Contents available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation

Oxidative stress markers in saliva and plasma differ between diet-controlled and insulin-controlled gestational diabetes mellitus

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ARTICLE INFO

Article history:
Received 28 August 2018
Received in revised form
8 November 2018
Accepted 29 November 2018
Available online 6 December 2018

ABSTRACT

Objectives: The aims of the study were as follows: to investigate possible differences between plasma oxidative status (OS) in late-onset GDM and well-characterized healthy pregnant women (oral health, diet); to verify the existence of possible differences between GDMG1 (diet-treated) and GDMG2 (insulin-treated GDM); to determine whether oxidative stress markers could be detected in saliva.

Material and methods: A total of 89 pregnant women ($n = 89$; 59 with GDM and 30 controls) were evaluated. Malondialdehyde (MDA), total antioxidant capacity (ORAC), inactivation of aldehyde dehydrogenase (I_{ALDH}), activity of glutathione peroxidase (GPx) and glutathione transferase (GST)) in plasma and/or saliva were analyzed.

Results: The activity of GPx and GST in plasma was higher in GDMG2 as compared to GDMG1 and controls. Also, in GDMG2, elevated concentrations of salivary MDA and higher I_{ALDH} were observed. In contrast, GDMG1 had higher plasma ORAC and lower GPx activity as compared to controls, probably due to low-energy diet, high in antioxidants and fibers. Salivary and plasma OS were correlated and most significant for ORAC.

Conclusion: Oxidative stress were not observed in GDMG1 but were confirmed to be moderate in GDMG2. However, large variability of the analyzed markers in GDM groups encourages screening of all patients, regardless of the treatment option. Saliva may be considered useful for the estimation of oxidative stress levels in GDM populations

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https://doi.org/10.1016/j.diabres.2018.11.021
0168-8227/

1. Introduction

Gestational diabetes mellitus (GDM) is a well-recognized, common metabolic complication of pregnancy. In Europe, GDM occurs in 3.8–7.8% of all pregnancies [1]. Traditionally, GDM is defined as glucose intolerance with onset or first recognition during pregnancy. Glucose levels can be controlled with appropriate diet (GDMG1) in most cases of GDM [2]. If glycemic goals are not achieved, insulin therapy is initiated (GDMG2) [3]. In GDMG1 patients, glycemia is usually better controlled and the risk of negative outcomes is lower [4].

Pregnancy is associated with increased susceptibility to oxidative stress (OS) due to high energy demand, elevated oxygen utilization, and excessive production of reactive oxygen species (ROS) [5]. OS is also believed to play an important role in the development and progression of GDM, and the related complications [6]. Different sources of ROS in diabetes have been proposed, including the sorbitol pathway, the induction of NAD(P)H oxidases, and some biochemical pathways strictly associated with hyperglycemia (glucose autooxidation, protein glycation) [7]. The literature offers numerous reports on oxidative stress and diabetes [8–10], but only some concerning GDM [11–13], and almost all of them combine pre-existing DM detected in pregnancy and late-onset GDM.

Malondialdehyde (MDA), a lipid peroxidation marker whose concentration in plasma is higher in GDM than in the control group, is one of the most frequently examined OS markers [11,14]. Total antioxidant capacity in GDM was shown to be lower [11,14]. Other frequently analyzed OS parameters include glutathione peroxidase and catalase activities [15,16]. Oxidative stress was observed in GDM not only in blood, but also in the amniotic fluid, cord plasma, and the placenta [11]. Nowadays, medicine aims to offer non-invasive tests for the diagnosis and the prognosis. Saliva seems to be an appropriate material, with lots of advantages over blood, providing a cost-effective approach for the screening of large populations. Saliva does not clot and can be more easily collected, transported and stored than blood. It is also safer in terms of potential infectious hazard [17]. Whole saliva provides information not only about the oral but also systemic health [18], e.g. salivary estriol test had been successfully used in pregnant women to assess the risk for preterm labor before other, cheaper methods were developed. Despite some reports on the changes in the antioxidant profile, i.e. concentration of the uric acid, MDA, vitamin C and E, activity of salivary peroxidase, salivary aldehyde dehydrogenase, superoxide dismutase and total antioxidant capacity in saliva of pregnant women, we were not able to find any studies on saliva of GDM patients [19–21]. One of the reasons might be the need for a careful examination of the oral cavity, performed by a dentist, before saliva collection due to the possible influence of blood on the results [22].

This study describes the antioxidant defense system and OS markers, but also dietary data was used for result interpretation. We aimed to determine whether oxidative status of GDM subjects and controls differs noticeably, and whether any differences were visible between GDMG1 and GDMG2. Additionally, in this study, we investigated whether OS markers were visible only in blood or also in saliva.

2. Patients and methods

In this paper, OS parameters and antioxidant defense system in GDM and healthy pregnant women were analyzed. Subjects with pre-gestational DM, even if diagnosed in pregnancy, were excluded. The GDM group was divided into two subgroups: GDMG1 – nutritional therapy only, and GDMG2 – patients who failed to maintain glycemic targets with nutritional therapy and received insulin analogues. Two diagnostic materials were used: standard (plasma) and alternative (saliva). The following were determined in plasma: (1) antioxidant enzyme activity (glutathione peroxidase (GPx), glutathione S-transferase (GST)), (2) total antioxidant capacity (ORAC), and (3) malondialdehyde (MDA) levels, and in saliva: (1) inactivation degree of aldehyde dehydrogenase (ALDH), which protects the oral cavity from aldehydes (from food, generated in oxidative stress), (2) ORAC, and (3) MDA concentrations. Since diet influences OS, dietary nutrient intake was calculated using a Semi-Quantitative Food Frequency Questionnaire (SFFQ). Oral health was assessed to eliminate patients with bleeding, severe periodontitis, and other pathologies which might affect salivary OS.

2.1. Study group

Fifty-nine pregnant women with GDM (GDMG1 = 44, GDMG2 = 15) and 30 women with uncomplicated pregnancy (between 26 and 41 weeks of gestation) were enrolled. The study was conducted between January 2011 and January 2013. Gestational age was established based on the date of the last menstrual period and confirmed by first-trimester ultrasound scan. The study group was selected from pregnant women routinely screened for GDM between 24 and 28 weeks of gestation based on the oral glucose tolerance test (OGTT) results. Inclusion criteria for the study group (GDM) were based on the International Association of Diabetes and Pregnancy Study Groups criteria, i.e. at least one value of plasma glucose concentration equal to or exceeding 5.1, 10.0, 8.5 mM (92, 180, 153 mg/dL) for fasting, 1 h and 2 h post glucose load, respectively. Controls were selected randomly and matched with GDM patients for maternal and gestational age, gravidity, parity, and BMI. The inclusion criteria for the control group were as follows: normal glucose tolerance test, age: 19–40 years, no history of pre-pregnancy diabetes and concomitant systematic diseases. Women with a history of pre-pregnancy diabetes or with pre-existing hypertension, renal disease, autoimmune disorders and smokers were excluded both, from controls and the study group. Only singleton pregnancies and live births were included. All patients included had regular medical check-ups at the First Department of Obstetrics and Gynecology, Medical University of Warsaw. GDM patients were under strict control in a diabetic outpatient clinic and, as a result of the daily diaries, strictly followed the diabetic diet. No other dietary plans and no other diets were used.

Local Ethics Committee approved of the study. All patients gave their written informed consent.

2.2. Sample collection

Blood and saliva samples were collected in the morning after an overnight fast (at least 6 h). All blood samples were collected into test tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Whole saliva samples were obtained simultaneously to blood samples between 8 and 9 am. Patients were asked to rinse mouth with warm water, sit, relax and rest for 5 min before sample collection. Saliva was allowed to accumulate in the floor of the mouth and the subject spited it out into test tubes. The samples were immediately centrifuged (saliva: 10,000 g, 10 min; blood: 3,000 g, 10 min). Plasma was separated, aliquoted and stored at −80 °C till analyzed. Salivary supernatant was analyzed up to 2 h after collection for inactivation of ALDH. Other saliva aliquots were stored at −80 °C till further analysis. The storage of materials did not exceed 30 days.

2.3. Biochemical analysis

Results are the average of three independent measurements. Detailed description of the methodology is presented in Supplementary Materials.

2.3.1. Enzyme activity

Total glutathione peroxidase activity (GPx) was measured spectrophotometrically, using the method developed by Paglia and Valentine, modified by Wendel, at the wavelength of 340 nm [23]. Glutathione S-transferase activity (GST) was measured spectrophotometrically at the wavelength of 340 nm, using the method described by Habig [24].

Inactivation degree of salivary aldehyde dehydrogenase (I_{ALDH}) was calculated using the following formula:

$$I_{ALDH}[\%] = \left(1 - \frac{V_{GSH}}{V_{DTT}}\right) \cdot 100\%$$

where V_{GSH} and V_{DTT} are reaction rates determined in the presence of 1 mM GSH and 0.5 mM DTT (Fluka), respectively. The reaction rates were measured using the fluorometric method [25].

2.3.2. ORAC assay

The ORAC-fluorescein (ORAC) fluorometric assay was performed based on the procedure of Ou et al. [26]. The ORAC values were expressed in Trolox equivalents.

2.3.3. MDA concentration

The assay was based on the reaction of MDA with thiobarbituric acid (23 mM, (Sigma-Aldrich)) in the presence of 13 mM sodium dodecyl sulfate (SDS, Sigma-Aldrich), 3 mM EDTA (Sigma-Aldrich) and acetate buffer (pH = 3.5, (Sigma-Aldrich)) [27]. The reaction product was extracted with butanol and measured using high-performance liquid chromatography with fluorometric detection.

2.4. Diet

Dietary nutrient intake was calculated using SFFQ, consisting of a list of foods with standard serve sizes commonly consumed by Polish adult population. The participants were asked to report how often they consumed each of the food items listed (number of times per day, per week, per month) during the previous month. The reported frequency for each item was then converted to a daily nutrient intake. Portion sizes of the consumed foods were converted to grams using household measures. The nutritional value of the diet was calculated using “Dieta 5” software developed by the Food and Nutrition Institute.

As far as diet in GDM is concerned, we followed the PTGP (Polish Society of Gynecologists and Obstetricians) guidelines on low-glycemic diet without caloric restriction [28], since it is a healthy alternative and improves pregnancy outcomes [29]. In Poland, a low-glycemic diet is most frequently recommended [28]. The daily meal plan should consist of 40–45% of carbohydrates (predominantly from bread, cereal, fruit, non-starchy vegetables, whole wheat bread, grains), 30% of protein (1.3 g/kg body weight), 20–30% of fat (predominantly polyunsaturated fat). Daily caloric requirement in normal weight diabetic pregnant women should be similar to healthy pregnant women - 1500–2400 kcal, or 35 kcal per kg of body weight.

2.5. Oral health

Each patient received a complete oral and periodontal examination. The controlled oral health indicators are shown in Table 1. In this study, the regular plaque index determination was modified. The teeth were examined for the presence of any plaque or calculus, clean teeth were marked as 0, teeth with any plaque as 1. The final value was the sum of all teeth with plaque or calculus. The patients were also assessed for periodontal lesions or prosthetic restorations. All patients had healthy and lesion-free oral mucosa. Four patients used non-removable restorations (prosthetic bridge or crown), one patient used removable prosthesis, and one had an orthodontic retainer.

2.6. Statistical analysis

Statistical evaluation of the results was performed with STATISTICA version 13.1 for Windows. Normal distribution of the results was evaluated by the Shapiro-Wilk test. In case of normal distribution the t-Student test and ANOVA were used, otherwise the Mann-Whitney U and Kruskal-Wallis one-way tests were applied. Differences between nominal variables were tested using the chi-square test. Correlations were presented as Spearman correlation coefficients. The p-value below 0.05 was considered as significant. The principal component analysis (PCA) was used to visualize and explore the obtained data.

3. Results

3.1. Characteristics of the study population

Clinical characteristics of the study group are presented in Table 2. Mean maternal age in the study group was 31 years (min. 19, max 40 years). No statistically significant differences between the study groups were found regarding age, parity,

Table 1 – Oral health indicators.

P	Teeth with caries
U	Missing teeth
W	Teeth with fillings
BI	Bleeding index: 0 - healthy gums; 1 - visually healthy, bleeding after probing; 2 - change of color, bleeding after probing; 3 - change of color, shape, bleeding after probing; 4 - change of color, shape, edema, bleeding after probing; 5 - change of color, shape, spontaneous bleeding, edema/ulceration
GI	Gingival index: 0 - healthy gums, 1 - inflammation, no bleeding, 2 - mild inflammation, bleeding on probing, edema 3 - severe inflammation, spontaneous bleeding, ulcerations
PD	Pocket depth
PI	Plaque index 0 - clean teeth, 1 - teeth with any plaque

gestational age, and body mass index. White blood cell count and the number of infants with macrosomia were the only observed differences. No statistically significant differences in oral health parameters were observed.

3.2. Diet

Basic dietary parameters for the entire study population are presented in Table 3. In the GDM group, the percentage of energy from saturated fats ($p = 0.0226$) and the amount of fiber ($p = 0.0307$) (both adjusted to energy intake) were significantly higher than in non-diabetic women. Moreover, controls consumed a higher number of calories than GDM patients ($p < 0.0001$), and more vitamin C (adjusted to energy intake). Higher vitamin E and folate consumption (not adjusted to energy intake) in that group was due to higher energy intake by healthy pregnant women. Comparing GDMG1 and GDMG2 flavonoids (adjusted to energy intake), the consumption was higher ($p = 0.0444$) but total energy input lower in GDMG2 ($p = 0.0446$). No other significant differences in the diet between both GDM groups were observed.

3.3. Biochemical analysis

Median activity of GPx, GST, ALDH inactivation, value of ORAC and concentration of MDA in plasma and/or saliva are presented in Table 4.

In GDMG2, plasma levels of GPx ($p < 0.0001$) and GST ($p = 0.0236$) were higher than in GDMG1. Also, elevated concentration of salivary MDA ($p = 0.04989$ *vs.* non-diabetic women, *vs.* GDMG1 $p = 0.04991$), higher inactivation of ALDH ($p = 0.0032$ *vs.* non-diabetic women, $p = 0.0002$ *vs.* GDMG1) and tendency for lower ORAC ($p = 0.0665$ *vs.* non-diabetic women $p = 0.0673$, *vs.* GDMG1) in saliva were observed. In con-

Table 2 – Selected clinical characteristics of the study population.

	GDMG1	GDMG2	Controls
Number	44	15	30
Gestational age[±†] [week]	36 ± 5[a]	38 ± 5[a]	34 ± 5[a]
Gestational age range[±] [week]	26–39	32–39	26–41
Estimated fetal weight[±†] [g]	1500 ± 2600[a]	2500 ± 1500[a]	1700 ± 2700[a]
C-reactive protein[†] [mg/L]	3.7 ± 1.9[a]	8.3 ± 3.8[a]	4.7 ± 3.3[a]
White blood cells x10^3/μL	8.8 ± 4.4[a]	9.6 ± 6.5[ab]	11.2 ± 6.3[b]
Body mass index[†] [kg/m^2]	28.3 ± 5.4[a]	28.4 ± 7.9[a]	27.1 ± 5.0[a]
Teeth with caries[†]	2.6 ± 1.9[a]	2.2 ± 2.2[a]	2.5 ± 1.8[a]
Missing teeth[†]	1.7 ± 1.5[a]	1.3 ± 1.2[a]	1.2 ± 1.0[a]
Teeth with fillings[†]	6.8 ± 3.7[a]	5.6 ± 3.7[a]	5.3 ± 3.7[a]
Bleeding index[†]	1.0 ± 0.8[a]	1.0 ± 1.0[a]	1.1 ± 1.0[a]
Gingival index[†]	0.9 ± 0.7[a]	0.9 ± 1.0[a]	1.2 ± 0.9[a]
Pocket depth[†] [mm]	2.9 ± 1.0[a]	2.8 ± 1.4[a]	2.7 ± 1.3[a]
Fetal weight[†] [g]	3230 ± 420[a]	3320 ± 880[a]	3330 ± 440[a]
Macrosomia [%][#]	0[a]	13[b]	10[b]
Low birth weight [%]	4.5[a]	13[a]	10[a]
Apgar score[†] [points]	9.8 ± 0.6[a]	9.7 ± 0.5[a]	9.3 ± 1.5[a]

[abc] Represent homogenous group according to Kruskal Wallis, ANOVA or Chi2 tests.
[#] Newborns weighing more than 4000 g.
[±] At the time of sample collection.
[†] Results expressed as mean ± standard deviation or median ± interquartile range depending on data distribution.

Table 3 – Energy and selected nutrient content (median ± interquartile range) in the entire study population.

	GDMG1	GDMG2	Controls
Energy [kcal]	1050 ± 550[a]	810 ± 470[c]	1580 ± 610[b]
- from carbohydrates [%]	60 ± 16[a]	60 ± 22[a]	63 ± 14[a]
- from fats [%]	25 ± 13[a]	28 ± 15[a]	29 ± 15[a]
- from saturated fats [%]	10.6 ± 6.3[ac]	10.0 ± 9.7[bc]	11.8 ± 8.9[b]
Flavonoids [mg]	608 ± 802[b]	630 ± 690[ab]	670 ± 480[a]
Flavonoids/kcal [μg/kcal]	0.50 ± 0.75[a]	0.62 ± 0.87[c]	0.34 ± 0.54[a]
Fiber/kcal [mg/kcal]	18 ± 10[a]	22 ± 12[a]	15 ± 10[b]
Fiber [g]	21 ± 1[a]	20 ± 21[a]	27 ± 15[a]
Vitamin E [mg]	3.9 ± 2.1[a]	3.2 ± 1.3[a]	5.2 ± 5.8[b]
Vitamin E/kcal [mg/kcal]	0.0034 ± 0.0010[a]	0.0037 ± 0.0022[a]	0.0034 ± 0.018[a]
Vitamin C [mg]	45 ± 52[a]	37 ± 35[a]	100 ± 110[b]
Vitamin C/kcal [mg/kcal]	0.040 ± 0.031[a]	0.032 ± 0.036[a]	0.053 ± 0.061[b]
Folate [μg]	170 ± 78[a]	150 ± 120[a]	250 ± 140[b]
Folate/kcal [μg/kcal]	0.162 ± 0.049[a]	0.191 ± 0.061[a]	0.162 ± 0.052[a]

[abc] Represent homogenous group according to the Kruskal Wallis test.

Table 4 – Median and interquartile range of the entire study population.

	GDMG1	GDMG2	Controls
Plasma GPx [U/mL]	25.3 ± 7.9[a]	31 ± 20[b]	28.3 ± 9.2[c]
Plasma GST	26.2 ± 8.4[a]	32 ± 19[b]	26.6 ± 8.5[a]
Plasma ORAC [mM]	15 ± 31[a]	9 ± 16[b]	8 ± 25[b]
Plasma MDA [μM]	0.95 ± 0.74[a]	1.4 ± 1.2[b]	1.3 ± 1.2[b]
ALDH inactivation [%]	54 ± 27[a]	73 ± 27[b]	56 ± 30[a]
Saliva ORAC [mM]	2.9 ± 4.0[a]	1.0 ± 2.5[a]	2.5 ± 5.1[a]
Saliva MDA [μM]	0.38 ± 0.37[a]	0.43 ± 0.52[b]	0.34 ± 0.46[a]

[abc] Represent homogenous group according to the Kruskal Wallis test.

trast, GDMG1 had higher plasma ORAC ($p = 0.0001$) and lower GPx activity ($p = 0.0002$) as compared to non-diabetic women.

As far as the correlations of biochemical parameters in plasma and saliva are concerned, the highest correlation was observed for ORAC ($r = 0.42$, $p < 0.0001$). The correlation was even stronger in GDM ($r = 0.74$, $p < 0.0001$). Salivary ORAC was also correlated with MDA level in plasma ($r = -0.36$, $p = 0.0006$). In non-diabetic women, the strength of the relation was higher ($r = -0.57$, $p = 0.0014$) than in GDM (−0.28, $p = 0.0334$). No correlations between MDA and I_{ALDH} in saliva and MDA or enzymes activities in plasma were found. Salivary MDA correlated only with ORAC in plasma.

3.4. *PCA analysis*

First and second principal components (PC1 and PC2) accounted for 29% and 20% of the total variation, respectively. PC1 was correlated mainly with salivary ORAC ($r = -0.71$), plasma ORAC($r = -0.66$), plasma MDA ($r = 0.59$) and activity of GPx ($r = 0.57$) and GST ($r = 0.61$). PC2 was correlated with the activity of GPx ($r = 0.70$) and GST ($r = 0.63$). The distribution of the subjects on a score plot (PC1 *vs.* PC2) is shown in Fig. 1. As far as the GDMG2 group is concerned, two separate equinumerous subgroups may be distinguished. One is located near the non-diabetic women and GDMG1 patients. Others are clearly separated (G2_H_PC1), and were marked with the dashed green line. They differ in the antioxidant enzyme activity in blood, i.e. GPx (45 ± 14 (G2_H_PC1) *vs.* 26.9 ± 3.8, $p = 0.0022$) and GST (42 ± 13 (G2_H_PC1) *vs.* 24.5 ± 3.5, $p = 0.0011$). The first subgroup with high PC1values has high enzyme activity as well as and low CRP (1.8 ± 2.6 *vs.* 4.8 ± 2.7, $p = 0.0259$). In GDMG1, also one subgroup differed (solid blue line, G1_L_PC1) with high ORAC values both, of saliva (3.1 ± 3.0 *vs.* 2.8 ± 3.3, $p = 0.0050$) and plasma (20 ± 67 *vs.* 28 ± 3, $p = 0.00501$), and low GPx (21.1 ± 6.4 *vs.* 27.4 ± 8.5, $p = 0.0007$) and GST (21.1 ± 6.6 *vs.* 28.6 ± 8.1, $p < 0.0001$) activities. No differences in other clinical parameters or diet within the group were observed. Moreover, seven of the G1 patients (G1_H_PC1, yellow line) had moderate OS: higher GPx (34.9 ± 2.4 *vs.* 27.4 ± 6.1, $p < 0.00001$) and GST activities (35.1 ± 1.9 *vs.* 27.8 ± 4.2, $p < 0.00001$), MDA concentration (1.8 ± 1.0 *vs.* 1.3 ± 0.7, $p = 0.04997$), and lower ORAC (1.4 ± 1.5 *vs.* 5.4 ± 5.6, $p = 0.0035$). In controls, PC1 was not correlated with any parameters, whereas PC2 was associated with EFW (−0.73) and WBC (0.50).

4. Discussion

We hypothesized oxidative stress levels were different in GDMG1 and GDMG2 as these medical conditions are different in terms of metabolic status. Compared to diet-treated women, women treated with insulin have a higher metabolic

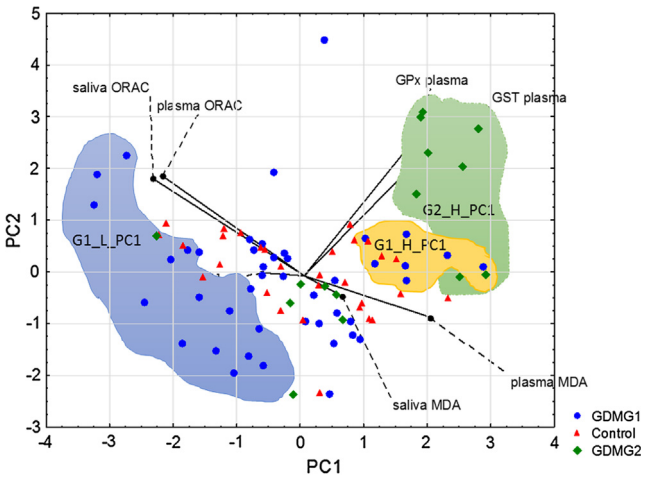

Fig. 1 – Distribution of the study group on the score plot (principal component 1 *vs.* principal component 2). G2_H_PC1 (green line) is a subgroup with higher enzyme level in plasma than other patients with GDMG2. G1_L_PC1 (blue line) is a subgroup with higher ORAC values, both in saliva and plasma, and lower enzyme level in plasma than other patients with GDMG1. G1_H_PC1 (yellow line) is a subgroup with higher enzyme activity in plasma, MDA concentration and lower ORAC than other patients with GDMG1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

risk profile and higher risk of negative outcomes [30]. GDMG1 and GDMG2 are also differed in both metabolome [31] and in transcriptome. Thus, in our study the groups were not merged, but analyzed separately. As expected in GDMG1 lower OS marker (MDA) in plasma was observed, not only comparing to GDMG2, but also to controls. Also, GDMG1 patients had higher levels of the mediator related to the presence of antioxidant activity, ORAC and lower plasma GPx. Low levels of enzyme activity, together with high ORAC and low MDA, indicate the absence of OS in GDMG1. One of the reason can be a diet, since it has great influence on OS markers and antioxidant enzyme activity [32]. High intakes of macronutrients can promote oxidative stress and subsequently contribute to inflammation *via* NF-κB. Dietary carbohydrates of high glycemic index may especially contribute to long-term consequences of nutritionally mediated inflammation. Also, long-chain saturated fatty acids were shown to promote proinflammatory endothelial cell phenotypes [33]. In contrast, fibers, some vitamins and flavonoids were shown to reduce ROS levels. Flavonoids and vitamins react with and inactivate ROS, whereas fibers influence gut microbiota, which affects immune function, e.g. through the production of short-chain fatty acids. The compounds inhibit oxidative stress and inflammation [34,35]. In our research we observed lower caloric intake in the GDM as compared to controls. It probably results from fear of hyperglycemia, strict calorie count, and balancing diet and activity. In our research we observed lower caloric intake in the GDM as compared to controls. It probably results from fear of hyperglycemia, strict calorie count, and balancing diet and activity. Dietary nutrient intake was calculated using SFFQ, which has some major limitations. Some patients find it difficult to report the actual and accurate number, type and size of meals from the previous days and the whole month. Thus, the energy intake in SFFQ is frequently underestimated [36]. Some patients would also knowingly or inadvertently avoid reporting the actual caloric intake not admitting diet mistakes, which can be other source of underestimation of calorie intake. In our Clinic patients are encouraged to maintain healthy diet without caloric limitation. We also follow up patients and fetal wellbeing by regular weight checks and fetal growth scans. In cases of major weight loss, abnormal fetal growth, maternal hyperglycaemia or hypoglycaemia we educate patients again and change insulin administration if needed. However, the results of such low calorie intake are alarming and should be addressed in future research concerning development of more accurate calorie intake calculation tool." In our study GDM patients more frequently consumed whole grain bread and legumes so fiber content in their meals was higher. Lower saturated fat content in the GDM group was also observed. The content of antioxidants such as vitamin C was lower, whereas vitamin E and folate content was comparable to controls (all adjusted to energy intake). To conclude, the diet of GDM patients in our study was more 'healthy' and had greater antioxidant and anti-inflammatory potential than the standard diet followed in Poland. So, it can be a reason of lack of OS in GDMG1. But, even the diet of the GDMG2 had even more factors reducing OS, i.e. lower energy input and higher flavonoid consumption, moderate oxidative stress was observed in GDMG2. When OS is classified as mild to moderate, Nrf2 enhances expression of antioxidant enzymes coded by ARE-responsive genes. Suppression the nuclear factor-kappa B- (NFκB) activation and the inflammatory response is observed as well. When OS is severe, Nrf2 can promote ROS generation. NFκB becomes active. NFκB has been known to inhibit Nrf2 as well, so in heavy OS the ARE genes are no longer upregulated. The proteins produced as a result of NF-κB induction include proinflammatory cytokines: tumor necrosis factor-α (TNF-α) and IL-6 (which induces CRP production) (38). However, the activation of NF-κB might also occur in another way.

Apart from diet with even higher antioxidant properties than in GDMG1, the main markers of OS in GDMG2, comparable to controls, were plasma ORAC and MDA levels. In that group, upregulation of antioxidant enzyme expression was observed. Higher activity of GPx and GST (enzymes upregulated by Nrf2/ARE) as compared to controls was also noted, although not in all women with GDMG2. We observed a subgroup with the level of enzymes comparable to controls (Fig. 1). That finding was probably associated with lower OS due to lack of differences in ARE-regulated enzyme activity as compared to non-diabetic women. Since in GDM oxidative stress is observed mainly due to unsatisfactory glycemic control, we assume that the subgroup included patients with better glycemic control. Similarly, to GDMG2, the GDMG1 group was not homogeneous, as it included patients with no signs of OS (G1_L_PC1) or with moderate OS (G1_H_PC1).

Other researchers also observed enhanced OS in GDM. Fadia et al., Wdowiak et al., and Li et al., reported moderate OS, which is similar to our findings [33,37,38]. However, high, and not moderate, OS was observed most frequently. So,

higher activity of enzymes upregulated by ARE activation was not observed in blood of the GDM patients. It might be caused by chronic inflammation, which results in enzyme inactivation (oxidation in active sites) by ROS. In these patients, high level of oxidative markers and low level of antioxidant enzymes upregulated by ARE activation were noted. In most papers, no separate evaluation of women with DM diagnosed in the first trimester and women with late-onset GDM, which is a mild form of gestational diabetes, was performed [11,39]. Moreover, we found no studies where similar oxidative parameters in diet-treated GDM and insulin-treated GDM groups were presented.

Apart from analyzing the conventional diagnostic material – blood, we decided to analyze OS markers in saliva, which is an attractive diagnostic tool for the diagnosis of systemic illnesses, superior to other body fluids [17,18]. However, traces of blood and pathologies in the oral cavity may influence or give erroneous results [17,40]. Therefore, before including the women in our study, a dentist carefully evaluated the state of their teeth and gums. Most patients, both in controls and the study group, presented BI and GI one or more, meaning their oral hygiene was not sufficient and several gum or periodontal problems were found. On the other hand, according to epidemiological data, the patients presented better results than their age-peers from the general population. Patients with gingival index (GI) > 2 and blood index (BI) > 3 were excluded from the study. We did not observe the influence of GI, BI and PI in our study population on ORAC, MDA and inactivation of ALDH in saliva, which might indicate that the undertaken exclusion criteria were sufficient.

We demonstrated saliva to be a good material in the evaluation of systemic OS in GDM. In GDMG2, MDA concentration was significantly higher than in GDMG1 and controls. Moreover, higher inactivation degree of salivary ALDH due to oxidation of sulfhydryl groups in the active site of the enzyme was also observed. The inactivation of the enzyme might result in lower protection of the oral cavity from aldehyde generated due to OS, air pollution and food. High oxidative stress (high MDA, inactivation of ALDH) in the oral cavity was a surprising finding. In plasma of these women, no elevation of MDA was observed and an elevated activity of the ARE related enzymes was the only marker of OS. There are three possible explanations of this phenomenon. First, plasma ORAC value is at least three times lower than salivary ORAC. It means plasma can neutralize three times more free radicals compared to the same volume of saliva. Thus, the antioxidant capacity of saliva in GDMG2 might be too low to neutralize ROS. Second, only free MDA passes through blood to saliva. Thus, the results can be associated with the difference between total and free MDA fraction in plasma. The third possible explanation is that in saliva we find average MDA levels, depending on OS in the system and the oral cavity. Diabetes, especially poorly controlled, is related to increased OS in the oral cavity (detected in non-pregnant women with DM) [41]. Several soft tissue abnormalities, e.g., periodontitis and gingivitis, have been observed in diabetic populations. Moreover, salivary dysfunction (leading to reduced salivary flow, changes in salivary composition and taste dysfunction) has been reported in non-pregnant patients with DM [42]. To the best of our knowledge, no data on OS in the oral cavity of GDM patients are available. We observed that, in contrast to GDMG2, OS in the oral cavity of GDMG1 subjects was similar to controls. The explanation of differences in MDA levels between two matrices might be that plasma (comparable to controls) and saliva (higher than in controls) can be similar as in GDMG1. Regardless of all differences, we can conclude that the analysis of the salivary samples provided us with information about OS prevalence in GDMG2 and lack of OS in GDMG1. Thus, this diagnostic material can be used for screening purposes due to the correlation between salivary ORAC and both, ORAC (strong) and MDA in plasma (low) levels. For example, in Fig. 1, it is possible to see a GDMG1 subgroup with high ORAC in saliva. No OS in that group was noted due to low antioxidant enzyme activity and high ORAC in plasma but a subgroup with low ORAC in saliva among GDMG1 can be observed. That subgroup suffered from moderate OS, having high activity of antioxidant enzymes, high MDA concentration (statistically higher than controls and GDMG2), and low ORAC in plasma.

Monitoring OS in pregnancy, especially complicated by GDM, might be beneficial for diagnostic and therapeutic purposes. OS is associated with intrauterine growth retardation and diabetic embryopathy. It influences the development and maturity of the placental villi and can cause fetal compromise (hypoxia) [43]. Some *in vivo* studies have shown that antioxidative treatment diminishes oxygen radical-related tissue damage [44]. Thus, diet rich in antioxidants and fiber in pregnancy, especially complicated by OS-related pathologies, seems justified.

5. Conclusions

To the best of our knowledge, this study has been the first to analyze OS in GDM using dietary data.

OS was rarely detected in late-onset gestational diabetes. OS was not observed in diet-treated GDM, and was moderate in insulin-treated GDM. However, large variability of the analyzed markers in GDM groups encourages screening of all patients, regardless of the treatment option. Moreover, the beneficial effect of diet on the oxidative parameters should be highlighted (higher plasma ORAC in GDM comparing to OGTT normal women). The oxidative status of plasma was correlated with those of saliva. Salivary ORAC may be considered as a systemic marker of oxidative stress. Salivary MDA and inactivation of ALDH is strongly influenced by local environment of the oral cavity and should be considered as a marker of OS in the oral cavity.

Acknowledgements

The authors wish to express their sincere gratitude to Agnieszka Zylka, M.D., Ph.D., from the Department of Oncological Endocrinology and Nuclear Medicine, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Warsaw, Poland for her contribution and invaluable comments about the manuscript.

Conflict of Interest

The authors declare no conflict of interest regarding the content of this article.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2018.11.021.

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