

Correlations of six related purine metabolites and diabetic nephropathy in Chinese type 2 diabetic patients

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Abstract

Objectives: The assessment of the clinical significance of adenosine, adenine, inosine, xanthine, hypoxanthine and uric acid concentrations in patients with type 2 diabetes mellitus (T2DM) and diabetic nephropathy (DN) for the detection of the relationship between purine metabolites and disease.

Design and methods: The study group consisted of 119 subjects which were divided into three groups: control ($n=31$), type 2 diabetes without nephropathy (DM, $n=23$) and with nephropathy (DN, $n=65$). Levels of related metabolites were measured in plasma of all participants.

Results: There is a significant increase of levels of adenosine ($P<0.001$), inosine ($P<0.001$), xanthine ($P=0.012$) and uric acid ($P=0.016$) with DN compared to DM. The level of xanthine oxidase (reflected by the uric acid: xanthine) did not change.

Conclusion: The levels of adenosine, inosine, uric acid and xanthine may be useful for monitoring the progression of DM and evaluating the treatment.

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Keywords: Type 2 diabetes; Diabetic nephropathy; Biomarker; Uric acid; Xanthine; Adenosine; Inosine

Introduction

In the recent years, a dramatic increase in the number of people diagnosed with T2DM is being observed. In 2002, it was estimated that 150 million people in the world had diabetes. This number is expected to increase to 300 million by the year 2025; most of these cases will be type 2 diabetes [1]. The growing epidemic of diabetes will ultimately affect more people than any other disease. The most important problem in diabetic people is the development of the vascular complications related to micro- and macro-angiopathy. Diabetic nephropathy, a serious vascular complication is observed in approximately one third of patients with diabetes. It has been the most common cause of end-stage renal disease. In addition, DN is associated with considerably increased cardiovascular disease risk and mortality. Thus, the

public health burden from DN is enormous [2]. Despite intensive research carried out by numerous groups, albumin excretion rate remains the standard risk factor of diabetic nephropathy development. However, it is generally acknowledged that overt microalbuminuria occurs at late stages of the pathologic changes in kidneys. Therefore, it is of tremendous importance to detect other markers of kidney damage that would precede its occurrence.

It is well established that purine metabolic pathway is strongly associated with the development of DN. Among related metabolites, adenosine plays an important role in water–electrolyte metabolism, such as renal blood flow, renin release and tubuloglomerular feedback [3,4]. Adenosine is phosphorylated into adenine and deaminated rapidly into inosine, which is converted to hypoxanthine. Then xanthine oxidase converts hypoxanthine to xanthine. Xanthine also acts as a substrate for xanthine oxidase and enhances superoxide generation [5], which plays a major role in microvascular dysfunction and exerts direct tissue damage, leading to lipid peroxidation.

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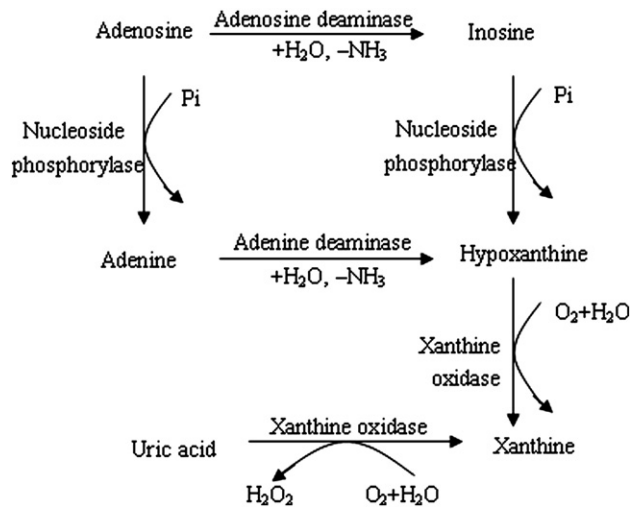


Fig. 1. The related purine metabolic pathway. Major components including adenosine, inosine, adenine, hypoxanthine, xanthine and uric acid were measured in this study.

Xanthine is metabolized to uric acid, the final product of purine degradation in humans. The high level of uric acid is associated with renal disease [6], but it is usually considered a marker of

renal dysfunction rather than a risk factor for progression. There is a controversy that plasma uric acid concentration is a cause or a result of renal disease [7,8]. Recent studies have reported that uric acid might be a true mediator of renal disease and progression. Uric acid can accelerate renal disease in experimental animals and epidemiologically is associated with progressive renal disease in humans [6]. In 2002, direct evidence was provided that uric acid induced vascular disease. The effect of uric acid on vascular smooth muscle cells in culture was also examined [7]. Metabolic pathways involved in these related metabolites are depicted in Fig. 1.

In the present paper, we assessed levels of the adenosine, adenine, inosine, xanthine, hypoxanthine, uric acid and xanthine oxidase (reflected by the uric acid: xanthine) in plasma in patients with DM and DN (Fig. 2). We also observed the relationship between the related metabolites and biochemical and clinical parameters.

Patients and methods

Plasma samples of 88 patients (52 males and 36 females, age 57.79 ± 8.66 years) in Beijing, China were collected as cases and 31 plasma samples of healthy people (22 males and 9 females, age

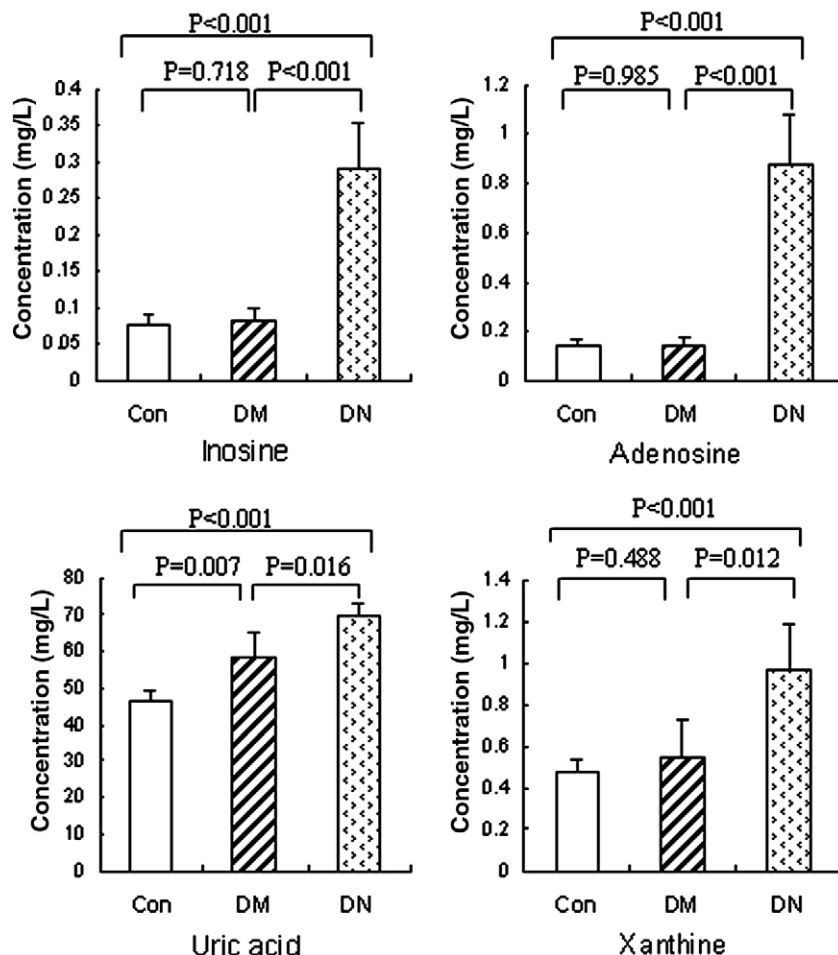


Fig. 2. Comparison of plasma potential risk factor concentrations among the groups of control (Con), DM and DN. DN subjects had significantly higher mean plasma concentrations of inosine ($P<0.001$, $P<0.001$), adenosine ($P<0.001$, $P<0.001$), uric acid ($P<0.001$, $P=0.016$) and xanthine ($P<0.001$, $P=0.012$) compared to the group of Con and DM. The group of DM had significantly higher mean plasma concentrations of uric acid ($P=0.007$) compared to the group of Con.

55±7.5 years) in the same area as controls. All study participants had given their informed consent. Type 2 DM was defined in accordance with the criteria of the American Diabetes Association. Details such as age, sex, and, in diabetes subjects, duration of diabetes, and other details of diabetic therapy were recorded; a complete clinical examination was conducted for all subjects.

Nephropathy was diagnosed if the patient had either persistent proteinuria (≥ 30 mg/day) or microalbuminuria (if albuminuria estimated by the albumin creatinine ratio (ACR) exceeded 30 $\mu\text{g}/\text{mg}$ of creatinine) in the absence of a urinary tract infection. In 65 patients, typical symptoms of diabetic nephropathy were proven.

Biochemical and clinical parameter analysis

The biochemical and clinical parameters of 88 patients were reviewed and approved by the Clinical Medicinal Research Institute, Sino-Japanese Friendship Hospital, Beijing, P. R. China. Body mass index (BMI) was defined as weight (kg) divided by height (m^2) — i.e., $\text{BMI} = W/H^2$. BP was taken in the seated position using standardized sphygmomanometers. The urinary albumin excretion was expressed as the average of three 24-h collections obtained during 6 months prior to the enrolment in the study. A written informed consent has been obtained from all patients and healthy controls. The clinical and biochemical analysis of all the cases was carried out in the year 2007. Serum creatinine, hemoglobin A_{1c} (HbA_{1c}), fasting glucose, lipid levels, and 24-h urinary protein levels were measured using standard automated clinical chemistry techniques.

Blood collection and preparation

Blood samples were collected from 6 to 9 o'clock after an overnight fast. All blood samples were centrifuged to obtain plasma in the hospital within 30 min and sent to our laboratory, where they were stored at -80°C prior to analysis. All measurements were done on the same sample of blood. Informed consent was obtained from all study subjects and the institutional ethics committee approved the study.

All blood samples were centrifuged to obtain plasma in the hospital and sent to our laboratory, where they were stored at -80°C until sample preparation. Before analysis, 800 μL of methanol were added to 200 μL aliquots of serum, vortexed for 2 min, and then centrifuged at 10,000 rpm for 15 min at 4°C . The clear supernatant was transferred to a 1.5 mL polypropylene tube, and dried under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 100 μL of a mixture of methanol–water (1:1, by volume), and stored at 4°C before analysis.

Detection of related metabolites in plasma

Plasma concentrations of adenosine, adenine, inosine, xanthine, hypoxanthine and uric acid were measured by high-performance liquid chromatography coupled to ultraviolet and tandem mass spectrometry method (HPLC-UV/MS/MS), using an Applied Biosystems (Toronto, Canada) API 3000 triple

quadrupole tandem mass spectrometer, equipped with a Turbo Ionspray interface and an Agilent 1100 binary HPLC system. Among the six metabolites, adenosine, adenine and uric acid were detected using UV detector for their good separation and others were detected using MS detector. They were quantified simultaneously in a single analysis. The method has been validated according to the requirement of analytical chemistry. Calibration curves suitable for the analysis of plasma were linear ($r^2 > 0.998$) with limits of detection (LOD) from 10 to 100 ng mL^{-1} . Intraday relative standard deviation (RSD) and interday RSD were both lower than 10%.

Statistical analysis

The results were analyzed using SPSS statistical package (version 14.0; SPSS Inc., USA). Some of the clinical characteristics of DM and DN subjects were compared with the Chi-square (χ^2) test and Fisher's exact test. To achieve normality, concentration data were log-transformed before analysis. Normality of the transformed data was verified by using the Kolmogorov–Smirnov test. The differences between the groups were calculated with Student's *t*-test or the non-parametric Mann–Whitney *U* test. The Spearman or the Pearson correlations were examined. In this study, a value of $P < 0.05$ was considered statistically significant. The metabolites, the levels of which have statistically significant differences between two groups of the three groups (control, DM, and DN) were defined as potential biomarkers.

Results

Clinical characteristics of type 2 DM

Table 1 shows the clinical characteristics of the study groups. DN patients were older, had significantly longer duration of the

Table 1
Clinical parameters of diabetic patients with (DM) and without nephropathy (DN)

Parameters	DM (<i>n</i> =23)	DN (<i>n</i> =65)	Statistical significance
<i>N</i> (male/female)	23(12/11)	65(41/24)	<i>P</i> =0.201
Age (years)	57.74±8.85	58.05±8.87	<i>P</i> =0.888
Duration of DM (years)	10.70±4.66	13.43±6.68	<i>P</i> =0.038
BMI (kg/m^2)	24.24±3.03	25.01±3.71	<i>P</i> =0.344
HbA _{1c} (%)	8.12±1.71	8.77±2.51	<i>P</i> =0.245
Albumin excretion rate ($\text{mg}/24\text{ h}$)	76.43±19.97	2407.82±565.42	<i>P</i> <0.001
Creatinine in serum ($\mu\text{mol}/\text{L}$)	78.45±8.45	362.75±83.82	<i>P</i> <0.001
Fasting blood glucose (mmol/L)	8.07±1.33	7.43±2.71	<i>P</i> =0.381
Triglycerides (mmol/L)	2.40±1.49	2.46±0.54	<i>P</i> =0.939
Total cholesterol (mmol/L)	5.41±0.66	5.20±0.44	<i>P</i> =0.619
HDL-cholesterol (mmol/L)	1.20±0.16	1.20±0.09	<i>P</i> =0.947
LDL-cholesterol (mmol/L)	3.43±0.36	2.84±0.32	<i>P</i> =0.023
Systolic blood pressure (mm Hg)	131.95±5.54	144.11±5.58	<i>P</i> =0.003
Diastolic blood pressure (mm Hg)	80.32±3.50	79.48±2.78	<i>P</i> =0.710
Urea nitrogen	5.73±0.55	14.70±2.48	<i>P</i> <0.001

Table 2
Related metabolites in DM patients with and without nephropathy and control group

Parameters	Control (n=31)	DM (n=23)	DN (n=65)	Statistical significance
Uric acid	46.53±3.01	58.59±6.36	69.38±4.09	$P=0.007^a$ $P<0.001^b$ $P=0.016^c$
Hypoxanthine	0.29±0.037	0.29±0.082	0.31±0.06	$P=0.911^a$ $P=0.726^b$ $P=0.862^c$
Xanthine	0.48±0.06	0.55±0.18	0.97±0.22	$P=0.488^a$ $P<0.001^b$ $P=0.012^c$
Inosine	0.077±0.012	0.081±0.017	0.29±0.065	$P=0.718^a$ $P<0.001^b$ $P<0.001^c$
Adenine	0.17±0.037	0.15±0.060	0.17±0.037	$P=0.357^a$ $P=0.750^b$ $P=0.374^c$
Adenosine	0.14±0.029	0.14±0.035	0.88±0.20	$P=0.985^a$ $P<0.001^b$ $P<0.001^c$
Uric acid: xanthine	139.5±46.96	141.2±25.49	131.5±31.22	$P=0.496$ $P=0.442$ $P=0.197$

^a P value from *t*-test between Control and DM.

^b P value from *t*-test between Control and DN.

^c P value from *t*-test between DM and DN.

disease, higher HbA_{1c}, creatinine in serum, LDL, albumin excretion rate, systolic blood pressure, postprandial blood glucose and urea nitrogen as compared to DM patients. No statistically significant differences of BMI, fasting blood glucose and HDL between the groups were observed.

Plasma xanthine, uric acid and uric acid: xanthine levels

The levels of uric acid and xanthine in the group of DN was significantly higher as compared with DM ($P=0.016$, $P=0.012$) and control group ($P<0.001$, $P<0.001$) (Fig. 2). Additionally, also, group of DM had significantly higher level of uric acid as compared to the healthy controls ($P=0.007$). For level of uric

acid: xanthine, no statistically significant differences between the groups were observed (Table 2).

Plasma adenosine and inosine levels

The levels of inosine and adenosine in the group of DN were significantly higher as compared with DM ($P<0.001$, $P<0.001$) and control group ($P<0.001$, $P<0.001$) (Fig. 2). But no statistically significant differences of levels of inosine and adenosine between the group of control and DM were observed.

Plasma adenosine and adenine levels

For levels of hypoxanthine and adenine, no statistically significant differences between the groups were observed.

Relationship between correlative metabolites and other parameters

Table 3 shows the relationship between levels of adenosine, adenine, inosine, xanthine, hypoxanthine, uric acid and clinical parameters including age, BMI, SBP, HbA_{1c}, and various other variables in all patients of both the DM and DN groups. Uric acid, xanthine and adenosine correlated positively with SBP and serum creatinine. In addition, xanthine and adenosine correlated positively with urea nitrogen.

Discussion

To better understand the role played by specific components leading to DM and DN, the metabolic profiling of them in the current study is dependent on an integrated technical platform composed of hyphenated LC-UV/MS/MS analysis, conventional clinical assays and classical statistical analysis. Compared with routine biochemical approaches, the highly selective and sensitive LC-UV/MS/MS method permits simultaneous determination of six relevant components.

Current evidence suggests that both genetic and environmental factors determine susceptibility to develop of DN [9–11]. Hypertension, poor glycemic and lipid control and smoking

Table 3
Correlation between metabolites and clinical parameters

	Uric acid	Hypoxanthine	Xanthine	Inosine	Adenine	Adenosine
BMI	0.006	0.042	0.150	0.007	0.155	0.256
Duration	0.120	0.032	0.227	−0.101	0.153	0.092
Age	0.190	−0.030	−0.172	0.006	0.136	0.124
SBP	0.392 **	0.206	0.292 *	0.084	0.065	0.485 **
DBP	0.055	0.065	0.117	−0.127	−0.077	0.240
Urea nitrogen	0.204	0.386 **	0.410 **	0.207	0.286 **	0.658 **
Serum creatinine	0.330 **	0.394	0.443 **	0.112	0.185	0.699 **
HbA _{1c}	0.273	−0.048	−0.240	0.043	−0.172	0.240
Cholesterol	−0.218	−0.173	0.018	−0.003	−0.196	0.010
Triglyceride	−0.288 *	−0.114	−0.008	−0.052	−0.108	−0.127
HDL	0.027	−0.035	−0.134	0.019	0.050	0.135
LDL	−0.167	−0.136	0.081	−0.030	−0.201	−0.049
Fasting blood glucose	−0.262 *	−0.118	−0.210	−0.135	−0.117	−0.238

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

increase the risk for development of DN [12]. Epidemiologic studies have shown that DN is strongly clustered in families and that race has a major effect on DN susceptibility and rate of progression, firmly establishing the importance of genetic risk factors in the development of DN [13,14]. Despite rapid research progress, robust predictors to assess prospectively with high precision the risk for DN in individuals with diabetes are still lacking. Thus, it is necessary to set out the study of biomarker discovery, especially for the low molecular weight metabolites, which are important and easy to be measured. In the present study, four potential biomarkers (adenosine, inosine, xanthine and uric acid) of nephropathy were showed.

In this study, the level of adenosine in the group of DN was significantly higher as compared with DM ($P<0.001$) and control ($P<0.001$). Adenosine correlated positively with urea nitrogen and serum creatinine. So we suppose that it may be a potential biomarker for diagnosis of nephropathy and evaluation of treatment. Adenosine, renal-circulation regulator, plays an important role in water–electrolyte metabolism, such as renal blood flow, renin release and tubuloglomerular feedback. It is suggested that the elevation of adenosine contributes to the renal vasoconstriction in the ANG II-induced hypertension. Adenosine is produced by two different enzymes, *S*-adenosylhomocysteine (SAH) hydrolase and 5′-nucleotidase. The extracellular metabolism of adenosine is mediated by two mechanisms. First, adenosine is taken up quickly and efficiently by red blood cells, via an equilibrative facilitated diffusion system [15]. Second, adenosine is deaminated rapidly into inosine by adenosine deaminase (ADA). ADA is found in large amounts particularly in mononuclear cells [16,17], where it plays a major role in adenosine concentration regulation in both extracellular [17] and intracellular spaces [18,19]. Some researchers had suggested that it was mediated by a decrease in the activity and expression of ADA, increased production of adenosine, and an induced imbalance in adenosine receptors. Moreover, type 2 DM is characterized by insulin resistance, a failure of the beta cell to produce enough insulin to overcome the resistance. The deficiency of insulin may lead to inappropriate immunoresponses even immune defect. A normal ADA activity level prevents adenosine (a strong immunosuppressive agent [20–22]) accumulation and thus ensures normal lymphocyte development and function [23]. The decrease in ADA activity, which induces high adenosine concentration in body fluids, causes severe combined immunodeficiency syndrome [21].

High level of uric acid is associated with renal disease, but it is usually considered a marker of renal dysfunction rather than a risk factor for progression. There is always a controversy that plasma uric acid concentration is a cause or a result of renal disease. Recently, many researchers set out an intense controversy that the contribution of uric acid is positive or negative in DM. D. Pitocco proposes that uric acid has a protective role and that hyperuricaemia is not the cause of mortality or causal observation, but probably is a consequence of the lack of NO production and a compensatory mechanism in order to reduce oxidative stress [24]. But A. G. Ioachimescu believes that uric acid may causally/mechanistically contribute to vascular disease [25]. The evidence is that hyperuricaemia

decreased nitric oxide (NO) production in bovine aortic cells [26], stimulated human vascular muscle cells proliferation [27] and increased C-reactive protein expression in these cells. However, several lines of evidence suggest that increased serum uric acid may be a significant risk factor of renal disease. Animal model experiments demonstrate that high level of uric acid in normal rats induced by the uricase inhibitor, oxonic acid (OA), results in hypertension, intrarenal vascular disease, and renal injury [7]. This led to the hypothesis that uric acid may contribute to progressive renal disease. In this study, we found significantly increased concentration of uric acid in the plasma of DM patients ($P=0.007$). It demonstrated that the high level of uric acid was not only a marker of renal dysfunction. In addition, we found that the level of plasma uric acid correlated positively with serum creatinine ($r=0.330$, $P<0.01$) and SBP ($r=0.392$, $P<0.01$). There was also a statistically significant difference of the level of plasma uric acid between the group of control and DN ($P<0.001$), as well as the group of DM and DN ($P=0.016$). The results demonstrated that uric acid might be a risk factor for DM and DN. Additionally, the results were consistent with some physiologic functions of uric acid, including activation of the rennin angiotensin system, and direct actions on endothelial cells and vascular smooth muscle cells. Hence, strategies to control and decrease plasma uric acid level may have a beneficial effect on improving kidney function or slowing the progression of renal diseases in clinical practice.

Xanthine acts as a substrate for xanthine oxidase and enhances superoxide generation. When xanthine oxidase converts hypoxanthine to xanthine in the presence of molecular oxygen, superoxide radicals (O_2^-) are released. Thereby, reactive oxygen species (ROS) generate, which play a major role in microvascular dysfunction and exert direct tissue damage, leading to lipid peroxidation, denaturation of proteins, and oxidation of DNA [28]. Many direct evidences have demonstrated that ROS was one of the most important mechanisms of DN. In this study, a significant difference between the group of control and DN ($P<0.001$), as well as the group of DM and DN ($P=0.012$) was presented. Additionally, the result of relationship study confirmed the well-recognized positive relationship between xanthine with renal function, as reflected by plasma creatinine. Therefore, xanthine may be a potential marker for monitoring the progression of DN. Previous studies on the effect of xanthine or xanthine oxidase as inducers of renal injury have produced inconsistent results. Galat et al. found that infusion of xanthine into isolated perfused kidneys increased the generation of oxygen free radicals and impairs renal function [29]. Another study by Linas et al. found that xanthine oxidase depletion improved renal function after reperfusion [30]. In this study, the level of xanthine increased with the progression of DN, while the level of xanthine oxidase (reflected by the uric acid: xanthine) did not change. So we suppose that the inducer of renal injury is not xanthine oxidase but xanthine itself. We also see that there is little change of the level of plasma hypoxanthine while an accrescent level of xanthine. Thanks to the invariant activity of xanthine oxidase, the increase of xanthine may come from guanine with the catalysis of guanine deaminase (GDA). GDA is an enzyme that catalyzes the

hydrolytic deamination of guanine to xanthine. Increased levels of GDA have been detected in cancerous kidney and serum of patients with endemic nephropathy [31]. Regrettably, in this study, the levels of guanine and guanine deaminase were not measured. So the hypothesis should be authenticated by the next study.

Acknowledgments

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