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The employment of high-sensitivity biomarkers for the early detection endothelial dysfunction in general and more specifically for the renal endothelial dysfunction seems to represent a major step ahead towards an improvement in the management algorithms of diabetes and its severe complications.

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*Strictly as per the compliance and regulations of:*

# The Relevance of a Significant Correlation Between ET-1 and Clinical Markers Such as Microalbuminuria and Fundus Oculi Changes in Early Detection of Diabetic Nephropathy in Type 2 Diabetes

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Purpose The aim of our study is to discover the correlations of endothelin-1 (ET1) with known clinical markers of endothelial dysfunction such as microalbuminuria (MA) and fundus oculi (FO) findings in order to help an early detection of renal damage and consequently preventing or slowing progress of diabetic nephropathy (DN).

*Materials and methods:* This is a prospective study where some eighty type 2 diabetes patients were recruited and were dichotomized in 2 groups. In the first group were included forty patients with normoalbuminuria (urinary albumin 0-30 mg/24 hours) while in the second were included the remaining forty patients with microalbuminuria (urinary albumin 30-300 mg/24 hours). Plasma ET-1 levels and 24 hour urinary excretion of albumin were measured. Diabetic retinopathy assessment was made according to the International Clinical Diabetic Retinopathy Disease Severity Scale which includes 5 severity scales. The first scale without retinopathy, the second of light retinopathy non proliferative, the third of moderate non proliferative and the fourth severe retinopathy non proliferative and the fifth one of proliferative retinopathy.

*Results:* We found a statistically significant correlation between ET-1 and MA ($p<0.001$) and ET-1 with fundus oculi ($p<0.032$), where the higher values of ET-1 were observed in the group with diabetic retinopathy changes. The level of changes between FO and ET-1 were proportional (higher ET-1 responded to higher scale of retinopathy) and of MA with fundus oculi ($p<0.001$).

*Keywords: biomarkers, endothelin-1, microalbuminuria, fundus oculi, diabetes, diabetic nephropathy.*

## I. Introduction

Diabetic nephropathy (DN) represents one of the most frequent complications of diabetes and recently it has been baptized as a worldwide spread medical catastrophe (Dr. E Ritz)[1].

Microalbuminuria (MA) is an early clinical marker of DN[2] being an essential parameter in establishing tubular and glomerular damage. MA represents an expression of systemic capillary damage which starts with endothelial dysfunction[3,4].

Prolonged hyperglycemia and insulin resistance in type II diabetes are the main factors contributing to the damage of the vascular endothelium (endothelial dysfunction) leading to micro and macroangiopathy[5] which result in significant amounts of damage towards many internal organs frequently being life threatening for the diabetic patient.

Vascular endothelium acts as a potent and active barrier[5,6] involved in preserving the vasomotor balance and the delicate homeostasis of the vascular tissue continuously reacting to different chemical and physical stimuli through the modification of the vessel diameter and by producin[7,8,9] several vasoactive substances, various substances involved in intravascular coagulation, inflammatory and anti-inflammatory mediators, etc.

In order to ensure an early detection of the endothelial dysfunction in the medical environment are being successfully employed different clinical markers such as MA and Fundus Oculi (FO) examination and different biomarkers such as endothelin-1 (ET-1) which has shown to be fundamental in detecting earlier this dysfunction.

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ET-1 was discovered back in 1988 as a peptide with potent vaconstrictor effects[10]. It was first discovered in the coronary arteries and afterwards in muscle cells, renal tubular epithelium, glomerular mesengial cells, nervous glial cells, macrophages, etc.

Endothelin exists in three forms: ET-1, ET-2, and ET-3. The first one is known as the most effective vasoconstrictive[11] substance amongst them. Under normal conditions the endothelium preserves the balance by maintaining the equilibrium between the production of vasodilatatory substances such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF) and vaconstrictive substances such as endothelin, angiotensin II, etc[12].

## II. Materials and Methods

In the study were recruited eighty (80) patients with type II diabetes that were receiving oral antidiabetic treatment. Patients were examined at the Specialties Polyclinic N. 3 in Tirana, Albania between september 2010 and december 2013. The diagnosis of type II diabetes was made according to the criteria published by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (WHO13 criteria). Patients were dichotomized in 2 groups. In the first group were included forty (40) patients with normoalbuminuria while in the second were included the remaining forty (40) patients with microalbuminuria. Patients were selected on the basis of diabetes duration (2-5 years) and the level of urinary albumin excretion during the 24 hours (where normoalbuminuria consists of urinary albumin excretion of 0-30 mg/24 hours and microalbuminuria consists of urinary albumin excretion of 30-300 mg/24 hours). All patients underwent dilated FO examination by indirect ophthalmoscopy and the changes observed were divided in 5 severity scales consisting in: grade 1 - No apparent Retinopathy; grade 2 - light retinopathy non proliferative; grade 3 - Moderate non-proliferative Diabetic Retinopathy; grade 4 -Severe Non-Proliferative Diabetic Retinopathy; grade 5 - Proliferative Diabetic Retinopathy14.

In order to evaluate ET-1 levels the patients underwent 5 cc of blood sampling while they were sober. Blood sampling was made with K3EDTA tubes and was centrifuged at a speed of 2500 rpm. ELISA test kit (DRGR Free PSA ELISA (EIA-1550) – DRG International) technology with calibration curve of three was employed as analytic kit. Measurements were done with ELISA HUMAN HS (Human Germany Company) with lecture filter 450 nm, correction filter 650 nm, where the measuring unit is nanograms per mililiter (ng/ml) and a standards number of 5.

The values of the standards were as follows: S1 0.01ng/ml, S2 0.1ng/ml, S3 1.0ng/ml, S4 10ng/ml, S5 100ng/ml.

## III. Statistical Analysis

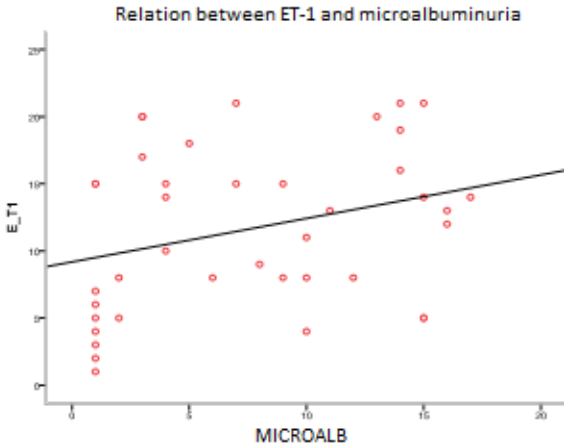
Continuous data were presented as mean value ± standard deviation (SD). Discrete data were presents in absolute value and percentage. Data were displayed on different tables and graphics such as bar and scatter diagram, surface graphic. Differences between continuous variables obtained in the 2 groups were analyzed with student <t> test and the ANOVA analysis of variance when comparing more than 2 groups. The differences observed between the groups regarding discrete variables were calculated by Chi-squared test. The relation between variables was analyzed through Pearson correlation coefficient and Kendall's tau coefficient. A P value less than 0.05 was considered significant. SPSS 19.0 was employed as a statistical software program in order to analyze data.

## IV. Conclusion

The study included 80 patients with a mean age of 55.78 ± 7.72 (SD) years. Of those 59% were males and 41 % females.

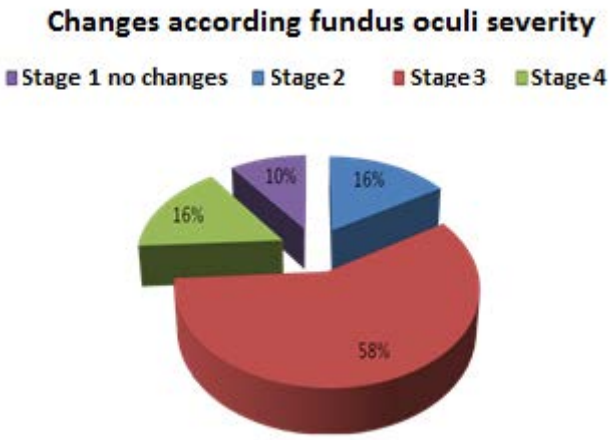
| Variables | Normoalbuminuria | Microalbuminuria | Total | F value | p* value |
|---|---|---|---|---|---|
| Age | 55.18±9.325 | 59.70±7.928 | 55.78±7.722 | 6.094 | 0.004 |
| Years with diabetes | 3.00±1.342 | 3.90±1.210 | 2.27±2.029 | 76.207 | <0.001 |
| ET_1 | 1.20±0.485 | 1.23±0.504 | 1.18±0.519 | 7.315 | 0.032 |

**Analysis ANOVA*




By analyzing the average values for the variables above, through one-way ANOVA analysis, Bonferoni procedure, it was observed that there was a statistically significant difference between groups regarding age (average age was higher in the group associated with MA), diabetes duration (the group associated with MA had a longer duration of diabetes) and the biomarker of endothelial dysfunction where ET-1 mean values resulted higher in the group associated with MA.

| Changes in Fundus Oculi according to the different grades of diabetic retinopathy (DR) | | |
|---|---|---|
| Fundus Oculi | Nr. Of patients | Percentage |
| No apparent retinopathy – severity scale I | 8 | 10 |
| Severity scale II | 13 | 16 |
| Severity scale III | 46 | 58 |
| Severity scale IV | 13 | 16 |
| Severity scale V | 0 | 0 |




By analyzing the relation between ET-1 levels and FO findings we found that ET-1 levels were higher in patients whose retinas were affected by DR as compared to those patients whose retinas were unaffected by DR.

## V. Discussion

*a) Endothelin-1 and albuminuria*

In type II diabetes there is a long term operating stress15 mediated by hyperglycemia and insulinresistancy resulting in increased quantities of vasoconstrictive substances which undermine the delicate balance and create the conditions for the presence of endothelial dysfunction. This is a finding which first appears in glomerular endothelium after the break down glomerular filtrating barrier (starts with glycocalix)[16]. This is followed by MA, whose rate of excretion runs parallel with the degree of damage.

MA is a measurable parameter and allows us to select it as a first choice biomarker in the assessment of glomerular endothelial dysfunction and consequently this appears to be the main reason why we included two groups of patients with and without MA in our study. The ET-1 levels in the normoalbuminuric group were 1.20±0.485, in the microalbuminuric group they were 1.23±0.504. In the light of this result we can assume that ET-1 levels begin to rise in the first years after diabetes' appearance suggesting also the presence of endothelial dysfunction during this period. These findings try to elucidate the role of hyperglycemia on the vascular endothelium and the associated changes on its homeostasis.

We found a statistically significant correlation between ET-1 and MA ($p<0.001$). This is a fundamental result of our study and allows us in affirming its important role in evaluating renal endothelial dysfunction.

Hyperglycemia[17] affects the metabolism by helping the production of free radicals and reactive oxygen species (ROS), by increasing oxidative stress, by activating protein kinase C (PKC). This cascade of events has a negative influence on the production of a known vasodilatory and antiatherogenic substance such as NO by reducing the quantity of eNOS cofactors. By decreasing NO production hyperglycemia helps in increasing ET-1 expression and its potent vasoconstrictive effects. The reduction of NO biodisponibility leads to an increase in the number of adhesion molecules which on the other hand exert a chemotactic effect mostly on neutrophils and macrophages. The breakdown of glomerular endothelial glycocalyx in the kidney by proinflamatory cytokines such as tumor necrosis factor α (TNF-α)[18] shall render the renal endothelium unable to preserve it negative charge leading thus to albumin excretion in the urine.

Endothelin receptors are subdivided into A and B types[19]. Type A receptors are blamed of causing sodium-dependent systemic arterial hypertension while acting in the kidneys. This type of hypertension can also

include inflammatory nitric oxide synthetase (iNOS) and type B receptors.

One of the mechanisms contributing to endothelin-mediated glomerular damage is its influence on nephrin protein that has a direct impact on renal filtrating barrier.

The cascade of events leading to the damage of the filtrating barrier begins with the glomerular glycocalyx and continues with renal endothelium which gradually goes toward dysfunction and albumin excretion whose grade depends on the severity of damage suffered by the endothelial cells, a factor majorly accounting for the increasing plasmatic levels of reacting endothelial substances such as ET-1. This close relationship between ET-1 and albuminuria is confirmed by other papers such as year 2008 in which this finding was statistically significant in 279[20] diabetic patients. In another paper it was found to exist a statistically significant correlation between ET-1, von Wilenbrand (vW) factor and albuminuria[21].

*b) ET-1 and Fundus Oculi*

All patients underwent dilated FO examination by indirect ophthalmoscopy.

Our data shows that in the group of patients with normoalbuminuria only 10% of the patients did not show signs of (DR) stage 1- (no apparent retinopathy), 16% of the patients had stage 2 DR (Mild Non-Proliferative Diabetic Retinopathy), 58% had stage 3 DR (Moderate Non-proliferative Diabetic Retinopathy), and only 16% of the patients had stage 4 DR (severe non proliferative diabetic retinopathy) and none of fifth grade. The 16% of patients with third grade diabetic retinopathy were normoalbuminuric patients suffering from arterial hypertension.

In the group of patients with MA 12% of patients had stage 1 DR, 65% had stage 2 DR and 23% had stage 3 proliferative DR. These data suggest that changes in the fundus oculi are related to retinal endothelial dysfunction and may be visible even in the first 2 years of diabetes appearance. Moreover these changes have a tendency to be more prominent by the presence of other risk factors such as arterial hypertension, a finding which was frequent in a subgroup of our patients. The strong relation observed between FO and the excreted amount of albumin where ($p<0.001$) and the changes depicted in FO and ET-1 where ($p<0.032$) bare suggestive hints regarding nature of this dysfunction, which seems to affect in the same way the vessels of small caliper (renal and retinal endothelium), and at the same time reconfirms the importance of these clinical markers in evaluating this dysfunction.

This can be explained with the functional changes suffered by the endothelium of small vessels in the retinal and glomerular tissue due to endothelial dysfunction. Hyperglycemia exerts its effects mainly through 4 elucidated mechanisms in MA[22] (polyol path, AGE (Advanced Glycation end Products, PKC (Creatine Phospho Kinase) and hexamine) which lead to an increase in inflammatory cytokines, vascular endothelial growth factor (VEGF) production and consequently to hyperpermeability and neoangiogenesis phenomenon. VEGF inhibits renal and retinal hypertrophy. It prevents cellular dysfunction regarding intracellular NO production[23,24]. The predominance of substances inhibiting production of NO from arginine25 and the reduction of NO biodisponibility shall lead to an important endothelial dysfunction in the retina and kidney manifesting with urinary excretion of albumin.

## VI. Conclusion

Based on the results of our study we can affirm that ET-1 is a very significant biomarker in the early detection of renal endothelial dysfunction. Raised plasmatic ET-1 levels in type 2 diabetic patients are a major clue in helping the general practitioner uncover this dysfunction and to intervene timely in order to prevent or slow DN from its early stages.

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