*Physiol. Res. 51: 597-604, 2002*

# Advanced Glycation End-Products and Advanced Oxidation Protein Products in Patients with Diabetes Mellitus

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*Received October 22, 2001*
*Accepted April 2, 2002*

## Summary

Accelerated glycoxidation takes part in the development of diabetic complications. We determined advanced glycation end-products (AGEs) and advanced oxidation protein products (AOPP) in the sera of 52 patients with diabetes mellitus (DM) – 18 with DM Type 1 and 34 with DM Type 2 and examined their relationship to the compensation of the disease. AGEs were estimated spectrofluorimetrically (350 nm/440 nm) whereas AOPP were determined spectrophotometrically (340 nm). AGEs were elevated only in DM Type 2 (DM2 $5.11\pm1.15x10^3$ AU/g vs controls $4.08\pm0.71x10^3$ AU/g, $p<0.001$, vs DM1 $4.14\pm0.86x10^3$ AU/g, $p<0.005$, DM1 vs controls were not significant). AOPP were elevated significantly in both types of DM with higher levels in DM Type 2 (DM2 157.50±75.15 μmol/l vs healthy subjects 79.80±23.72 μmol/l, $p<0.001$, vs DM1 97.50±30.91 μmol/l, $p<0.005$, DM1 vs controls $p<0.05$). There was a tight correlation between AGEs and AOPP in both types of DM (DM1 $r=0.75$, DM2 $r=0.47$ ($p<0.05$)) and both AGEs and AOPP correlated with triglycerides. In DM Type 1 only, AGEs correlated with HbA1c $r=0.47$ ($p<0.05$) and with blood glucose. Slight but not significant differences in AGEs and AOPP levels were observed in patients with or without diabetic complications. Oxidative stress is increased in both types of DM, more in Type 2 where it contributes to the formation of glycoxidation products.

**Key words**

Advanced glycation end-products • Advanced oxidation protein products • Diabetes mellitus • Oxidative stress

## Introduction

Diabetes mellitus is associated with hyperglycemia and thus with accelerated non-enzymatic glycation (Maillard 1912, Vlassara 1997), oxidative stress (imbalance between free radicals and reactive oxygen and nitrogen species, and antioxidants in favor of free radicals), and carbonyl stress (increase of reactive carbonyl compounds caused by their enhanced formation and/or decreased degradation or excretion) (Miyata *et al.* 1999, Baynes and Thorpe 1999). Through these mechanisms, advanced glycation end-products (AGEs – among them pentosidin and carboxymethyllysine are best known) and advanced oxidation protein products (AOPP) are formed, and biologically important compounds are damaged (Witko-Sarsat *et al.* 1996, Miayta *et al.* 1999).

Accumulation of AGEs has several toxic effects. AGEs can modify proteins, directly damage the structure and metabolism of extracellular matrix or act *via* their specific receptors (e.g. RAGE – receptor for advanced



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glycation end-products). AGE-RAGE interaction activates nuclear factor NF-κB, stimulates the transcription of genes for cytokines, growth factors and adhesive molecules, induces migration of macrophages and has further toxic effects (Bierhaus *et al.* 1998). In addition to circulation in the blood, AGEs accumulate in tissues and thus take part in the development of diabetic complications – nephropathy, neuropathy, retinopathy and angiopathy. They cause damage to biological membranes and the endothelium. Moreover, they modify LDL particles and together with vascular damage, they are involved in the acceleration of atherosclerosis (Vlassara 1997). Apart from diabetes mellitus, AGEs are elevated in patients with renal failure (more than in diabetic patients) and play a role in the development of accelerated atherosclerosis, dialysis related amyloidosis and participate in the damage to peritoneum of patients treated with continuous ambulatory peritoneal dialysis (Miyata *et al.* 2000). Moreover, they accumulate in the nervous tissue in neurodegenerative diseases (Alzheimer´s disease) (Munch *et al.* 1998).

Advanced oxidation protein products were described by Witko-Sarsat *et al.* (1996) for the first time. They are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (produced by myeloperoxidase in activated neutrophils). They are supposed to be structurally similar to AGE-proteinss and to exert similar biological activities as AGEs, i.e. induction of proinflammatory cytokines and adhesive molecules. They are elevated in patients with renal insufficiency and have the highest levels in patients on renal replacement therapy (Witko-Sarsat *et al.* 1996). Moreover, increased levels were found in HIV-positive patients where they correlated with markers of monocyte activation (Witko-Sarsat *et al.* 1998), and in hypoxic premature newborns (Buoncore *et al.* 2000).

The aim of this study was to determine AGEs and AOPP in patients with diabetes mellitus type 1 and type 2 in order to examine their relationship to other metabolic parameters (blood glucose, HbA1c, serum lipids), blood pressure and complications of diabetes mellitus.

## Patients and Methods

*Patients*

The studied group consisted of 52 patients with diabetes mellitus (DM) - 18 patients with DM type 1 (DM1) and 34 patients with DM type 2 (DM2).

Patients with DM type 1 (8 males and 10 females), mean age 46±12 years (22-71 years) were treated for diabetes mellitus for 23±13 years (2-43 years). Their biochemical parameters were as follows: blood glucose concentration 9.6±4.01 mmol/l, glycated hemoglobin HbA1c 7.5±1.04 %, serum total protein 76.1±4.58 g/l, cholesterol 5.1±0.62 mmol/l, HDL-cholesterol 1.5±0.49 mmol/l, LDL-cholesterol 3.0±0.53 mmol/l and triglycerides 1.2±0.53 mmol/l. Their blood pressure was 123±13/80±7 mm Hg (100-150/65-90). Thirteen of these patients had microvascular complications and one patient had macrovascular ones (both micro- and macrovascular complications).

Patients with DM type 2 (26 male and 8 female), mean age 60±8 years (46-77 years) were treated for diabetes mellitus for 10±7 years (1-37 years). Their biochemical parameters were as follows: blood glucose concentration 9.3±3.21 mmol/l, glycated hemoglobin HbA1c 7.5±1.18 %, serum total protein 75.0±6.16 g/l, cholesterol 5.8±1.05 mmol/l, HDL-cholesterol 1.2±0.42 mmol/l, LDL-cholesterol 3.6±0.9 mmol/l and triglycerides 2.1±1.02 mmol/l. Their blood pressure was 139±9/86±6 mm Hg (125-155/75-100). Twenty of these patients had microvascular and 13 patients macrovascular complications.

All patients had serum creatinine lower than 100 μmol/l, were in stabile clinical status without signs of acute infection and were not taking any antioxidants. They gave their informed consent prior to entering this study.

*Control group*

The control group consisted of 24 healthy subjects (12 males and 12 females), mean age 59±11 years (41-82 years), total serum protein 74.8±3.77 g/l. They were not taking any antioxidants and gave their informed consent prior to entering this study.

*Blood samples*

Blood samples were collected from the cubital vein. Blood was centrifugated at 1450x*g* at 4 °C for 10 min (Hettich). Serum was stored at –20 °C and determination of the samples occurred within 3 months.

*AGEs assay*

Determination of AGEs (i.e. some fluorescent products from the family of AGEs) was based on the spectrofluorimetric detection according to Henle *et al.* (1999) and Munch *et al.* (1997) in our modification (Kalousová *et al.* 2001). Blood serum was diluted 1:50

with PBS pH 7.4 and fluorescence intensity was recorded at the emission maximum (~440 nm) upon excitation at 350 nm (spectrofluorimeter Fluoromax-3, Jobin Yvon Horiba, USA). Fluorescence intensity was expressed in arbitrary units (AU) and in AU/g protein.

*AOPP Assay*

Determination of AOPP (i.e. some oxidation products with characteristic absorbance) was based on spectrophotometric detection according to Witko-Sarsat *et al.* (1996) in our modification (Kalousová *et al.* 2001). 200 µl of blood serum diluted 1:5 with PBS, 200 µl of chloramin T (0-100 µmol/l) for calibration and 200µl of PBS as blank were applied on a microtiter plate. 10 µl of 1.16 M KI and 20 µl of acetic acid were added and absorbance at 340 nm was measured immediately (spectrophotometer Multiskan Ascent, Labsystems, Finland). Concentration of AOPP is expressed in chloramine units (µmol/l).

*Other biochemical parameters*

Blood glucose, glycated hemoglobin HbA1c, total protein, triglycerides and cholesterol and its fractions (HDL- and LDL-cholesterol) were determined using routine clinical chemical assays recommended by IFCC.

*Statistics*

All results are expressed as mean ± standard deviation (SD). The statistical significance was evaluated using unpaired Student´s t-test and correlation coefficient r. Results were considered significant at $p<0.05$.

**Table 1.** Advanced glycation end-products and advanced oxidation protein products in the patients with diabetes mellitus and in healthy subjects.

| | **Patients with diabetes mellitus n=52** | | **Healthy subjects n=24** |
|---|---|---|---|
| | **Diabetes mellitus type1 n=18** | **Diabetes mellitus type2 n=34** | |
| AGEs (AU) | $3.56\pm0.74x10^5$** | | $3.06\pm0.56x10^5$ |
| | $3.15\pm0.68x10^5$ | $3.78\pm0.68x10^5$*** # | |
| AGEs (AU/g) | $4.77\pm1.12x10^3$* | | $4.08\pm0.71x10^3$ |
| | $4.14\pm0.86x10^3$ | $5.11\pm1.25x10^3$*** # | |
| AOPP (µmol/l) | 136.7±69.3*** | | 79.80±23.72 |
| | 97.5±30.9* | 157.5±75.2*** # | |

*Data are means ± SD. *** p<0.001, ** p<0.005, * p<0.05 vs healthy subjects, # p<0.005 vs diabetes mellitus type 1*

## Results

We found the elevation of advanced glycation end- products in the patients with diabetes mellitus in comparison with healthy subjects, which is significant only in the patients with diabetes mellitus type 2 (DM2 vs healthy subjects $p<0.001$, DM2 vs DM1 $p<0.005$, DM1 vs healthy subjects not significant) (Table 1).

AOPP were elevated significantly in the patients with both types of diabetes mellitus and had higher levels in the patients with diabetes mellitus type 2 in comparison with healthy subjects (DM2 vs healthy subjects $p<0.001$, DM2 vs DM1 $p<0.005$, DM1 vs healthy subjects $p<0.05$) (Table 1).

We did not find any significant correlation of AGEs (or AOPP) with age or duration of treatment of diabetes mellitus (neither in the patients with DM type 1, nor in those with DM type 2). On the other hand, there is a significant correlation between AGEs and AOPP ($r = 0.75$ ($p<0.05$) in DM type 1 (Fig. 1) and $r = 0.47$ ($p<0.05$) in DM type 2 (Fig. 2)). Additionally, the serum levels of advanced glycation end-products in patients with diabetes mellitus correlated with parameters of compensation of the disease only in diabetics type 1 (AGEs/g prot. vs HbA1c $r=0.47$, $p<0.05$) (Fig. 3), whereas the relationship of AGEs with blood glucose was not significant ($r = 0.45$). Such correlation was not observed in patients with diabetes mellitus type 2. Correlation of AOPP with glucose or HbA1c was not found in any group of patients. A significant correlation of both AGEs and AOPP with triglycerides (TG) but no correlation with cholesterol, including HDL and LDL cholesterol, was observed in both types of diabetes mellitus (more pronounced in DM1). (DM1 AGEs vs TG

r = 0.63 (p<0.05), AGE/g prot. r = 0.68 (p<0.05) (Fig. 4), AOPP vs TG r = 0.57 (p<0.05), DM2 AGEs vs TG r = 0.40 (p<0.05), AGE/g prot. r = 0.33 (not significant), AOPP vs TG r = 0.65 (p<0.05)). No correlation with either systolic or diastolic pressure was found in either of the studied groups of diabetic patients.

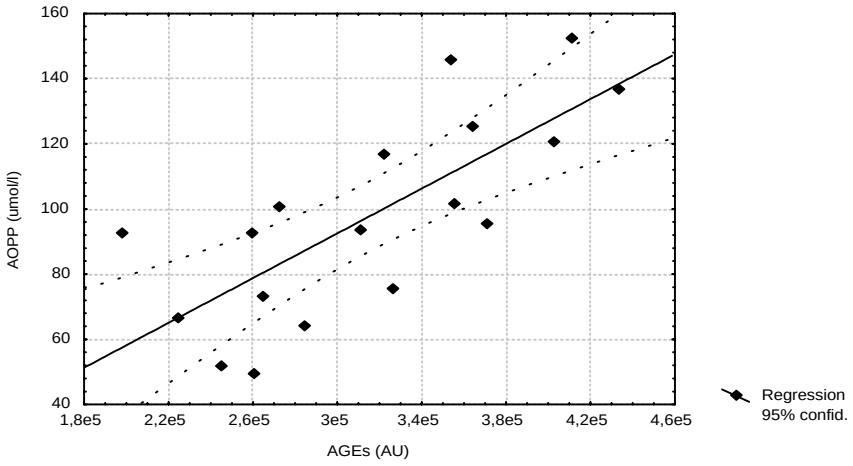


**Fig. 1.** *Correlation between AGEs and AOPP in diabetes mellitus type 1 (r=0.75, p<0.05, y = –10.50 + 0.00034 * x)*

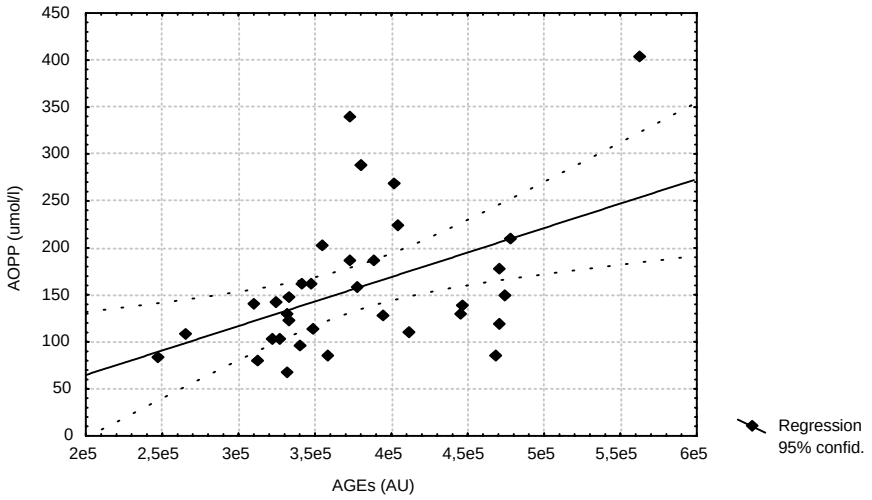


**Fig. 2.** *Correlation between AGEs and AOPP in diabetes mellitus type 2 (r=0.47, p<0.05 y = –39.52 + 0.00052 * x)*

Slight but not statistically significant differences in AGEs and AOPP levels were observed in diabetic patients with and without microvascular or macrovascular complications. (Tables 2 and 3). Unfortunately, only one patient with diabetes mellitus type 1 and macrovascular complications (both micro and macrovascular ones) was included in our study.

This did not enable the statistical evaluation of this group. Nevertheless, both AGEs and AOPP were higher in this patient in comparison with patients without macrovascular complications (AGEs $4.33x10^5$ AU, AGEs/g prot. $5.29x10^3$ AU/g, AOPP 136.7 μmol/l, vs AGEs $3.08±0.63x10^5$ AU, AGEs/g prot. $4.07±0.84x10^3$ AU/g, AOPP 95.2±30.23 μmol).

**Table 2.** Relationship of AGEs and AOPP levels to diabetic complications in diabetes mellitus type 1 – microvascular complications.

| **Microvascular complications** | **Present (n=13)** | **Absent (n=5)** |
|---|---|---|
| AGEs (AU) | $3.26±0.69x10^5$ | $2.85±0.60x10^5$ |
| AGEs (AU/g) | $4.33±0.87x10^3$ | $3.65±0.70x10^3$ |
| AOPP (μmol/l) | 100.50±34.15 | 89.70±21.38 |

*Data are expressed as means ± SD. The differences between groups are not statistically significant.*

**Table 3.** Relationship of AGEs and AOPP levels to diabetic complications in diabetes mellitus type 2.

| | **Microvascular complications** | | **Macrovascular complications** | |
|---|---|---|---|---|
| | **present n=20** | **absent n=14** | **absent n=11** | **absent n=23** |
| AGEs (AU) | 3.87±05.58x10$^5$ | 3.65±0.80x10$^5$ | 3.97±0.59x10$^5$ | 3.69±0.71x10$^5$ |
| AGEs (AU/g) | 5.35±1.13x10$^3$ | 4.78±0.80x10$^3$ | 5.23±0.85x10$^3$ | 5.05±1.42x10$^3$ |
| AOPP (μmol/l) | 157.6±68.42 | 157.4±86.56 | 172.5±81.86 | 150.3±72.51 |

*Data are expressed as mean ± SD. The differences between corresponding groups are not statistically significant.*

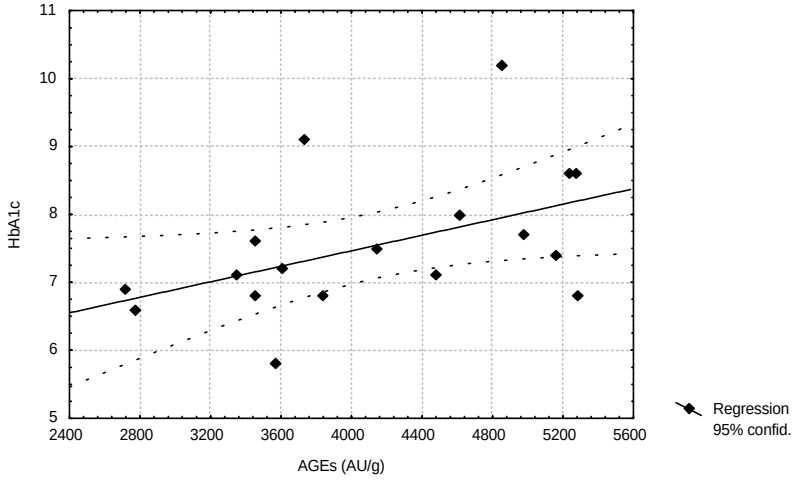


**Fig. 3.** *Correlation between advanced glycation end-products and HbA1c in diabetes mellitus type 1 (r=0.47, p<0.05. y = 5.1816 + 0.00057 * x)*

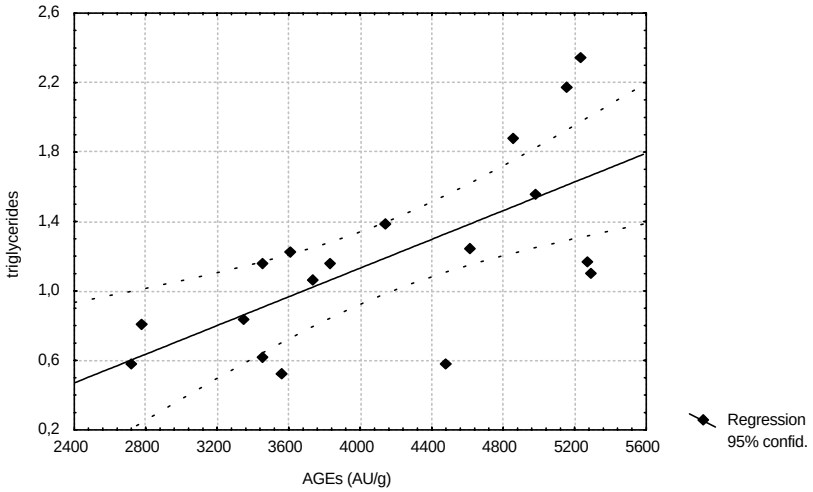


**Fig. 4.** *Correlation between advanced glycation end-products and triglycerides in diabetes mellitus type 1 (r=0.68, p<0.05, y = –0.5224 + 0.00041 * x)*

## Discussion

Our results show a slight elevation of advanced glycation end-products in patients with diabetes mellitus and significant differences between patients with diabetes mellitus type 1 and 2. The elevation of AGEs was found only in DM2 but not in DM1 when compared with healthy subjects, although there was no significant difference in blood glucose or HbA1c between these groups. Our observations are in accordance with Dolhofer-Bliesener *et al.* (1995), who described a small but significant increase of AGEs in diabetic patients, with Schleicher *et al.* (1997), who found elevation of $N^{\varepsilon}$-carboxymethyllysine (CML – one of described AGEs) and with Odani *et al.* (2001), who refers higher levels of $N^{\omega}$-carboxymethyl-arginine (CMA – a new AGE-

product) in diabetics. On the other hand, Daimon *et al.* (1999) found elevated pentosidine but not carboxymethyllysine in type 2 diabetics, whereas Wagner *et al.* (2001) demonstrated normal levels of carboxymethyllysine and AGEs (fluorescence) also in patients with type 2 diabetes mellitus who had normal renal function. Buongiorno *et al.* (1997) presented normal AGEs levels in diabetic patients type 1 and type 2 in good metabolic control during pregnancy.

AGE levels correlate with renal function – Wagner *et al.* (2001) described a significant negative correlation of serum CML and AGE-fluorescence levels with creatinine clearance and significant positive correlation with serum creatinine in diabetic patients with impaired renal function. However, these authors did not find any correlation of AGEs with plasma glucose levels, fructosamine and hemoglobin A1c. Nevertheless, a significant correlation of advanced glycation hemoglobin (AGE-hemoglobin) with hemoglobin A1c as an early glycation product was demonstrated by Turk *et al.* (1998) who determined these parameters both in patients with diabetes mellitus type 1 and 2 (without comparison of these groups). However, we found a correlation of AGEs with a parameter of glycemic control (HbA1c) only in the patients with diabetes mellitus type 1. Additionally, unlike Wagner *et al.* (2001), we describe a significant correlation with serum triglycerides which supports the idea that diabetes mellitus is a complex metabolic disorder. Moreover, reactive carbonyl compounds, which are known as precursors of carbonyl stress end-products (both advanced glycoxidation end-products and advanced lipoperoxidation end-products), can be generated from carbohydrates as well as from lipids (Miyata *et al.* 1999).

Reviewing Medline since 1996, when the first description of AOPP was made by Witko-Sarsat, there are only restricted data about AOPP in diabetic patients. Witko-Sarsat *et al.* (1996) reported increased levels of AOPP in hemodialyzed patients without differences between diabetics and non-diabetics, but we did not find anything about AOPP in diabetic patients with normal renal function. In our study, advanced oxidation protein products were increased in the patients with both types of diabetes mellitus with higher serum levels in patients with diabetes mellitus type 2. It was interesting that AOPP values are elevated in comparison with healthy subjects (1.7-2 fold in DM type 2 and 1.2 fold in DM type 1) more than AGEs (1.2 fold increase only - due to the elevation in patients with diabetes mellitus type 2). In hemodialyzed patients, the increase of serum AOPP and AGEs is similar in comparison with blood donors (3.65 fold and 3.8 fold, respectively) (Kalousová *et al.* 2001). Furthermore, a correlation of AOPP with triglycerides was found. Witko-Sarsat *et al.* (1996) considered the relationship of lipids and AOPP as still unclear but admitted a connection of AOPP to oxidative modification of LDL thus leading to atherosclerosis. Our finding is in line with clinical and experimental evidence that the generation of reactive oxygen species is increased in both types of diabetes mellitus (Rosen *et al.* 2001). Wagner *et al.* (2001) did not find a relationship between serum AGEs level (AGE-fluorescence and CML) and diabetic complications, although the toxic effect of AGEs has been known and accumulation of AGEs has been found in damaged tissues (Stitt *et al.* 1997, Bierhaus *et al.* 1998, Baynes and Thorpe 1999). We observed only slightly higher levels of both AGEs and AOPP in the patients with micro- or macrovascular complications, but this difference was not statistically significant. However, other reports have described a correlation of both CML and pentosidine (HPLC) with the severity of diabetic complications (Furth 1997).

We can conclude that both AGEs and AOPP are elevated in patients with diabetes mellitus with marked differences between patients with diabetes mellitus type 1 and type 2. The increase of both parameters is more pronounced in patients with diabetes mellitus type 2. In diabetics of type 1, there is no significant elevation of AGEs (which correlate significantly with HbA1c but not significantly with blood glucose concentration) but a statistically significant increase of AOPP. This might indicate the presence of oxidative stress in both types of diabetes mellitus with its increase in type 2. Oxidative stress probably also plays a more important role in AGEs formation in diabetes mellitus type 2 than in type 1, where AGEs formation depends more on the compensation of the disease (blood glucose and HbA1c).

## Acknowledgements

The study was supported by grant IGA MH CZ NB 7035-3 and by research project MSM 1111002 (206017-01).

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