Original Article

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Can Visfatin be Considered as a Diagnostic Marker for Diabetic Nephropathy?

Diyabetik Nefropati için Tanısal Belirteç Olarak Visfatin Düşünülebilir mi?

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Abstract

Purpose: In this study, we investigated the role of visfatin in early diabetic nephropathy and its association with oxidative stress, paraoxonase (PON) and arylesterase.
Material and Method: Twenty five diabetic patients with microalbuminuria, 25 diabetic patients with normoalbuminuria and 25 healthy individuals were enrolled in the study. Serum total antioxidant status (TAS), total oxidant status (TOS), PON, arylesterase, free sulfhydryl (SH) group, lipid hydroperoxide (LOOH) and visfatin levels were measured. The ratio of TOS to TAS was accepted as oxidative stress index (OSI).
Results: Serum visfatin levels were higher in microalbuminuric diabetic group compared to that in normoalbuminuric diabetic group and healthy control group (p<0.001, for each). Visfatin levels in normoalbuminuric diabetic group were significantly higher compared to healthy control group (p=0.001). Correlation analysis yielded that plasma visfatin levels were negatively correlated with SH, arylesterase, and PON levels (r=-0.444, p<0.001; r=-0.340, p=0.004; r=-0.322, p=0.006, respectively) and that they had a positive correlation with LOOH and OSI levels (r=0.252, p=0.034; r=0.622, p<0.001; respectively). According to logistic regression analysis model, the increased levels of OSI and serum visfatin OSI index clarify 51% of developing microalbuminuria in diabetic patients (sensitivity 78.3% and specificity 83.7%).
Discussion: The data of this study reveal that increased serum levels of visfatin have a role in the development of diabetic nephropathy. Visfatin has a significant correlation with oxidative stress.
Keywords: Diabetic nephropathy, visfatin, oxidative stress index, paraoxonase, arylesterase

Öz

Amaç: Bu çalışmada erken diyabetik nefropatide visfatinin rolünü ve bunun oxidatif stres, paraoksanaz (PON) ve arilesteraz ile ilişkisini araştırmaktır.
Gereç ve Yöntem: Çalışmaya mikroalbuminurisi olan diyabetik 25 hasta, normoalbuminurisi olan diyabetik 25 hasta ve 25 sağlıklı kişi alındı. Serum toplam antioksidan kapasite (TAK), toplam oksidan kapasite (TOK), PON, arilesteraz, serbest sülfidril (SH) grup, lipid hidroksiperoksid (LOOH) ve visfatin düzeyleri ölçüldü. TOK'un TAK'a yüzde olarak oranlaması oksidatif stres indeksi (OSİ) olarak kabul edildi.
Bulgular: Serum visfatin düzeyleri mikroalbuminurik diyabetik grupta normoalbuminurik diyabetik grup ve sağlıklı kontrol gruba göre daha yüksekti (her biri için, p<0,001). Korelasyon analizi visfatin düzeyleri SH, arilesteraz ve PON düzeyleri ile negatif korelasyon (sırasıyla, r=-0,444, p<0,001; r=-0,340, p=0,004; r=-0,322, p=0,006) ve LOOH ve OSİ düzeyleri ile pozitif korleasyon olduğunu vermiştir (sırasıyla, r=0,252, p=0,034; r=0,622, p<0,001). Lojistik regresyon analiz modeline göre serum visfatin ve OSİ düzeylerindeki artış diyabetik hastalarda mikroalbuminuri gelişimini %51 oranında açıklamaktadır (duyarlılık %78,3, özgüllük %83,7).
Tartışma: Bu çalışmanın verileri serum visfatin düzeyleri artışının diyabetik nefropati gelişiminde bir rolü olduğunu ortaya koymuştur. Visfatin, oksidatif stres ile önemli korelesyon göstermektedir.
Anahtar kelimeler: Diyabetik nefropati, visfatin, oksidatif stress indeksi, paraoksanaz, arilesteraz

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Introduction

Diabetic nephropathy is one of the important complications of diabetes mellitus (DM) and is the most common cause of end-stage renal failure in clinical practice (1). Early treatment of diabetic nephropathy depends on understanding the underlying mechanism of the disease.

Though microalbuminuria has been identified as the most effective indicator of early diabetic nephropathy, some structural changes might have already occurred by the time microalbuminuria is detected (2).

Visfatin, a insulin mimetic adipokine produced by visceral adipose tissue, is also synthesized in renal glomerular mesangial cells (3,4). Various studies have shown that the level of visfatine increases in obese and type 2 DM patients (5,6). Song et al. (4) reported that visfatine levels were upregulated in renal glomerular mesangial cells by glucose stimulation and, consequently, glucose uptake was increased. Additionally, the synthesis of profibrotic molecules including transforming growht factor-β, plasminogen activator inhibitor-1 and type I collagen was found to be increased by visfatin treatment and visfatin-induced upregulation of profibrotic molecules was inhibited with pretreatment with cytochalasin B.

It has been suggested that increased oxidative stress, an important parameter for DM, could be responsible for developing diabetic nephropathy (7). It has been postulated that the oxidant/antioxidant balance is deformed before developing renal lesions and oxidation degree is increased in parallel to the progression of the disease (8). Oxidative stress has been found to increase in vascular endothelium, skeletal muscle and human malignant melanoma cells after exogenous administration of visfatin (9,10,11). These data made us think that visfatin may have a role in developing diabetic nephropathy via increasing oxidative stress.

The role of visfatin levels in early-stage diabetic nephropathy and the relationship of oxidative stress with antioxidant enzymes, arylesterase and paraoxanase (PON) were investigated.

Materials and Methods

The study was initiated upon obtaining approval from local ethics committee. All participants were informed and an informed consent was obtained prior to participation in the study.

Patient Group and Study Protocol

A total of 75 participants were included in the study: 25 type 2 diabetic patients with microalbuminuria (median age=51.0 years; IQR:10.0; 13 females, 12 males); 25 type diabetic patients with normoalbuminuria (median age=47.0 years; IQR:9.0; 14 females, 11 males); and 25 healthy individuals (median age= 48.0 years; IQR:12.5; 15 females, 10 males). Individuals with systemic diseases, such as infectious diseases, inflammatory diseases, hypertension, liver failure, cardiovascular diseases, malignancies, neurodegenerative diseases, cerebrovascular diseases; those on antioxidants, such as antihypertensive medications, lipid-lowering medications, and vitamin E; and smokers were excluded from the study. Eight patients with microalbuminuria and 3 patients with normoalbuminuria were receiving insulin therapy. Other patients were taking at least 2 oral antidiabetic drugs.

Evaluation of Nephropathy and Duration of Diabetes Mellitus

The mean urine albumin/creatinine ratio was measured in the spot urine collected on 3 different days. Urine contaminated with bacteria, red blood cells, and white blood cells were removed. Urinary albumin concentration was measured via latex turbidimetric immunoassay method using commercial kits. When albumin/creatinine ratio was 30-300 mg/g in type 2 DM patients, it was considered microalbuminuria. The staging criteria recommended by Mogensen et al. (12) were used for the diagnosis of early diabetic renal disease.

For the duration of DM, the time of initial symptoms associated with the disease was considered beginning of the disease. If there were no symptoms, the time of diagnosis was considered as the beginning. The diagnostic criteria for the diagnosis of DM established by the American Diabetes Association in 2010 were used for the diagnosis of DM (13).

Measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP), height, and weight were measured in each participant. Body mass index (BMI) was calculated as body mass (kg)/height (m)^2.

Blood samples were collected in the morning hours after an 8-hour fasting period. Serum samples were stored at -80 °C until total antioxidant status (TAS), total oxidant status (TOS), PON, arylesterase, free sulfhydryl (SH) group, lipid hydroperokside (LOOH) and visfatin levels were measured. Urine microalbumin and creatinine values were measured via turbidimetric method by a Cobas Integra 800 model auto-analyzer (Roche®). Hemoglobin A1c (HbA1c) levels were tested by using the Celldyn 3700 (Abbott®, USA) auto-analyzer commercial kit. Serum urea and creatinine values were measured spectrophotometrically by routine biochemical methods using Cobas Integra 800 model auto-analyzer (Roche®). Serum triglyceride (TG), total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured using an auto-analyzer (Aeroset, Abbott®, USA) commercial kit (Abbott®, USA).

Measurement of Total Oxidant/Antioxidant Status

Serum total oxidant status (TOS) was determined using a novel automated measurement method developed by Erel (14). Oxidants present in the study sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed as mmol H_2O_2 Equiv./l.

Serum TAS was determined using a novel automated measurement method developed by Erel (15). In the method, hydroxyl radical, the most potent biological radical, is produced first. In the assay, reagent 1 containing ferrous ion solution is mixed with reagent 2, which contains hydrogen peroxide. The sequentially produced radicals, such as brown colored dianisidinyl radical cation produced by the hydroxyl radical, are

also potent radicals. The anti-oxidative effect of the study sample against the potent-free radical reactions, which are initiated by the produced hydroxyl radical, was measured. The assay has excellent precision values, lower than 3%, and the results are expressed as mmol Trolox Equiv./l. OSI percentage ratio of TOS to TAS level was accepted as OSI [OSI (Arbitrary Unit) = TOS (mmol H_2O_2 Equiv./l)/TAS (mmol Trolox Equiv./l)] (16).

Serum LOOH levels were measured with the ferrous ion oxidation–xylenol orange assay. The principle of the assay depends on the oxidation of ferrous ion to ferric ion through various oxidants and the produced ferric ion is measured with xylenol orange. LOOHs are reduced by triphenyl phosphine (TPP), which is a specific reductant for lipids. The difference between with and without TPP pretreatment gives LOOH levels (17).

Measurement of total free SH groups was briefly as follows: 1 ml of buffer containing 0.1 MTris, 10 mMEDTA, pH 8.2, and 50 l serum was added to a cuvette followed by 50 l 10 mMDTNB in methanol. Blanks were run for each sample as a test, but there was no DTNB in the methanol. Following incubation for 15 minutes at room temperature, sample absorbance was read at 412 nm on a Cecil 3000 spectrophotometer. Sample and reagent blanks were subtracted. The concentration of SH groups was calculated using reduced glutathione as free SH group standard and the result was expressed as millimolars (15).

Measurements of Paraoxonase and Arylesterase Activities

PON and arylesterase activities were measured with commercially available kits (Relassay, Gaziantep, Turkey). PON measurement was performed either in the presence (salt-stimulated) or in the absence of NaCl. Paraoxon hydrolysis rate (diethyl-p-nitrophenyl phosphate) was measured by monitoring increased absorption at 412 nm at 37 °C. The amount of generated p-nitrophenol was calculated from the molar absorption coefficient at pH 8.5, which was 18.290/M per cm (18). PON activity was expressed as U/l serum. The coefficient of variation (CV) for individual samples was 1.8%. Arylesterase activity was measured using phenyl acetate as substrate. Enzymatic activity was calculated from the molar absorption coefficient of the produced phenol, 1310/M per cm. One unit of arylesterase activity was defined as 1 mmol phenol generated per minute under the above conditions and expressed as U/l (19). The CV for individual serum samples was 4.1%. The sensitivities of both tests were over 98%.

Measurement of visfatin levels: Serum visfatin levels were determined by Sandwich ELISA (RayBiotech®, Inc. Norcross, GA, USA).

Statistical Analysis

The Shapiro-Wilk test was used to test continuous variables for normality. Measurements of normally distributed variables (TAS, OSI, HDL, LDL, urea and arylesterase) are presented as mean ± standard deviation. Those with non-normal distributions are presented as median and interquartile range (IQR). Student's t-test was used in comparison of 2 independent groups of normally distributed variables; one-way analysis of variance (ANOVA) test was used when comparing more than 2 groups; and Fisher's Least Significant Difference (LSD) test was used for paired comparisons to identify the group that the difference was caused by. For non-normally distributed variables, the Mann-Whitney U test was used to compare 2 independent groups and the Kruskall Wallis test was used to compare more than 2 independent groups. Dunn's test was used for post-hoc comparisons. Spearman's correlation analysis was done to identify associations between the parameters. Multiple linear regression analysis was conducted to identify the variables impacting the visfatin level. Multiple logistic regression analysis was performed to evaluate the relationship of microalbuminuria with OSI and visfatin in diabetic patients. SPSS for Windows version 15 software was used for statistical analyses. The level of significance was set at $p \leq 0.05$.

Results

The mean age of the participants in all the 3 groups and their gender distribution were similar (p=0.280 and p=0.898, respectively). There was no significant difference in body mass index, systolic blood pressure, DBP, LDL-C, HDL-C, blood urea nitrogen (BUN), and creatinine levels between the groups (p=0.245, p=0.677, p=0.648, p=0.425, p=0.124, p=0.120, and p=0.634, respectively). Diabetic patient groups with and without microalbuminuria were not different in terms of duration of DM, insulin use, and HbA1c levels (p=0.105, p=0.072, and p=0.728, respectively; Table 1).

TAS level was lower in microalbuminuric and normoalbuminuric diabetic groups compared to that in healthy control group (p<0.001 and p=0.005, respectively). Although the TAS levels were lower in microalbuminuric diabetic group compared to normoalbuminuric diabetic group, the difference was not significant (p=0.096).

TOS levels were higher in microalbuminuric diabetic group than in normoalbuminuric diabetic group and healthy control group (p=0.002 and p<0.001, respectively). However, there was no significant difference between normoalbuminuric group and healthy control group in terms of TOS levels (p=0.902).

OSI levels were higher in microalbuminuric diabetic group compared to normoalbuminuric diabetic group and healthy control group (p<0.001, for each). The OSI levels in normoalbuminuric group were higher than those in healthy control group, however, the difference could not reach the level of significance (p=0.058).

PON levels were lower in microalbuminuric diabetic group and normoalbuminuric diabetic group compared to that in healthy control group (p=0.005, for each). The groups with and without microalbuminuria did not differ significantly with respect to PON levels (p=0.930).

Visfatin levels were higher in microalbuminuric diabetic group than in normoalbuminuric diabetic group and healthy control group (p<0.001, for each). Visfatin levels in normoalbuminuric group were higher than those in healthy control group (p=0.001)

Arylesterase levels were lower in microalbuminuric diabetic group compared to healthy control group (p=0.021). On the other hand, there was no significant difference between diabetic groups with and without microalbuminuria or between normoalbuminuric group and healthy control group (p=0.844 and p=0.089, respectively).

SH levels were lower in diabetic groups with and without microalbuminuria compared to that in healthy control group

(p<0.001 and p=0.002, respectively). SH levels in microalbuminuric group were lower than those in normoalbuminuric, but the difference was not significant (p=0.127).

LOOH levels were higher in microalbuminuric diabetic group compared to normoalbuminuric diabetic group and healthy control group (p<0.001, for each). LOOH levels in normoalbuminuric group were lower than those in healthy control group, however, the difference could not reach the level of significance (p=0.093).

Correlation analysis yielded that serum visfatin levels were negatively correlated with SH, arylesterase, and PON levels (r=-0.444, p<0.001; r=-0.340, p=0.004; r=-0.322, p=0.006, respectively) and that they positively correlated with LOOH and OSI levels (r=0.252, p=0.034; r=0.622, p<0.001; respectively). These associations were confirmed in the multiple regression analysis (Table 2).

Multiple logistic regression analysis results demonstrated that microalbuminuria development was significantly associated with oxidative stress and serum visfatin levels in diabetic individuals (Table 3). According to logistic regression analysis model, the increased levels of serum visfatin and OSI clarify 51% of microalbuminuria development in diabetic patients (sensitivity 78.3% and specificity 83.7%) in our study.

Discussion

It has been reported that elevated visfatin levels were associated with the progression of diabetic nephropathy and other vascular complications of diabetes (20). Plasma visfatin levels were positively correlated with urinary albumin excretion, and were inversely correlated with creatinine clearance as well as plasma adiponectin (21). It has been suggested that increased visfatin synthesis from cultured mesangial cells may have a role in diabetic nephropathy via aggravation of metabolic alterations (4). However, the relationship of visfatin with oxidative stress, PON and arylesterase in diabetic nephropathy has not been evaluated yet.

Various studies have shown that oxidative stress develops due to a disturbance in the balance between oxidants and antioxidants (22). The role of oxidative stress in the development of diabetic nephropathy has been demonstrated (23). In this study, it has been shown that there was a significant correlation between visfatin and oxidative stress. Oxidative stress has been shown to occur

Table 1. Clinical and metabolic parameters of the mikroalbuminuric diabetic, normoalbuminuric diabetic and healthy control groups

Parameters	Microalbuminuric diabetic (n=25)	Normoalbuminuric diabetic (n=25)	Healthy control (n=25)	p	d
Age (years)*	51.0±10.0	47.0±9.0	48.0±12.4	0.280	
Sex (Female/Male)	13/12	14/11	15/10	0.898	
BMI (kg/m^2)*	27.5±4.2	27.3±3.2	27.1±2.82	0.245	
Duration of DM (years)*	5±7	5±6		0.105	
Insülin use (years)	8	3		0.072	
SBP (mmHg)*	120.0±20.0	120.0±20.0	120.0±20.0	0.677	
DBP (mmHg)*	80.0±20.0	80.0±20.0	75.0±20.0	0.648	
FPG (mg/dl)*	174.0±98.0	176.0±110.0	90.0±20.0	<0.001	
LDL (mg/dl)	112.1±28.4	109.8±26.3	120.0±32.3	0.425	
HDL (mg/dl)	42.2±6.7	44.4±5.8	46.4±5.8	0.124	
BUN (mg/dl)	32.2±9.3	29.7±8.1	30.2±5.9	0.120	
Cr (mg/dl)*	0.8 ±0.3	0.9±0.2	0.9±0.1	0.634	
Urinary microalbumin/Cr (mg/L) *	52.0±68.0	11.0±9.0	8.0±11.0	<0.0001	
Visfatin (ng/ml)*	102.0±30.0[a,b]	36±13.0[c]	28±13.0	<0.001	
TAS (µmol H_2O_2)	0.9±0.2	1.0±0.1	1.2±0.2	<0.001	
TOS (mmol Equiv./l)*	20.2±9.4	16.2±2.6	16.4±2.2	<0.001	
OSI (arbitrary unit)	2.3±0.4[a,b]	1.7±0.4	1.4±0.3	<0.001	
PON (U/ml)*	106.0±118.0[d]	115.0±101.0[e]	195.0±133.5	0.005	
Arylesterase (U/ml)	196.4±27.9[f]	202.1±38.9	223.9±34.8	0.016	
SH (mmol/L)*	0.2±0.1[b]	0.2±0.1	0.3±0.1	<0.001	
LOOH (mmol H_2O_2 Equiv./l)*	12.1±6.1[a,b]	9.4±2.1	9.9±1.3	<0.001	

*Data in which non-parametric tests were used and expressed as median (Inter quarter range). DM: Diabetes Mellitus, BMI: Body mass index, FPG: Fasting plasma glucose, HDL/LDL: High-density lipoprotein/Low-density lipoprotein, BUN: Blood urea nitrogen, Cr: Creatinin, OSI: Oksidative stress index, PON: Paraoxonase, SBP/DBP: Systolic blood pressure/Diastolic blood pressure, TAS: Total antioksidant status, TOS: Total oxidative status, SH: Sulfhydryl, LOOH: Lipid hydroperoxide P<0.001, [a]Microalbuminuric diabetic versus normoalbuminuric diabetic, p<0.001, [b]Microalbuminuric diabetic versus control, p=0.001, [c]Normoalbuminuric diabetic versus control, p=0.005, [d]Microalbuminuric diabetic versus control, p=0.005, [e]Normalbuminuric diabetic versus control, p<0.021, [f]Mikroalbuminuric diabetic versus

as a result of disturbed oxidant/antioxidant balance in type 2 DM and to have a role in the development of diabetic nephropathy (7,8). TAS, TOS, OSI, SH and LOOH levels were analysed as oxidant status markers in this study. OSI and LOOH levels were significantly higher in microalbuminuric diabetic group compared to both normoalbuminuric diabetic and healthy control groups. TAS and SH levels were significantly lower in diabetic groups with and without microalbuminuria compared to healthy control group. However, SH levels in microalbuminuric group were lower than those in normoalbuminuric group, however, the difference was not significant. These results show that the oxidant-antioxidant balance in diabetic nephropathy may be started by an increase in oxidants following with a decrease in antioxidants.

Various studies reported an increase in oxidative stress following exogenous admistration of visfatin (9,10,11). The administration of APO866, a visfatin inhibitor, decreased the tissue damage related to oxidative stress through a mechanism involving NADPH oxidase stimulation in myocardial infarction-induced rats (24). This study revealed firstly the relationship between visfatin and oxidative stress in patients with diabetic nephropathy. According to logistic regression analysis model, the increased levels of serum visfatin and OSI clarify 51% of microalbuminuria development in diabetic patients (sensitivity 78.3% and specificity 83.7%) in our study.

PON-1, which has PON and arylesterase activities, is a HDL-bound antioxidant enzyme that protects LDL-C and HDL-C against oxidative damage. Reduced PON1 enzyme activity is associated with several arteriosclerosis-related diseases. Studies have determined lower PON-1 activities in patients with type 1 and 2 DM than in healthy individuals (25,26,27). In accordance with other studies, in our study, PON-1 levels was lower in both diabetic groups with and without microalbuminuria compared to that in healthy control group (p=0.005 and p=0.005, respectively).

Table 2. Multiple linear regression analysis between visfatin and oxidant and antioxidant markers

	β **coefficients**	**p value**
SH	0.255	0.013*
LOOH	0.345	0.015*
Arylesteraz	0.088	0.373
PON-1	0.224	0.018*
OSI	0.673	0.0001*

*Significant at p<0.05, SH: Sulfhydryl, LOOH: Lipid hydroperoxide; OSI: Oxidative stress index; PON: Paraoxonase

Table 3. Results of logistic regression models predicting microalbuminuria

	Microalbuminuria vs normal	
	OR (95% CI)	**p value**
Visfatin	1.097 (1.002-1.200)	0.042*
OSI	0.092 (0.018-0.484)	0.005*

*Significant at p<0.05, OSI: Oxidative stress index, OR: Odds ratio CI: Confidence Interval

The role of PON-1 activities in the development of DM-related complications has been investigated. A negative correlation was detected between PON-1 activities and presence of vascular complications (26). Diabetic patients with neuropathy were demonstrated to have lower PON-1 levels compared to diabetes patients without neuropathy (28). Another study has shown an association between PON-1 expression and development of diabetic nephropathy in type 2 DM patients (29). In this study, PON and arylesterase levels were lower in microalbuminuric diabetic group compared to the normoalbuminuric diabetic group, however, the difference was not significant (p=0.930). Correlation analysis yielded that PON1 levels were negatively correlated with visfatin. Multiple regression analysis showed that decreased PON levels was an independent risk factor for increased serum visfatin levels.

Consequently, visfatin level is increased in early stage type 2 diabetic nephropathy. Serum visfatin levels showed a strong correlation with oxidative stress whereas a mild correlation with PON and arylesterase, the antioxidant enzymes. Further studies on this topic will help us better understand the pathogenesis of early diabetic nephropathy and to develop new treatment options.

Ethics

Ethics Committee Approval: The study were approved by the Harran University of Local Ethics Committee, Informed Consent: Consent form was filled out by all participants, Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Suzan Tabur, Hakan Korkmaz, Tevfik Sabuncu, Design: Suzan Tabur, Mehmet Ali Eren, Tevfik Sabuncu, Data Collection or Processing: Suzan Tabur, Hakan Korkmaz, Mehmet Ali Eren, Nurten Aksoy, Analysis or Interpretation: Suzan Tabur, Hakan Korkmaz, Nurten Aksoy, Seval Kul, Literature Search: Suzan Tabur, Hakan Korkmaz, Elif Oğuz, Writing: Suzan Tabur, Hakan Korkmaz, Conflict of Interest: No conflict of interest was declared by the authors, Financial Disclosure: The authors declared that this study received no financial support.

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