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Association between early oxidative DNA damage and iron status in women with gestational diabetes mellitus

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ABSTRACT

This study aims to assess the relationship between oxidative DNA damage and iron status in women with gestational diabetes mellitus (GDM) compared to those with normal glucose tolerance in the first and the second trimesters of pregnancy. Maternal serum and urine samples were collected in the 11th-14th weeks and the 24th-28th weeks of gestation. In addition to oral glucose tolerance test in the second trimester, fasting blood glucose, HbA1c, ferritin and hemoglobin levels were measured in blood samples. Urinary levels of oxidative DNA damage products 8-hydroxy-2′-deoxyguanosine (8−OH-dG) and 8,5′-cyclo-2′-deoxyadenosines (*S*-cdA, *R*-cdA) were determined using liquid chromatography-tandem mass spectrometry with isotope-dilution. In the first trimester, urinary 8−OH-dG levels were found higher in the GDM group (n = 33) than in the control group (n = 84) (p = 0.006). *R*-cdA and *S*-cdA levels were not significantly different between the two groups (p = 0.794 and p = 0.792 respectively). When the cases were stratified according to their first trimester ferritin levels, women with ≥50th centile (≥130 ng/mL) demonstrated higher levels of 8−OH-dG and *R*-cdA than those under <50th centile (p = 0.034, p = 0.009). In the GDM group, there was a positive correlation between the second trimester 8−OH-dG and ferritin and 1st-hour glucose levels (p = 0.014, p = 0.020). This is the first study where oxidative DNA damage is evaluated in both early and late periods of pregnancy. Our findings reveal an association between GDM and iron status and oxidative DNA damage.

1. Introduction

Gestational diabetes mellitus (GDM) refers to varying levels of glucose intolerance not becoming overt before gestation and diagnosed in the second or third trimester of pregnancy [1]. GDM affects about 7 % of all pregnancies and its prevalence varies between 1 % and 14 % depending on the population examined and the diagnostic test utilized [2]. It creates a high risk of developing type 2 diabetes, metabolic syndrome and cardiovascular disease [2–4]. Furthermore, GDM accounts for 35 % of complications in all labors [3]. Fetal macrosomia, neonatal hypoglycemia, hyperbilirubinemia, shoulder dystocia and operative delivery frequently appear in pregnant women with GDM [5]. At present, the diagnosis of GDM is made in the second and third trimesters mostly with oral glucose tolerance test (OGTT). Nevertheless, adverse metabolic dysfunctions might have already produced negative effects on the mother and the fetus [6]. In addition, there is not a complete consensus on whether a single or two-stage OGTT should be used to diagnose GDM, whether screening and diagnostic methods should be utilized in all pregnant women or only in pregnant women at risk and what diagnostic threshold values should be adopted [7]. According to data from Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study published in 2008, International Association of Diabetes in Pregnancy Study Group (IADPSG) recommended that two-stage screening and diagnostic methods should be abandoned and that two-hour OGTT

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requiring 75 g glucose should be used in the 24th-28th gestational weeks instead [8]. This has caused scrutinizing the criteria for the diagnosis of GDM again [9]. All these suggest that, early signs of GDM have extreme importance.

Several studies have shown that oxidative stress plays a role in the pathogenesis of gestational and non-gestational diabetes as well as the mechanism of complications [10,11]. Oxidative stress emerges from an impaired balance between the increased production of free radicals mainly reactive oxygen species and cellular antioxidant defense systems [12]. During a healthy pregnancy, the placenta rich in mitochondria and the abundance of transition metals like iron predispose to the environment of oxidative stress [13]. Compared to normal pregnancy, oxidative stress levels have been shown to be higher in GDM accompanied by hyperglycemia a leading to the production of reactive oxygen species [14–20]. During pregnancy, insulin-producing pancreatic beta cells have a weak antioxidant defense and are vulnerable to the harmful effects of oxidative stress. Therefore, it is thought that high oxidative stress affects the beta cells and facilitates GDM development [21,22].

Iron deficiency is prevalent in pregnant women. In the literature, it is stated that the worldwide prevalence of anemia in pregnant women is 41.8 % [23]. However, surplus amounts of free iron are toxic and administration of iron supplements to iron replete pregnant women can cause iatrogenic iron loading [24,25]. It has been recently shown that iron is likely to play a role in the pathophysiology of diabetes [26]. While its molecular mechanism is not exactly known, high levels of tissue iron were shown to have a link with oxidative stress and diabetes [27,28]. In several prospective studies, high iron intake during pregnancy has been associated with an increase in GDM risk especially in women who are not anemic in the first trimester and doubts about routine iron supplementation have been mentioned [27,29,30].

Oxidative DNA damage is considered to mediate the relationship between iron intake and emergence of GDM symptoms [31]. The oxidative DNA damage product 8-hydroxy-2-deoxyguanosine (8−OH-dG), a dependable indicator of oxidative stress most frequently examined in the literature, appears as a result of oxidation of 2′-deoxyguanosine [32,33]. Other DNA damage products, (5′*R*) and (5′*S*)-8, 5′-cyclo-2′-deoxyadenosines (*R*-cdA and *S*-cdA), produced by hydroxyl radical attack on 2′-deoxyribose moiety of 2′-deoxyadenosine in DNA followed by 8,5′-intramolecular cyclization and oxidation, indicate concomitant damage to both sugar and base moieties of the same nucleoside in DNA and may play an important part in the development of several diseases [34]. Oxidized nuclear DNA in general undergoes repair, DNA repair products of oxidative DNA lesions, i.e. oxidized nucleosides and bases, are water soluble and excreted into the urine. Several soluble oxidatively modified products have been found in urine, including 8−OH-dG, *R*-cdA and *S*-cdA [35,36].

There have been few studies about oxidative DNA damage in GDM, and this damage has been examined in blood, urine and placental tissue samples from pregnant women with GDM and/or mild gestational hyperglycemia by using ELISA or comet assay in these studies [32,33,37,38]. While oxidative DNA damage is significantly increased in blood and urine samples (8−OH-dG, purine and pyrimidines) in GDM patients, there is no significant difference in 8−OH-dG levels in placental tissue between patient and control groups. In addition, these studies have been performed by using samples obtained in the 16th gestational week the earliest. None of the studies have examined the relationship between GDM, iron status and the levels of oxidative DNA damage in both the first (11th-14th weeks) and the second trimesters (24th-28th weeks) of pregnancy. In addition, no data are available on the changes in the levels of *R*-cdA and *S*-cdA in GDM. Qui et al. indicated that a single measurement of urinary 8−OH-dG in patients with GDM may not be sufficient to reflect oxidative stress that may appear over time throughout pregnancy [32]. Therefore, it is important to measure 8−OH-dG and other products at certain intervals to reveal the maternal oxidative stress status in pregnancy thoroughly.

Various analytical methods are used to measure oxidative DNA damage [39]. However, most of them are able to measure only a single product without a dependable spectroscopic evidence for their identification [40]. Methods using liquid chromatography with tandem mass spectrometry (LC–MS/MS) using isotope-dilution accurately measure and identify many products at the same time. European Standards Committee on Oxidative DNA Damage (ESCODD) compared measurements of 8−OH-dG levels with different methods and reported that measurements with LC–MS/MS are the gold standard [41]. There have not been any studies in which 8−OH-dG and *R*-cdA and *S*-cdA in pregnant women with GDM or non-GDM were simultaneously measured using LC–MS/MS.

This observational study aims to assess the relationship between the urinary oxidative DNA damage products and iron status in women with GDM and normal glucose tolerance in two trimesters of the pregnancy. The longitudinal design of the study was assumed to reveal the potential role of both oxidative DNA damage products and iron status determined in the first trimester screening period in predicting the future GDM.

2. Material and methods

2.1. Subjects

The women who applied to the Pregnancy Outpatient Clinic of Dokuz Eylül University Faculty of Medicine, Department of Obstetrics and Gynecology between March 2016 and August 2018 and who read and signed the informed consent form were included in this study. According to the study design, morning urine, serum and EDTA anti-coagulated whole blood samples were obtained from the women participating in the study in their 11th-14th and 24th-28th gestational weeks. The first samples were collected during the first trimester with the combined test between 11 weeks 0 days and 13 weeks 6 days and the second samples were obtained during the second trimester, at the visit scheduled for the oral glucose tolerance test between 24 weeks 0 days and 28 weeks 0 days. The study samples were collected from 117 pregnant women, of whom 33 had the diagnosis of GDM and 84 had normal glucose tolerance.

In addition to OGTT in the second trimester, fasting glucose, ferritin was analyzed in serum samples and complete blood count (CBC), glycosylated hemoglobin A1c (HbA1c) tests were performed on EDTA anti-coagulated whole blood samples on the day of sampling in Dokuz Eylul University Central Laboratory. The blood samples were obtained after an 8 -h-fasting and stored +4 ℃ until the measurements. The urinary samples were collected from the morning first urine and urinary creatinine measurements were also accomplished. The remaining urinary samples were stored at -80 ℃ until the completion of the recruitment period. Upon enrollment in the study, each pregnant woman filled in a detailed questionnaire including questions about maternal age, gravidity and parity, use of folic acid, iron and multivitamin supplementation, systemic diseases, smoking habits and drugs. Arterial blood pressure, weight and height were measured and recorded. The inclusion criteria were as follows: age ≥18 years, singleton pregnancy, usual findings at the 11–14 t h weeks' sonographic screening examination (cases with sonographic findings suggesting a vanishing twin or any suspected or confirmed fetal anomaly were excluded), not being insulin dependent or pregestational diabetes mellitus at the first prenatal visit, not to have infectious or non-communicable diseases, not to have alcohol intake habit (no amount of alcohol) and continuation of the follow-ups. The exclusion criteria were being under 18 years, multifetal pregnancy, inflammatory diseases, alcohol intake, high-risk combined test results and corticosteroid therapy for any indication. Also, the cases with one missing sample or with lack of any results for the studied variables were not included in the final analyses. Since the Obstetrics and Gynecology Department adopted a universal screening policy, one step 75 g glucose tolerance test was offered to all women and the GDM diagnosis was made according to the IADPSG criteria [42]. When any one of the parameters was abnormal, including fasting blood glucose

(FBG) level ≥92 mg/dL, the 1st hour blood glucose level ≥180 mg/dL, or the 2nd hour blood glucose level ≥153 mg/dL, the women were diagnosed as GDM. All LC–MS/MS measurements of 8−OH-dG, *R*-cdA and *S*-cdA were performed in the Medical Biochemistry Department of Dokuz Eylul University.

2.2. Materials

Nylon syringe filters (0.22 μm) were purchased from Labsolute (Geyer GmbH & Co., Germany). Oasis HLB Extraction Cartridges from Waters Corp. (Milford, Massachusetts, USA) were used for solid phase extraction of the urine samples. Nanosep Omega tubes with a molecular mass cut-off of 3 kDa were purchased from Pall (Pall Corporation, NY, USA). Alkaline phosphatase was purchased from Roche Applied Science (Indianapolis, Indiana, USA). Acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). The stable isotope-labeled internal standards 8−OH-dG-$^{15}N_5$, *R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$ were received from National Institute of Standards and Technology (NIST-Gaithersburg, Maryland, USA).

2.3. Measurement of 8−OH-dG, R-cdA and S-cdA by LC–MS/MS

In order to evaluate oxidative DNA damage, LC–MS/MS with multiple reaction monitoring (MRM) with stable isotope dilution (SID) was performed according to the protocol described by Jaruga et al. [43,44]. Aliquots of 8−OH-dG-$^{15}N_5$, *R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$ as internal standards were added to an aliquot of 1 mL of urine samples, which were then centrifuged at 1000xg for 15 min. Subsequently, supernatants were filtered using nylon syringe filters (0.22 μm). Extraction cartridges were activated with 1 mL methanol, dried and then washed with 2 mL of water for solid phase extraction of the urine samples. Filtered supernatant fractions were loaded onto extraction cartridges and washed with 2 mL of water. 1 mL of 30 % methanol was used for the elution of retained material. Extracted samples were dried in a SpeedVac (Thermo Scientific Marietta, Ohio, USA) and then dissolved in 100 μL digestion buffer (10 mmol/L Tris−HCl, 1 mol/L sodium acetate, pH 7.5). Subsequently, samples were hydrolyzed with 22 units of alkaline phosphatase at 37 °C for 1 h. All samples were filtered using Nanosep Omega tubes by centrifugation at 5000xg for 50 min. After filtration, 30 μL of the filtrates were used for LC–MS/MS analyses. LC–MS/MS analyses were performed using an HPLC system (Shimadzu, Kyoto, Japan) coupled with a triple quadrupole ion-trap mass spectrometer (4000 QTRAP Applied Biosystems, CA, USA) equipped with a TurboIonSpray™ source in the positive ionization mode, as described by Kant et al. [45]. Analysis by LC–MS/MS with MRM was performed using the mass/charge (*m/z*) transitions *m/z* 284→*m/z* 168 and *m/z* 289→*m/z* 173 for 8−OH-dG and 8−OH-dG-$^{15}N_5$, respectively and with *m/z* transitions *m/z* 250→*m/z* 164 and *m/z* 255→*m/z* 169 for both *R*- and *S*-diastereomers of cdA and cdA-$^{15}N_5$, respectively. The quantification was performed using integrated peak area ratios of analytes and internal standards. The results were normalized with urinary creatinine concentrations and expressed in nmol/mmol creatinine. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on signal-to-noise (S/N) ratios of 3 and 10, respectively. LOD for 8−OH-dG and *S*-cdA were calculated as 0.18 nmol/mL and 1.1 nmol/mL. LOQ levels for 8−OH-dG and *S*-cdA were determined as 0.58 nmol/mL and 4.4 nmol/mL. *R*-cdA and *S*-cdA are 5′*R* and 5′*S* stereoisomer cdA lesions, so that only used *S*-cdA standard for method optimization studies.

2.4. Biochemical analyses

Complete blood count analyses were performed by an automated COULTER® LH 780 Hematology Analyzer (Beckman Coulter, Inc., USA). HbA1c was measured using an HLC-723 G8 HbA1c analyzer (Tosoh Bioscience, Inc., Tokyo, Japan). Ferritin measurements were performed by the chemiluminescence immunoassay method using the DXI analyzer (Beckman Coulter, USA) and the urinary creatinine measurements were performed by a colorimetric method (Beckman Coulter AU5800, USA) in Dokuz Eylul University Central Laboratory.

2.5. Statistical analyses

Statistical analyses were performed by using SPSS 22.0 (SPSS Inc., USA). The values obtained from all measurements were presented as mean and standard deviation. Data about each variable was checked for normality of their distribution by the Shapiro–Wilk normality test. Square root transformations (for 8−OH-dG, *R*-cdA and *S*-cdA levels) were applied to provide the Gaussian distribution. Frequency analyses for categorical variables were analyzed using the Chi-Square test. Comparisons of the groups were made with independent samples *t*-test and paired samples *t*-test. The levels of 8−OH-dG, *R*-cdA and *S*-cdA were compared among study groups using the Univariate Analyses of Covariance (ANCOVA) models which include age, body mass index (BMI), presence of comorbidity (hypothyroidism, mild anemia, psoriasis, asthma, mitral valve prolapse hyperthyroidism, arrhythmia, kidney stones) and smoking status as covariates. Correlation analyses were performed with Pearson's test. A significance level of 0.05 was used for all statistical tests.

3. Results

In this study, out of a total of 117 pregnant women in the second trimester, 33 had GDM (GDM group) and 84 had normal glucose tolerance (control group comprising healthy pregnant women). The demographic characteristics of the groups were not statistically different; the mean maternal age was 30.4 ± 5.2 in the GDM group and 29.2 ± 5.7 years in the control group ($p = 0.239$). The mean BMI was 25.6 ± 3.5 in the GDM group and 25.4 ± 4.6 kg/m^2 in the control group ($p = 0.819$). The mean systolic arterial pressure was 103.5 ± 9.5 mm Hg in the GDM group and 105.2 ± 11.8 mm Hg in the control group ($p = 0.442$). The mean diastolic arterial pressure was 67.1 ± 7.3 mm Hg in the GDM group and 69.1 ± 8.2 mm Hg in the control group ($p = 0.228$). It was found that only one (0.85 %) of 117 pregnant women included in the study had GDM in their previous pregnancies. Nineteen of 117 pregnant women had hypothyroidism, fourteen had mild anemia, three had psoriasis, two had asthma and mitral valve prolapse. In addition to these pregnant women, three different pregnant women had hyperthyroidism, arrhythmia and kidney stones. In compliance with the study design, a reevalution of each participant was conducted during the second sample collection after GDM diagnosis. This reevalution revealed a previous diagnosis with asymptomatic status in three pregnant women (one with psoriasis and two with asthma) who had reported no medical history at recruitment with a long period without medication. Demographic features, medication and smoking status are shown in Table 1.

Fig. 1 illustrates the ion − current profiles of the mass transitions for *R*-cdA, *S*-cdA, *R*-cdA-$^{15}N_5$, *S*-cdA-$^{15}N_5$, 8−OH-dG, and 8−OH-dG-$^{15}N_5$, which were recorded during the LC−MS/MS analysis of a urine sample. Whether the presence of comorbidities, smoking, BMI and age affected the significance of the difference between the groups in terms of DNA damage parameters were tested by analysis of covariance (ANCOVA). Accordingly, the effect of comorbidities, smoking, BMI and age on DNA damage parameters in the groups were not found to be statistically significant ($p > 0.05$). In the first trimester, urinary 8−OH-dG levels were higher in the GDM group than those in the control group ($p = 0.006$) (Table 2, Fig. 2) and this difference remained significant even after the adjustment for age, BMI, presence of comorbidities and smoking status ($p = 0.013$). *R*-cdA and *S*-cdA levels were not significantly different between the groups (Table 2). There are not any reference values of 8−OH-dG for pregnant women. Therefore, the women in the control group were stratified based on 8−OH-dG levels in the 11th-14th gestational weeks and the 50th centile was considered as a cut-off value for 8−OH-dG (8−OH-dG>1.190 nmol/mmol creatinine). To

Table 1
Demographic features and medication status of GDM and control groups.

		Control group (n = 84)	GDM group (n = 33)
Age (years) mean ± SD		29.15 ± 5.74	30.45 ± 5.16
Systolic arterial pressure (mm Hg) mean ± SD		105.2 ± 11.8	103.5 ± 9.5
Diastolic arterial pressure (mm Hg) mean ± SD		69.1 ± 8.2	67.1 ± 7.3
BMI in 1st trimester (kg/m^2) mean ± SD		25.43 ± 4.62	25.59 ± 3.47
BMI during 1st trimester (kg/m^2) (n)	underweight (BMI < 18.50)	1	1
	normal (BMI = 18.50–24.99)	40	7
	overweight (BMI: 25.00–29.99)	18	14
	obese (BMI ≥ 30.0)	9	2
	no information	16	9
Iron supplementation during 1st trimester (n)	using	16	8
	not using	53	19
	no information	15	6
Folate supplementation during 1st trimester (n)	using	37	19
	not using	34	10
	no information	13	4
Smoking status (n)	smoking	8	2
	not smoking	60	24
	no information	16	7
Medication, Drugs, Other supplementation during 1st trimester (n)	no drugs or supplements	22	3
	multivitamin supplements	8	5
	thyroid hormone supplementation	9	3
	blood thinner	1	3
	progesterone (progestan)	2	1
	cortisol	1	–
	vitamin D	1	–
	nausea medicine	1	–
	unidentified drug	1	–
	lamictal (anti epileptic)	–	1
	beta blocker	–	1
	antacid (gaviscon)	–	1
	no information	13	4

The numbers represent the mean for the ages and BMI and the uncertainties are standard deviations. Iron and folate supplementations and smoking are shown by number of individuals and percentage. $p < 0.05$ was considered statistically significant between groups. SD: standard deviation, GDM: gestational diabetes mellitus, BMI: body mass index.

examine the relationship between urinary 8−OH-dG levels in the first trimester and the risk of GDM, when all the pregnant women were classified according to this cut-off, 51 of 117 pregnant women had urinary 8−OH-dG levels below the cut-off value and 66 of them had 8−OH-dG levels above the cut-off value. While 36.3 % of 66 pregnant women were diagnosed as GDM, 17.6 % of 51 pregnant women who had low levels of 8−OH-dG in the first trimester were diagnosed as GDM at the end of the second trimester. When the difference between these percentages was evaluated, the pregnant women who had high 8−OH-dG levels in the first trimester were found to have a significantly higher rate of GDM development than the pregnant women who had lower 8−OH-dG levels ($p = 0.038$). On the other hand, the change in the maternal urine oxidative DNA damage products from the first to the second trimester could not reach a statistically significant level in both the GDM and the control groups (Table 3, Fig. 2).

The comparison of the studied glucose and iron status parameters of the GDM and the control groups in two trimesters of pregnancy is presented in Table 3. FBG levels in the first trimester were significantly higher in the GDM group than in the control group ($p < 0.001$). Concerning FBG levels in the first trimester, 21 (22.8 %) of 92 pregnant women with FBG < 92 mg/dL were diagnosed as GDM in the second trimester, while 12 (48.0 %) of 25 pregnant women with FBG ≥ 92 mg/dL developed GDM. The GDM development rate was found to be significantly higher in the pregnant women with FBG ≥ 92 mg/dL in the first trimester compared to the women with FBG < 92 mg/dL ($p = 0.022$).

Serum ferritin levels were not significantly different between the GDM and control groups in the first and second trimesters (Table 4). When all the women were stratified according to their first trimester ferritin levels, the pregnant women with ferritin levels ≥ 50th centile (≥ 130 ng/mL) demonstrated significantly higher levels of 8−OH-dG and *R*-cdA than the pregnant women under < 50th centile ($p = 0.034$ and $p = 0.009$, respectively) (Table 5).

Twenty-four women reported taking iron supplements regularly in the first trimester. Their nucleoside damage parameters did not significantly differ from the parameters of 72 pregnant women who reported not taking any iron supplements in the first trimester (8−OH-dG; 1.394 ± 0.984 and 1.564 ± 0.832 nmol/mmol creatinine, $p = 0.315$; *S*-cdA; 0.030 ± 0.026 and 0.040 ± 0.039 nmol/mmol creatinine, $p = 0.141$; *R*-cdA; 0.015 ± 0.009 and 0.014 ± 0.005 nmol/mmol creatinine, $p = 0.798$, respectively). The rate of GDM development in the second trimester was higher in 24 pregnant women taking iron supplements than those not taking the supplements, though the difference was not significant ($p = 0.602$).

When routine biochemical parameters in the first trimester were compared with those in the second trimester, hemoglobin levels in both the GDM and control groups significantly decreased in the second trimester compared to those in the first trimester ($p < 0.001$ and $p < 0.001$, respectively). In addition, FBG, ferritin and HbA1c levels in the control group significantly decreased in the second trimester compared to those in the first trimester ($p < 0.001$, $p < 0.001$, $p = 0.035$, respectively). In the GDM group, FBG, ferritin and HbA1c levels were not significantly different between the first trimester and the second trimester ($p = 0.188$, $p = 0.101$ and $p = 0.369$, respectively) (Table 4).

In both the GDM group and the control group, *R*-cdA in the second trimester had a positive correlation with *S*-cdA in the second trimester ($r = 0.549$, $p = 0.003$ and $r = 0.504$, $p < 0.001$, respectively) (Figs. 3 and 4). There was a significant positive correlation between *R*-cdA and *S*-cdA and 8−OH-dG levels in the first trimester in the control group ($r = 0.456$, $p < 0.001$ and $r = 0.364$, $p = 0.001$, respectively). A positive correlation was also found between ferritin levels and *R*-cdA and 8−OH-dG levels during the first trimester in the control group ($r = 0.279$, $p = 0.011$ and $r = 0.256$, $p = 0.019$, respectively) (Fig. 4). In the GDM group, 8−OH-dG levels had a positive correlation with ferritin and the 1st hour glucose values on OGTT in the second trimester ($r = 0.466$, $p = 0.014$ and $r = 0.452$, $p = 0.020$, respectively) (Fig. 3).

In all the pregnant women; there was a positive correlation between *R*-cdA levels and *S*-cdA, 8−OH-dG and ferritin values in the first trimester ($r = 0.387$, $p = 0.000$, $r = 0.319$, $p = 0.000$ and $r = 0.264$, $p = 0.004$, respectively). A positive correlation was also found between *R*-cdA levels and *S*-cdA and 8−OH-dG levels in the second trimester ($r = 0.514$, $p = 0.000$ and $r = 0.365$, $p = 0.001$, respectively). In addition, ferritin levels in the second trimester had a significant correlation with 8−OH-dG in the second trimester and ferritin levels in the first trimester ($r = 0.302$, $p = 0.004$ and $r = 0.353$, $p = 0.001$, respectively) (Fig. 5).

4. Discussion

This is the first prospectively designed study to simultaneously measure urinary 8−OH-dG, *R*-cdA and *S*-cdA levels that represent cumulative oxidative damage and to determine their relations with the iron status in women with GDM and healthy women, both in the early (11-14th weeks) and late stages (24-28th weeks) of pregnancy. Although there are a number of studies on the association between oxidative stress and GDM [13–16,18–20,46], only a few focus particularly on oxidative

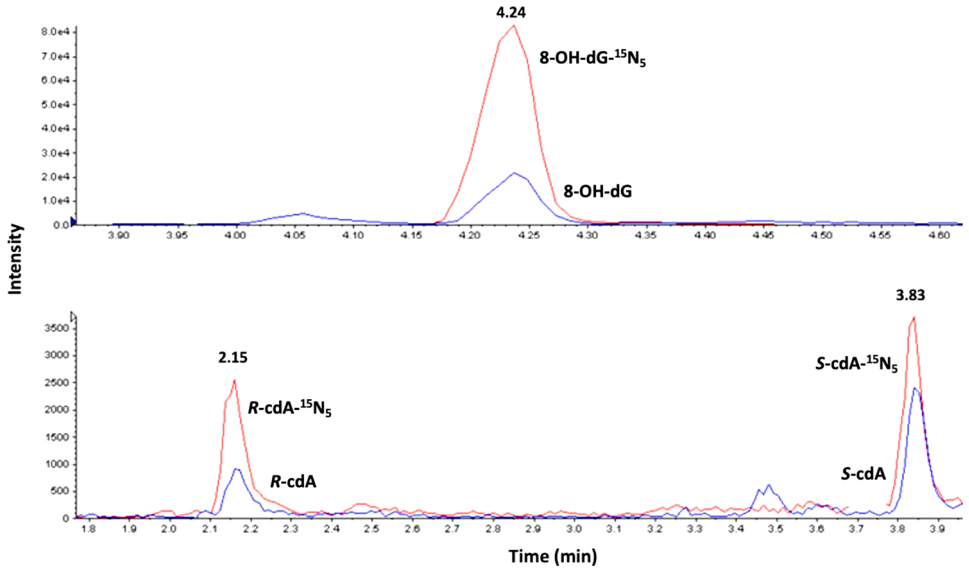

Fig. 1. Ion – current profiles of the *m/z* 250→164 (*R*-cdA and *S*-cdA), *m/z* 255→169 (*R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$), *m/z* 284→168 (8–OH-dG), and *m/z* 289→173 (8–OH-dG-$^{15}N_5$) mass transitions.

Table 2
The comparison of the oxidative DNA damage product levels measured in the maternal urine samples.

		GDM group (n = 33)	Control group (n = 84)	p value
11th–14th weeks of gestation	8-OH-dG (nmol/mmol creatinine)	1.847 ± 1.178	1.355 ± 0.581	0.006
	S-cdA (nmol/mmol creatinine)	0.038 ± 0.032	0.037 ± 0.035	0.792
	R-cdA (nmol/mmol creatinine)	0.014 ± 0.004	0.014 ± 0.007	0.794
24th–28th weeks of gestation	8–OH-dG (nmol/mmol creatinine)	1.487 ± 0.821	1.317 ± 0.557	0.414
	S-cdA (nmol/mmol creatinine)	0.040 ± 0.025	0.050 ± 0.059	0.608
	R-cdA (nmol/mmol creatinine)	0.014 ± 0.005	0.016 ± 0.008	0.301

DNA damage, which is a widely accepted reliable marker for systemic oxidative stress [32,33,35,36,45–47]. Furthermore, none of those studies are based on samples from the two critical periods of pregnancy. Previous studies on GDM were conducted using different laboratory techniques such as ELISA and comet assay for the measurement of DNA damage products in a range of biological specimens, such as blood and urine [32,33,37,38,47–49]. Oxidative DNA damage was investigated by identification and quantification of reliable biomarkers using the reference tandem mass spectrometric method in the present study. Using urine samples for 8–OH-dG, *R*-cdA and *S*-cdA measurements is a further strength of the present study since it is obtained more readily and non-invasively than other biological samples and fully reflect the oxidative load in the whole body [50,51]. From this perspective, the study has a unique design.

One of the most important results of this prospective study is that the pregnant women diagnosed as GDM on OGTT in the second trimester had a higher urine 8–OH-dG level in the first trimester compared to the control group. High levels of urinary 8–OH-dG in the first trimester of the women diagnosed as GDM in the second trimester is a strong evidence of the increased production of this compound early in pregnancy, most likely due to known augmented oxidative stress and inflammation, and consequently DNA damage in GDM. When the cut-off value for 8–OH-dG in the first trimester determined in this study was taken into account, the higher possibility of GDM development in the women with 8–OH-dG levels higher than the threshold value suggests that early urine 8–OH-dG levels can be predictive of the risk of GDM likely to appear later.

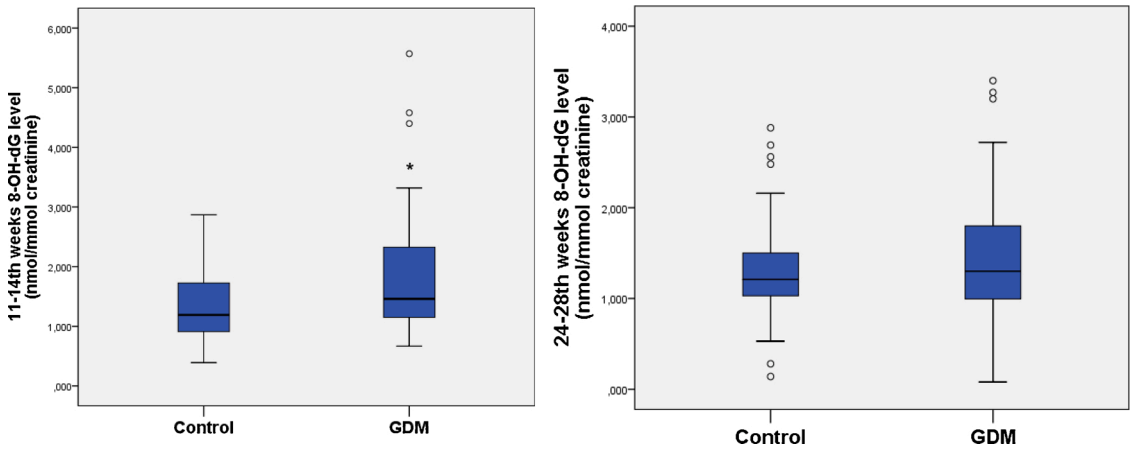

Fig. 2. 8–OH-dG levels in the 11th–14th weeks (A) and the 24th-28thweeks (B) in urine samples of the control group (n = 84) and the GDM patients (n = 33).

Table 3
The change in the maternal urine 8−OH-dG, *R*-cdA and *S*-cdA levels between the first and the second trimesters in the GDM and the control groups.

	GDM group 11th–14th weeks (n = 33)	GDM group 24th–28th weeks (n = 33)	p value	Control group 11th–14th weeks (n = 84)	Control group 24th–28th weeks (n = 84)	p value
8-OH-dG (nmol/mmol creatinine)	1.847 ± 1.178	1.487 ± 0.821	0.140	1.355 ± 0.581	1.317 ± 0.557	0.334
S-cdA (nmol/mmol creatinine)	0.038 ± 0.032	0.040 ± 0.025	0.711	0.037 ± 0.035	0.050 ± 0.059	0.175
R-cdA (nmol/mmol creatinine)	0.014 ± 0.004	0.014 ± 0.005	0.611	0.014 ± 0.007	0.016 ± 0.008	0.262

Table 4
The comparison of the glucose and iron status parameters of the GDM and the control groups in two trimesters of pregnancy.

	Laboratory tests	GDM group (n = 33)	Control group (n = 84)	p value
11th-14th weeks of gestation	Fasting blood glucose (mg/dL)	92.9 ± 19.5	81.8 ± 10.3	<0.001
	Ferritin (ng/mL)	20.0 ± 19.0	15.3 ± 10.7	0.182
	Hemoglobin (g/dL)	12.2 ± 0.9	12.1 ± 0.9	0.925
	HbA1c (%)	5.3 ± 0.4	5.1 ± 0.3	0.084
24th-28th weeks of gestation	Fasting glucose on OGTT (mg/dL)	87.4 ± 10.2	75.8 ± 7.2	<0.001
	1st hour glucose on OGTT (mg/dL)	184.7 ± 23.3	122.6 ± 27.2	<0.001
	2nd hour glucose on OGTT (mg/dL)	143.3 ± 24.6	102.9 ± 17.9	<0.001
	Ferritin (ng/mL)	12.8 ± 17.8	9.9 ± 7.8	0.256
	Hemoglobin (g/dL)	11.3 ± 1.2	11.3 ± 0.9	0.974
	HbA1c (%)	5.2 ± 0.5	5.1 ± 0.3	0.182

Table 5
Urinary oxidative DNA damage product levels according to the first trimester serum ferritin centile.

		Ferritin < 50th centile (n = 58)	Ferritin ≥ 50th centile (n = 58)	p value
11th–14th weeks of gestation	8-OH-dG (nmol/mmol creatinine)	1.354 ± 0.774	1.646 ± 0.848	0.034
	S-cdA(nmol/mmol creatinine)	0.032 ± 0.025	0.042 ± 0.041	0.182
	R-cdA (nmol/mmol creatinine)	0.013 ± 0.006	0.016 ± 0.006	0.009
24th–28th weeks of gestation	8-OH-dG(nmol/mmol creatinine)	1.428 ± 0.738	1.320 ± 0.569	0.694
	S-cdA(nmol/mmol creatinine)	0.056 ± 0.068	0.039 ± 0.026	0.124
	R-cdA (nmol/mmol creatinine)	0.016 ± 0.009	0.015 ± 0.006	0.541

During a normal pregnancy, oxidative stress stimulates antioxidant mechanisms that are capable of reacting through enzyme activity and non-enzyme free radical deactivators [52]. In contrast to DNA damage in the first trimester, DNA damage product levels in the 24-28th weeks found to be similar between the groups (Table 2) suggested a probable antioxidant and/or protective mechanism against increased oxidative stress in the second trimester. Thus, comparative studies of pregnant and nonpregnant patients [60] showed that total plasma antioxidant status in the first trimester of pregnancy is significantly lower. In the second and third trimesters of pregnancy, total plasma antioxidant capacity increases, and in the last week of pregnancy reaching values similar to those observed in non-pregnant women.

As well as 8−OH-dG, *R*-cdA and *S*-cdA in human urine offer alternative biomarkers for oxidative DNA damage [43]. In most studies focusing on oxidative DNA damage in GDM, this damage has been evaluated only by measuring 8−OH-dG levels. In the present study, in addition to 8−OH-dG, *R*-cdA and *S*-cdA were evaluated as unique tandem lesions. Although no statistically significant difference was observed between *R*-cdA levels and *S*-cdA levels in the two groups, *R*-cdA levels were found to be correlated with 8−OH-dG and *S*-cdA levels in all the studied pregnant women for the first and second trimesters. *R*-cdA, *S*-cdA and 8−OH-dG are typical products of reactions of hydroxyl radical (˙OH) with DNA components. However, the formations of 8−OH-dG and cdA vary with the O_2 concentration in the cell [34,53]. Such mechanistic differences between these two types of lesions and varying oxygen concentrations throughout pregnancy can explain the differences in levels of the two lesions. Therefore, further studies are needed to clearly demonstrate the role of these lesions in GDM.

In line with the results obtained in the current study, Qui et al. [32] reported higher levels of urinary 8−OH-dG in women who were later diagnosed as GDM than in controls. By comparing the pregnancies from the highest and lowest quartile values of 8−OH-dG, they also found a 3.79 relative risk for developing GDM. However, the authors studied maternal urinary samples in a later and only a single period of pregnancy, which was the 16th week, by using the ELISA method unlike the current study. Jamil et al. and Toljic et al. found an increased maternal serum 8−OH-dG levels only in the second trimester and these findings are in contrast with the results of the present study about levels of DNA damage products in the 24-28th weeks. However, the use of the ELISA method in the studies, more likely to cause a cross-reaction as compared with the mass spectrometric method, and whether serum samples reflect the whole-body oxidative stress load should be questioned. In another study conducted by Zein et al. [48] with 14 GDM and 79 control pregnant women, the results of the logistic regression analysis showed that increased DNA damage was associated with a six-fold increased risk of developing GDM. In their study, blood samples were collected in the 24-28th weeks and DNA damage was determined by the comet assay method. Due to the analytical measurement method they used, it is not possible to determine exact DNA damage products as well as to quantify them absolutely and therefore a relative quantification was performed in that study.

HAPO study described a continuous association between maternal glycemic levels and the perinatal morbidities [8]. Glycemic levels that even could not reach a “hyperglycemic” threshold increased the perinatal morbidities [5]. This finding emphasized the importance of close surveillance for near-threshold levels. In the present study, the mean first trimester FBG level in pregnant women likely to have GDM was close to normal limits (92.9 ± 19.5 mg/dL), whereas the high 8−OH-dG level in this period is thought to indicate that DNA damage might have started earlier, which is important for the diagnosis of GDM.

In our study, the unchanged HbA1c levels of the GDM group compared to the control group in both trimesters, reveals that although HbA1c is a beneficial biomarker for reflecting the last 2–3 months of the glycemic status, the use of HbA1c as a marker in pregnancy is not recommended, particularly from the second trimester due to the increased erythropoiesis during pregnancy and the alteration of erythrocyte turnover rate. On the the other hand, the decrease in ferritin and hemoglobin can be explained by hemodilution as well as the ascendant necessity for fetus during pregnancy.

Our fasting blood glucose level results in the healthy controls found

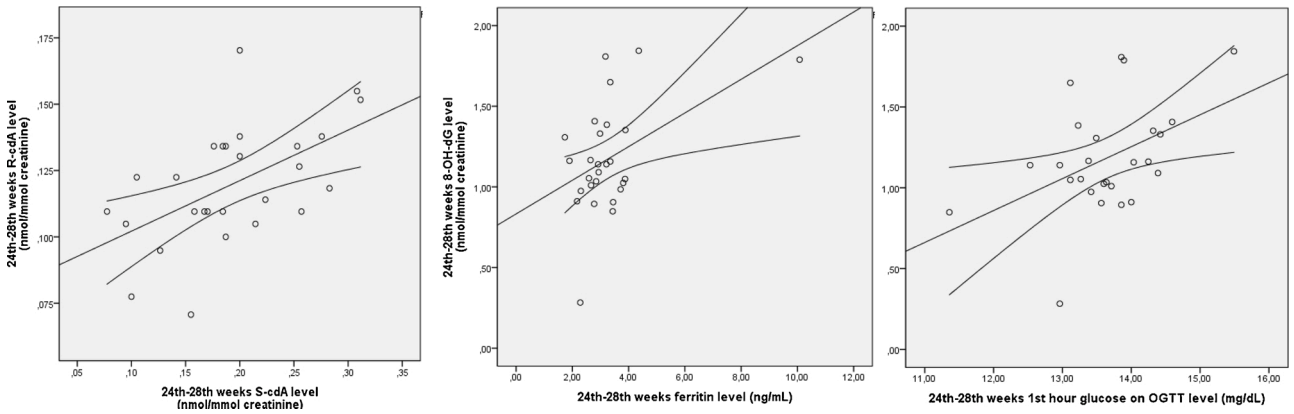

Fig. 3. Significant correlations of *R*-cdA level with *S*-cdA, and 8−OH-dG levels with ferritin and 1st hour glucose on OGTT level in second trimester in GDM patients.

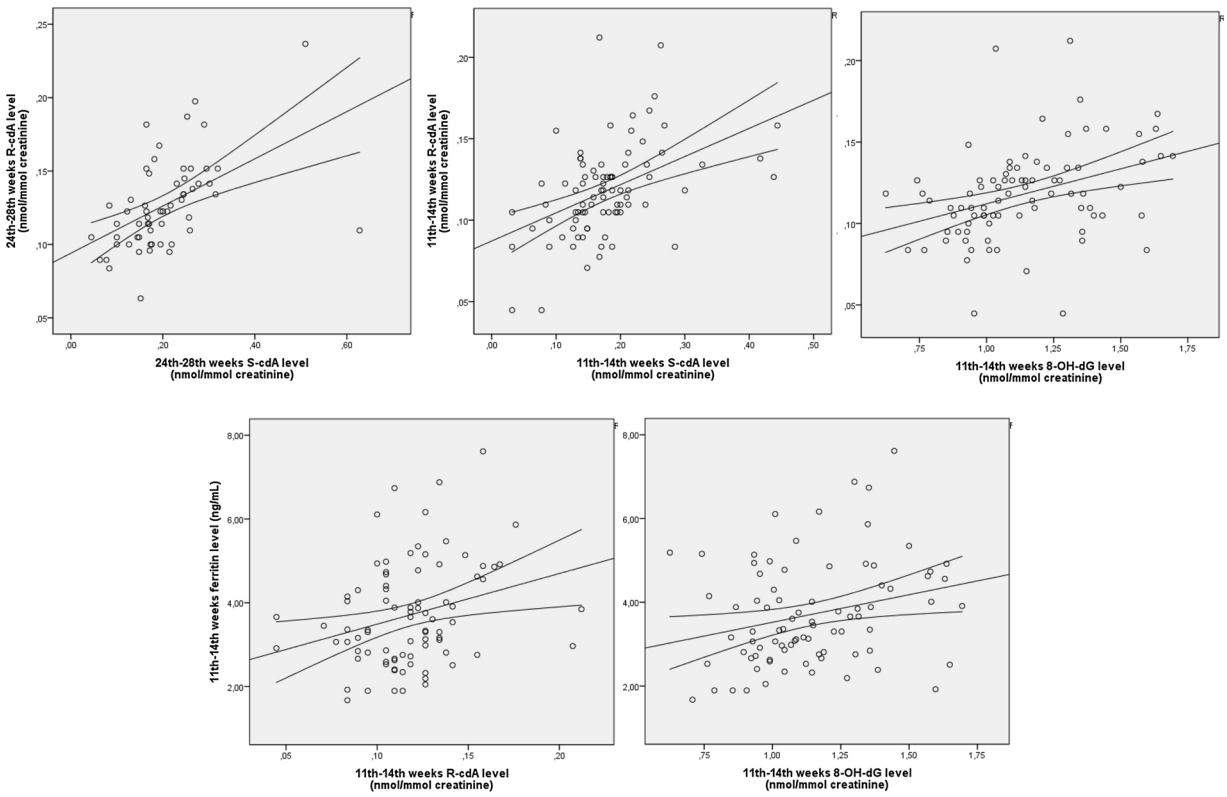

Fig. 4. Significant correlations of *R*-cdA with *S*-cdA level in second trimester, *R*-cdA with *S*-cdA and 8−OH-dG levels and ferritin with *R*-cdA and 8−OH-dG levels in first trimester in control group.

as decreased in the second trimester compared to the first trimester is most likely related to the fact that some of the pregnant women visited the clinic postprandial for routine analyses of the first trimester. This is a major limitation of the study. Other limitations of this study should be noted as follows: Firstly, the diet and haem/non-haem iron intake were not taken into account. Another hypothesis of this study was that iron supplementation could increase the GDM frequency and DNA damage product levels. However, the frequency of GDM diagnosis was not different between the women who took iron supplements and those who did not. Since iron supplementation data were available only for 96 of 117 pregnant women, only 96 cases of them were included in statistical analyses. Another limitation of the study is that BMI and smoking status data were not available for all women. We also could not evaluate the educational level, household income, food habits, and physical activity status which were missing in the questionnaire form.

Some studies also reported higher ferritin levels in GDM diagnosed pregnancies [54,55] and an increased risk of developing GDM for pregnant women with elevated ferritin levels [56]. Zein et al. showed in their study in 2015 that high ferritin levels in non-anemic women could predict glycemic status and found a strong correlation between increased ferritin levels and glucose values in the second hour on 75 g OGTT [57]. In another study in 2017, Zein et al. concluded that serum ferritin and iron status could be a modifying factor in the interaction between oxidative stress and glucose tolerance [48]. Ferritin concentrations have consistently been associated with GDM, and this relationship has continued at low and high ferritin levels. However, it is still unclear which cut-off value should be used for high level iron status [29,58,59]. In addition, if it is thought that iron storage levels decrease with increasing iron need in pregnant women, it may be beneficial to rearrange the reference range of ferritin for pregnant women. In the present

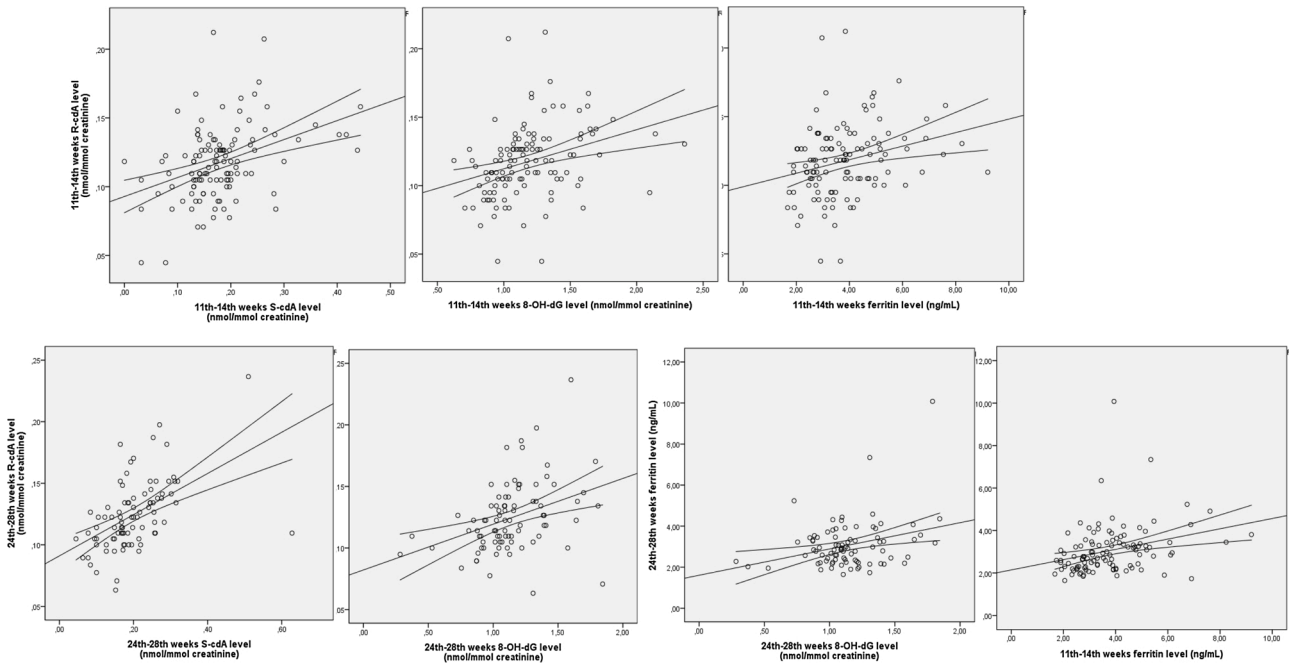

Fig. 5. Significant correlations of *R*-cdA with *S*-cdA, 8−OH-dG and ferritin levels in first trimester, *R*-cdA with *S*-cdA and 8−OH-dG levels in second trimester, and ferritin with 8−OH-dG level in second trimester and ferritin level in first trimester in all the pregnant women.

study, the relationship between the iron status and DNA damage in women with and without GDM was also investigated. Serum ferritin was measured to evaluate iron storage levels. When the cases were stratified according to their serum ferritin levels, the *R*-cdA and 8−OH-dG levels were higher in the ≥ 50th centile (ferritin ≥ 13.0 ng/mL) than in the < 50th centile groups as hypothesized at the beginning of the study. These findings suggest that there might be a relationship between relatively high iron storage levels and oxidative DNA damage. In addition, ferritin levels in the second trimester had a tendency to decrease compared to those in the first trimester in both the GDM and control groups ($p = 0.101$ for the GDM group; $p < 0.001$ for the control group). The same tendency was also true for DNA damage. This finding is suggestive of an association between ferritin and DNA damage and the presence of a pregnancy-related defense system as expected.

This study was based on the measurements of the oxidative DNA damage products and ferritin levels at two points; in the first trimester (11-14th weeks) and the second trimester on OGTT (24-28th weeks). One of the most striking findings of the present study was the presence of a significant positive correlation between 8−OH-dG levels and ferritin and glucose values in the 1st hour on OGTT at the time of GDM diagnosis. These findings support the possible interrelations between hyperglycemia, iron status and oxidative DNA damage. Focusing on assessing the oxidative effects of supplemented iron, Zhuang et al. [31] reported that supraphysiological or high doses of iron can induce lipid peroxidation both in vitro and in vivo animal studies. High iron status reflected by high serum ferritin or other various markers might contribute to increased risk of GDM via accelerated lipid peroxidation and/or DNA damage [31,61]. Despite being limited by the lack of stratification according to the iron supplementation dose, we could demonstrate a synchronous elevation of the key molecules (ferritin, glucose and 8−OH-dG) of these three pathways; iron dynamics, glucose metabolism and DNA damage.

In a previous study by the authors of this study, the levels of oxidative stress markers were investigated in patients with prediabetes and type 2 diabetes mellitus (T2DM) in comparison with healthy volunteers. Oxidative DNA damage and lipid peroxidation products were found to be elevated in patients with prediabetes [45]. These results indicated that oxidative macromolecular damage appears before the establishment of T2DM. The present study included pregnant women with impaired glucose tolerance to reveal whether they had elevated oxidative DNA damage levels long before GDM development similar to prediabetes patients. The lack of an international uniformity about the detection and diagnosis of impaired glucose tolerance in pregnancy creates a problem [2]. Therefore, in the present study adapting some reference criteria, the pregnant women with FBG levels and the first-hour blood glucose levels on 75g-OGTT below the cut-off value and with the second-hour blood glucose levels of 140−152 mg/dL were considered to have impaired glucose tolerance. Nevertheless, since there were only four pregnant women with these features, they were not included in the study. Oxidative DNA damage markers could be necessary to diagnose and differentiate impaired glucose tolerance and GDM earlier. Well-designed studies with a larger sample size are required on this subject.

Evaluation of GDM in the first trimester will be beneficial for early intervention in women who may be at risk of adverse pregnancy and long-term outcomes. In line with the results obtained from the present study, it can be suggested that measurement of oxidative DNA damage products, which have a place in the pathophysiology of GDM, especially in the first trimester, may contribute to the early detection, intervention strategies and improvement of maternal factor screening models of GDM.

5. Conclusion

This is the first study in which systemic oxidative DNA damage was measured with a reference method in both early and late periods of pregnancy. Women with GDM demonstrated increased levels of urinary 8−OH-dG indicating higher oxidative DNA damage in the 11-14th gestational weeks. Lower levels of oxidative DNA damage products in the pregnant women with a low level of ferritin suggested a relation between iron levels and oxidative stress as a modifying factor in the interaction between oxidative stress and glucose metabolism. First trimester non-invasive urinary oxidative DNA damage products may be helpful to predict GDM.

Author contributions

M.O.E., G.T., G.H.İ. and S.K. designed the study; S.K. and S.A. performed diagnostic interviews; M.O.E. and S.K. performed clinical assessments and paperwork; M.O.E. performed sample storage, M.O.E., G. T., M.K., M.A., N.E.D.B. and G.H.İ. performed the LC–MS/MS analysis. M.O.E., G.T. and G.H.İ. carried out statistical analysis and G.T., S.K. and G.H.İ. wrote the first draft. All authors contributed to the final manuscript.

Ethical approval

The study was approved by the ethics committee of Dokuz Eylul University School of Medicine (Ethics committee approval date: February 18, 2016, protocol number: 2016/05–37).

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Informed consent

Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2021.05.009.

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