuria.

Conclusions: Both AOPP and IMA may be helpful clinical markers for estimating kidney dysfunction, but AOPP is better able to identify diabetic patients with nephropathy. We suggest that AOPP is almost ideal for discriminating

between T2DM patients with micro- and macroalbuminuria.

In the plasma of diabetic patients, the albumin molecule is modified under conditions of chronic hypoxia, provoked mainly by the hyperglycaemia and oxidative stress (OS) existing in these patients. This leads to many changes in the structure and function of albumin including the formation of advanced oxidation protein products (AOPP) and ischemia-modified albumin (IMA).¹⁻⁴ AOPP were first detected in uremic and haemodialysed patients. They are formed during OS by the action of free radicals, mainly chloraminated oxidants (hypochlorous acid and chloramines) produced by myeloperoxidase in activated neutrophils. This leads to the formation of dityrosine residues and protein cross-linking, which results in abnormal albumin molecules in the plasma. AOPP are recognized as very good markers of oxidative damage to proteins and as indicators of disturbances in the oxidant/antioxidant balance.^{3,5,6} IMA is an albumin molecule modified by hypoxic conditions, which causes a

change in its inherent affinity to bind transition metals such as Co^{2+} , Ni^{2+} , and Cu^{2+} . The mechanism of formation of IMA may involve free radical release and damage to the N-terminus, although this remains to be proven *in vivo*. IMA can be measured in the laboratory spectrophotometrically by the loss of its ability to bind cobalt ions.^{1,7} IMA was first described as a marker of myocardial ischemia, but lately it is being intensively investigated in other disorders, including diabetes, and in other conditions associated with oxidative stress.^{8,9} Increased AOPP and IMA formation are observed in different diseases and have been recognized as early markers and/or prognostic factors for numerous pathological conditions, such as acute coronary syndrome, systemic sclerosis, rheumatoid arthritis, trauma, pulmonary embolism¹⁰⁻¹⁵.

In our previous reports, we showed significantly higher plasma levels of AOPP as well as IMA in patients with type 2 diabetes mellitus (T2DM) in comparison with healthy individuals.^{9,16} AOPP levels was related with poor glycaemic control, obesity, lipid disorders, duration of disease (longer than 10 years), and diabetic complications including nephropathy.^{4,16,17} IMA levels were also higher in diabetic patients, especially those with complications, and was related to the levels of HbA_{1c} .⁹

In diabetes, the most frequent complication is diabetic nephropathy (DN), which is the most common cause of end-stage renal disease, requiring dialysis, and it shortens human life. That is why DN should be detected and treated at the early albuminuria stage, especially in the microalbuminuria stage, while it is potentially reversible.^{18,19} Some authors suggest that microalbuminuria is not a good predictor of nephropathy development in individuals with diabetes because multiple mechanisms contribute to the development of nephropathy.²⁰ Therefore, additional markers are still being sought. Albumin concentration in 24-hour urine samples are used as a gold standard but this measurement is often difficult to carry out. Calculating the urinary albumin/creatinine ratio (UACR) of the first

morning urine sample has been recommended to determine the degree of advancement of DN.^{21,22}

AOPP and IMA levels are connected with diabetic complications and may be helpful in assessing the disease's development and predicting the severity of its course and perhaps even the effectiveness of therapy. This led us to compare the usefulness of these two oxidative modified forms of albumin in assessing kidney dysfunction in diabetic patients. The urinary albumin/creatinine ratio was applied as an indicator of the degree of albuminuria. We used Receiver Operating Characteristic (ROC) curve analysis to evaluate the clinical usefulness of plasma levels of AOPP and IMA.

Materials and Methods

This study was approved by the Bioethics Committee of Wroclaw Medical University and all subjects were informed of the aim of this study and gave their consent to participate in it. We studied 218 individuals, 153 patients with T2DM, diagnosed routinely on the basis of the National Diabetes Data Group guidelines from 2002 year and treated at the Clinic of Angiology, Hypertension, and Diabetology of Wroclaw Medical University, and 65 healthy adult controls. At the time of the study, none of the diabetic patients suffered from an acute illness or chronic inflammatory condition, which was determined both clinically and by biochemical markers (leukocyte count, ESR, CRP and fibrinogen). Similarly, the individuals who made up the control group had no evidence of an inflammatory disorder, abnormalities in lipid and carbohydrate metabolism, or kidney disorders in routine medical checkups.

All the examined parameters in the groups of patients and healthy controls are expressed as mean values and standard deviation. The main clinical features of the diabetic patients were age: 68.10 ± 8.50 years, sex: 54 males and 99 females, disease duration: 19.70 ± 8.50 years, plasma glucose: 9.23 ± 2.37 mmol/l, glycated haemoglobin: $9.42 \pm 2.70\%$, BMI:

28.05 $\pm$ 6.70 kg/m², blood pressure: 143.30 $\pm$ 21.50/80.40 $\pm$ 10.50 mmHg, serum creatinine: 131.71 $\pm$ 31.82 μ mol/l, and treatment: oral hypoglycaemic agents in 38, insulin in 27, combined therapy (oral hypoglycaemic agents + insulin) in 69, and diet in 19 patients. The main clinical features of the healthy people were age: 57.90 $\pm$ 15.70 years, sex: 24 males and 41 females, plasma glucose: 5.19 $\pm$ 0.27 mmol/l, glycated haemoglobin: 4.80 $\pm$ 0.75%, BMI: 24.70 $\pm$ 5.80 kg/m², blood pressure: 132.80 $\pm$ 16.50/74.80 $\pm$ 11.90 mmHg, and serum creatinine: 87.68 $\pm$ 22.98 μ mol/l.

Blood samples were drawn from the subjects of both groups in a fasting state (12 hours) into tubes containing heparin (250 units/ml). Glycated haemoglobin (HbA_{1c}) was determined in the blood haemolysates by a latex-enhanced turbidimetric immunoassay. The concentrations of plasma glucose were determined using routine clinical assays. First morning samples of urine from the same patients were taken. Both materials (plasma and urine) were frozen and stored at -86°C for no longer than two months.

Advanced oxidation protein products were measured by the spectrophotometric method according to Witko-Sarsat *et al.*⁵ This method is based on the color reaction of AOPP with a potassium iodide solution in an acidic environment. Absorbance was read at 340 nm and the results were expressed in chloramine T units (μ mol/l).

Ischemia-modified albumin was determined by a manual colorimetric assay described by Bar-Or *et al.*¹ This method consists of adding an excess of a known amount of exogenous Co²⁺ to a plasma sample and measuring unbound Co²⁺ colorimetrically using dithiothreitol (DTT). The results are given in absorbance units (ABSU).

Plasma and urine concentrations of creatinine were determined using the method described by Jaffe. The concentration of albumin in urine was determined according colorimetric method described by Shosinsky *et al.* based on the ability of albumin to bind to

bromophenol blue (BPB).²³ The results vary linearly with range of albumin concentration up to 6 g/l. The intra-assay and inter-assay CV was 3.2% and 6.5%, respectively. On this basis, we calculated the urinary albumin/creatinine ratio and divided individuals into groups with normoalbuminuria (UACR <30 mg/g), microalbuminuria (UACR 30-300 mg/g), and macroalbuminuria (UACR >300 mg/g).

All parameters (AOPP, IMA, UACR) were determined for each individual in duplicate and the results are expressed as mean values. The analysis was conducted by the Med Calc program for Windows, version 9. A p value of less than 0.05 was considered statistically significant.

The ROC curve was used to assess the diagnostic performance of AOPP and IMA and to compare these parameters as auxiliary tests for estimating diabetic kidney dysfunction. The sensitivity and specificity were plotted for each measurement and the area under the curve (AUC) was determined. The values of likelihood ratio (LR) and Youden's index (J) were also estimated.^{18,24}

Results

On the basis of the UACR, 99 samples were classified as normoalbuminuric (57 healthy and 39 diabetic), 87 as microalbuminuric (8 healthy and 79 diabetic), and 32 as macroalbuminuric (only diabetic). The mean plasma levels of AOPP and IMA of all examined subjects were 171.26 $\pm$ 98.75 μ mol/l and 0.487 $\pm$ 0.162 ABSU, respectively. In the individuals with normoalbuminuria (UACR <30 mg/g), the concentrations of both parameters (AOPP: 128.07 $\pm$ 37.81 μ mol/l, IMA: 0.381 $\pm$ 0.145 ABSU) were significantly lower (p<0.001) than those with "upper normal" albuminuria (UACR >30 mg/g) manifested both by micro- and macroalbuminuria (AOPP: 212.78 $\pm$ 108.56 μ mol/l, IMA: 0.578 $\pm$ 0.126 ABSU).

The ROC curves used to evaluate and compare the diagnostic performance of plasma levels of AOPP and IMA for differentiating between subjects with normal

TABLE 1. Comparison of the diagnostic performance of AOPP and IMA plasma levels for distinguishing between individuals with normal kidney function (normalalbuminuria) and those with “upper normal” albuminuria (both micro- and macroalbuminuria)

	AOPP	IMA
Mean AUC	0.765 ± 0.032	0.848 ± 0.026
<i>P</i> value (versus AUC=0.5)	0.0001	0.0001
95% Confidence interval (CI)	0.703 – 0.820	0.793 – 0.893
Cutoff value	121.25	0.504
Sensitivity and Specificity (%)	89.08 and 51.52	82.35 and 78.79
+LR and -LR	1.84 and 0.21	3.88 and 0.22
Youden’s index	0.406	0.611

kidney function (UACR <30 mg/g) and these with UACR >30 mg/g (both with micro- and macroalbuminuria) are shown in Figure 1. We observed a slightly, but statistically significant ($p=0.026$), larger area under the curve for IMA (0.848 ± 0.026) in comparison with AOPP (0.765 ± 0.032). This indicates that IMA has a slightly higher diagnostic potential in comparison with AOPP. This is confirmed by data on the overall performance, sensitivity, specificity, bootstrap 95% confidence interval, likelihood ratios of positive and negative test results, and Youden’s index of AOPP and IMA which are presented in Table 1. The optimum diagnostic cut-off point maximizing the sensitivity and specificity of these parameters was 121.25 $\mu\text{mol/l}$ for AOPP (89.08% and 51.52%, respectively) and 0.504 ABSU for IMA (82.35% and 78.79%, respectively).

Figure 2 shows the ROC curves for plasma AOPP and IMA for differentiating between diabetic patients with microalbuminuria (UACR 30-300 mg/g) and these with macroalbuminuria (UACR >300 mg/g). We observed a significantly ($p=0.001$) larger area under the curve for AOPP (0.934 ± 0.031) than for IMA (0.723 ± 0.056). The optimum diagnostic cut-off point maximizing the sensitivity and specificity of these parameters revealed that the AOPP had 100.00% sensitivity and 77.01% specificity for a cut-off of 208.80 $\mu\text{mol/l}$. The sensitivity and specificity of IMA for a

TABLE 2. Comparison of the diagnostic performance of AOPP and IMA plasma levels for distinguishing between diabetic patients with kidney dysfunction manifested by microalbuminuria and macroalbuminuria

	AOPP	IMA
Mean AUC	0.934 ± 0.031	0.723 ± 0.056
<i>P</i> value (versus AUC=0.5)	0.0001	0.0011
95% Confidence interval (CI)	0.873 – 0.971	0.634 – 0.801
Cutoff value	208.80	0.609
Sensitivity and Specificity (%)	100.00 and 77.01	75.00 and 71.26
+LR and -LR	4.35 and 0.00	2.61 and 0.35
Youden’s index	0.770	0.463

cut-off of 0.609 ABSU are 75.00% and 71.26%, respectively. The data on overall performance, sensitivity, specificity, bootstrap 95% confidence interval, likelihood ratios of positive and negative test results and Youden’s index for AOPP and IMA are presented in Table 2. These data indicate a higher diagnostic power of AOPP in comparison with IMP because of AOPP’s stronger ability to discriminate between diabetic patients with different stage of nephropathy as manifested by micro- and macroalbuminuria.

Discussion

The importance of oxidative stress in the development of diabetic complications, including nephropathy, has been discussed by many authors; however, the relationship between OS and the pathophysiology of kidney damage is still not well explained.^{25,26} The most popular indicator and predictor of DN and chronic kidney disease (CKD) is albuminuria.²⁰ Screening for albuminuria is recommended in the general population and especially in patients at increased risk for CKD, including those with hypertension, diabetes, cardiovascular disease, and a family history of CKD.^{27,28}

As mentioned earlier, in patients with type 2 diabetes mellitus, an increase in protein damage takes place under conditions of chronic OS and this manifests as increased plasma levels of advanced oxidation protein products as well as ischemia-modified

FIGURE 1. ROC curves (n=218) for plasma levels of AOPP ($\mu\text{mol/l}$; continuous line) and IMA (ABSU; dashed line) for distinguishing between individuals with normal kidney function (normalalbuminuria) and those with “upper normal” albuminuria (both micro- and macroalbuminuria)

FIGURE 2. ROC curves (n=119) for plasma levels of AOPP ($\mu\text{mol/l}$; continuous line) and IMA (ABSU; dashed line) for distinguishing between diabetic patients with kidney disturbances manifested by micro- and with macroalbuminuria

albumin.^{9,16} Interest in AOPP has greatly increased in recent years, in part because of their reported increase in different diseases, including diabetes mellitus and chronic renal failure.^{5,6,12,29} Keneda *et al.*³⁰ showed that patients with ischemic heart disease (confirmed by angiography) probably have increased AOPP values, similar to dialysed patients, and that the concentrations of these products in the two groups differ significantly from those of healthy volunteers. Sharma *et al.*³¹ examined patients with myocardial ischemia and end-stage renal disease (ESRD) resulting from various diseases, including diabetes, and referred for renal transplantation and found higher IMA plasma levels in comparison with healthy controls. In diabetic patients, correlation of IMA with the urinary albumin/creatinine ratio and plasma creatinine concentration was observed.³² Thus, IMA has been proposed as a biological marker of myocardial ischemia in suspected cases of acute coronary syndrome. Extracardiac oxidative stress could elevate IMA levels and thereby limit the usefulness of elevated IMA in the detection

of cardiac ischemia.^{7,11} Plasma levels of IMA in T2DM is connected with parameters of oxidative processes such as AOPP and thiol groups (SH).³²

The ROC curve has become popular in recent years for evaluating the discriminatory power of tests, methods or parameters, and displays graphically the relationship between sensitivity and specificity over all possible decision values. The ROC curve also allows for comparison of the sensitivity and specificity over a wide range of cut-off points and the selection of the best diagnostic criterion for the test, methods or parameters.²⁴ We compared AOPP and IMA not only by the area under the ROC curves, but also with the use of Youden's index and the likelihood ratio. Some authors suggest that AOPP may be used as a marker of oxidative stress and as a prognostic factor for severe forms of cardiovascular disease, specifically acute coronary syndrome, with 64% sensitivity and 71% specificity.¹² Others showed that the diagnostic power of relative AOPP (plasma AOPP divided by plasma albumin) in discriminating patients with inflammatory

states (inflammatory bowel disease) from non-diseased subjects was less than that of C-reactive protein (CRP), an often useful marker of inflammation.³²

Comparing the diagnostic usefulness of AOPP and IMA in estimating kidney dysfunction required evaluation of their ability to distinguishing individuals without any kidney disturbances (normoalbuminuria) from those with “upper normal” albuminuria manifested both by micro- and macroalbuminuria. According to these criteria, we observed that the mean AUC for IMA was larger than for AOPP but with appropriate cut-off values for AOPP and IMA, resulting from these ROC plots, we found similar sensitivity for these two parameters (87.38 and 81.55%, respectively), but different specificity (51.52 and 78.79%, respectively). This indicates that IMA can be used with higher specificity to reveal individuals with “upper normal” albuminuria. This was somewhat surprising because in our earlier investigation we found that the relative increase of AOPP concentration (almost twofold) in T2DM patients was larger than that IMA (about 75%) compared with healthy people.^{9,16} However, comparing the diagnostic performance of these two parameters in distinguishing patients with diabetic nephropathy manifested by micro- or macroalbuminuria we revealed that AOPP was of greater diagnostic value. The AOPP is almost ideal for differentiating these patients because of its high value of AUC (0.934) and 100% sensitivity and 77.01% specificity. These results are also confirmed by the high value of the likelihood ratios and the Youden’s indexes. A good LR is usually higher than 2, which corresponds to a high probability of having the disease, and the Youden’s index ranges from 0 (for a worthless test) to 1 (for a perfect test).²⁴

It is possible that the increase in the oxidatively modified forms of albumin may also result from the local overproduction of ROS in the diabetic kidney or the altered catabolism of modified albumin in the kidney⁶ but further investigation is required to discriminate between these two possibilities. In this study, all individuals had plasma levels of albumin within nor-

mal range. This excluded the possible influence of higher albumin concentration on increased levels of AOPP and IMA in diabetic patients. Ongoing inflammation can also lead to albumin modification but in the patients studied, the leukocyte count, ESR, CRP and fibrinogen were all within the normal range.

This study confirms that both AOPP and IMA may be helpful clinical markers in estimating kidney dysfunction in diabetic patients, although the lack of high specificity requires careful clinical interpretation of data. The possibility of the simultaneous measurement of both AOPP and IMA in the same samples of blood, with other routine biochemical parameters, makes them useful auxiliary markers in estimating diabetic nephropathy. AOPP has greater diagnostic performance in discriminating between patients with micro- and macroalbuminuria in comparison with IMA and seems to be almost perfect for diagnosing these individuals. Our study was somewhat limited by the relatively small number of individuals in the macroalbuminuria group. In the future, we would also like to evaluate whether combined assessment of AOPP and other parameters of kidney diseases that are recognized to be useful markers of kidney function, such as plasma creatinine, cystatin C, β_2 -microglobulin, or N-acetyl- β -D-glucosaminidase^{33,4,35}, might improve their diagnostic and/or discriminatory power as well as enhance their specificity, i.e. a lower rate of false-positive results.

Acknowledgement

The authors gratefully acknowledge of PhD Joanna Urbaniak for enable us to use the Med Calc program and MPharm Ewie Zurawskiej-Plaksej for skillful statistical assistance.

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