Lipid-lysine adducts and modified tyrosines as markers of oxidative stress in the second trimester of pregnancy and their association with infant characteristics

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Abstract. Pregnancy is a physiological state accompanied by excessive levels of oxidative stress (OS), due to the increased demand and utilisation of oxygen. There is increasing evidence that maternally augmented OS exerts an adverse effect on pregnancy outcome. The aim of the present prospective study was to determine the association between the urinary concentration of relatively novel OS markers measured in the second trimester of pregnancy and the infant characteristics at birth. The maternal levels of urinary hexanoyl-lysine (HEL), propanoyl-lysine (PRL), dityrosine (DiY) and 3-nitrotyrosine (NY) were evaluated in generally healthy pregnant subjects to determine their association with birth weight, gestation at delivery and Apgar score. The observed levels of the markers were in agreement with those measured in healthy non-pregnant subjects in a previous study. A positive correlation was detected between HEL and PRL, as well as between HEL and DiY. Although the absence of a correlation between NY and the other markers has been previously noted in a non-pregnant population, a positive correlation in the pair PRL-NY (r=0.367; P<0.001) was observed in the present study. Maternal cigarette smoking was associated with increased urinary PRL levels (P=0.034). The most notable observation in the present study was that high levels of PRL and NY were associated with low Apgar scores at 1 and 5 min after birth (OR, 1.098 and 2.084 for PRL and NY, respectively; P<0.05). However, poor predictive accuracy was shown. For NY, the following results were obtained: Area under the curve (AUC), 0.818; sensitivity, 100%; specificity, 57%; positive predictive value (PPV), 11.54%; and negative predictive value (NPV), 100%. For PLR the values were as follows: AUC, 0.802; sensitivity, 100%; specificity, 62.6%; PPV, 13.05%; and NPV, 100%. DiY was negatively associated with preterm birth risk (OR=0.703; P=0.028). In conclusion, the results of the present study indicated the presence of OS in the second trimester of pregnancy, which was detected with damage to lipids and proteins and associated with an adverse Apgar score; however, the selected urinary markers exhibited poor positive predictive efficacy.

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Introduction

Pregnancy is a physiological condition accompanied by dynamic metabolic changes in multiple organ systems, resulting in increased oxygen consumption. In the first weeks after conception, embryonic development occurs in a primarily hypoxic environment; however, during pregnancy a highly vascular placenta develops, which is rich in mitochondria and influences maternal homeostasis. After the first trimester of pregnancy, the formation and development of placental blood flow results in a rapid increase in partial oxygen pressure (1,2). Increased metabolic activity and reduced antioxidative activity in uncomplicated pregnancy may lead to exaggerated oxidative stress (OS) (3-5).

OS is defined as an imbalance between oxidants and antioxidants in favour of reactive oxygen species (ROS), and may lead to a disruption of redox signalling and control, and/or to molecular damage (6). ROS are produced endogenously by various physiological processes and in response to external factors, such as pathogens and environmental risk factors. Excessive physiological levels of ROS may lead to an increase in damage to the cellular components, including lipids, nucleic acids and proteins (7).

Oxidative damage to lipids is a complex process, which modifies cellular membranes and has an adverse effect on cellular function. The lipids that are most vulnerable to lipid peroxidation (LP) are polyunsaturated fatty acids. Lipid hydroperoxide and its corresponding aldehydes are generated at an early stage of LP. Hydroperoxides and aldehydes are highly reactive and may interact with proteins, nucleic acids or amino-phospholipids (8). Stable amide-type adducts, which

are a product of lipid hydroperoxide-derived protein modification, have been recently identified, including hexanoyl-lysine (HEL) and propanoyl-lysine (PRL). HEL is formed via the reaction of peroxidised n-6 fatty acid with protein lysine residues, while PRL is generated through the reaction of n-3 fatty acid with these residues (9). Previous studies have suggested that HEL levels are elevated in the urine of patients with diabetes (10-12), metabolic syndrome (13), rheumatoid arthritis (14) and systemic sclerosis (15).

Oxidative damage to proteins may alter various levels of the protein structure and activity. Aromatic amino acids, such as tyrosine, are highly susceptible to oxidation. The nitration of tyrosine in proteins by peroxynitrite, which is a strong oxidising agent generated during the reaction of nitric oxide and the superoxide anion, generates 3-nitrotyrosine (NY). NY is a promising biomarker for protein tyrosine nitration *in vivo*, and elevated levels of NY have been detected in the urine of diabetic patients (16). An additional stable and potential marker of protein oxidation is dityrosine (DiY), the formation of which is initiated with a tyrosyl radical and concludes with the intermolecular cross-linkage of two tyrosine-containing proteins (17,18).

Exaggerated oxidation of any of the aforementioned substrates may indicate and theoretically contribute to various complications and chronic diseases, thus posing a health risk to the mother and the fetus. Previous studies have demonstrated an association between exaggerated OS markers and pregnancy complications, including gestational diabetes, pre-eclampsia, intrauterine growth restriction, preterm birth and low birth weight (19-23). The extent of physiologically acceptable OS and the critical concentrations affecting fetal growth and health outcomes are unclear.

The aim of the present study was to investigate relatively novel potential oxidative markers in pregnant patients and to evaluate their association with and effect on the pregnancy outcome. The identification of novel biomarkers is a crucial challenge for the early diagnosis, prediction and prevention of pregnancy complications. The use of any marker requires knowledge of normal baseline levels and their association with clinical changes.

We hypothesised that the urine levels of markers of oxidative damage to lipids and proteins in the second trimester of pregnancy were associated with maternal and fetal characteristics and with an increased risk of adverse infant features. To the best of our knowledge, the present study is the first to investigate markers of the initial stages of lipid peroxidation (HEL and PRL) and of tyrosine moiety oxidation (NY and DiY) in the urine of pregnant women.

Materials and methods

Study procedure and subjects. The present study is part of a larger study with the aim of investigating the markers of OS in maternal urine, blood and amniotic fluid. The study was conducted at the Department of Obstetrics and Gynaecology of University Medical Centre Ljubljana (UKCLJ; Ljubljana, Slovenia). The study cohort consisted of women undergoing amniocentesis and a routine examination of karyotype during the second trimester of pregnancy, and that intended to deliver their infants at the UKCLJ. All of the subjects were previously assigned for the amniocentesis due to their own decision or increased risk for Down's syndrome, due to advanced maternal age, increased nuchal translucency and/or inadequate levels of serum Down's syndrome markers (β-hCG, AFP and UE3) or family history of karyotype abnormalities.

A total of 130 women were prospectively recruited into the study between January 2011 and December 2012. The study group consisted of healthy, singleton pregnant women, between 15 and 26 weeks of gestation, with no detectable structural or genetic fetal abnormalities. Urine samples were collected at enrolment and analysed for the selected biomarkers. All women signed written informed consent and completed a questionnaire, which included demographic and biological data, such as gynaecological history, smoking status and maternal body mass and height information. Infant status data was collected at delivery. Apgar scores were evaluated at 1 and 5 min after birth (24), while birth weight and gestation were recorded at delivery. Certain subjects were excluded due to missing data for essential variables, including maternal weight, infant birth weight, birth length, Apgar scores and biomarker concentrations, resulting in a final sample size of 114 women.

The study protocol was approved by the National Medical Ethics Committee of Slovenia (no. 108/09/09).

Sample preparation and quantitative analysis. Urine samples were collected at enrolment. Samples were immediately delivered to the laboratory of the Clinical Institute of Clinical Chemistry and Biochemistry (University Medical Centre Ljubljana, Ljubljana, Slovenia) and centrifuged at 219 x g for 10 min at 4˚C, then divided into 200 μl single-use aliquots and stored at -20˚C prior to analysis.

Biochemical analysis was performed at the laboratory of the School of Human Science and Environment (University of Hyogo, Himeji, Japan). Prior to the analysis, urine samples were defrosted and centrifuged at 877 x g for 10 min at 4˚C to remove all insoluble particles, and the supernatant was collected. The concentration of urinary PRL, HEL, NY and DiY was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), using a multiple reaction monitoring technique with stable-isotope dilution, as previously described (11,16). LC-MS/MS analysis was conducted using an API-3000 electrospray ionisation/quadrupole tandem mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA). HEL, PRL and NY were not detectable in their intact form and were therefore butylated (n-butanol/HCl) prior to the analysis, as previously described (11,16). Chromatography was performed using a Develosil C30-UG-5 (2x150 mm) column for HEL and PRL, an ODS-HG-3 (2x50 mm) column for DiY and an ODS-SR-5 (2x150 mm) for NY (all columns from Nomura Chemical Co., Seto, Japan), using an Agilent 1100 high-performance liquid chromatography system (Agilent Technologies, Inc., Santa Clara, CA, USA).

Urinary concentrations of the biomarkers were normalised against creatinine to account for the variations in urine flow and expressed as μmol/mol creatinine (μmol/mol Cr). Creatinine levels in the urine were evaluated using a Roche/Hitachi 917 automated chemistry analyser (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis. Statistical analysis was conducted using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA)

and MedCalc version 14.12.0 (MedCalc Software, Mariakerke, Belgium) for receiver operating characteristic (ROC) curve. General descriptive statistics were calculated for selected characteristics. Continuous variables are reported as the mean ± standard deviation and dichotomous as counts and percentages. Normal distribution was tested using the Kolmogorov-Smirnov test. All skewed variables are expressed as the median value with the interquartile range.

Creatinine-corrected concentrations (μmol/mol) were calculated by dividing the HEL, PRL, DiY and NY concentrations (μmol/l) by the creatinine concentration. A number of maternal characteristics, such as maternal age, parity, smoking status and prepregnancy body mass index (BMI) were categorised (Table I). Prepregnancy height and weight were used to calculate prepregnancy BMI, which was categorised according to the WHO classification as underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2). Smoking status was defined as active smoker or non-smoker. Parity was defined as nulliparous or parous (second pregnancy or more). Statistical differences between parameters for each characteristic were evaluated using the χ^2 test for dichotomous variables. Continuous variables were analysed using the independent sample *t*-test in the case of Gaussian, and non-parametric Mann-Whitney test in the case of non-Gaussian distribution.

As not all the parameters were normally distributed and certain parameters were ordinal, Spearman's correlation coefficient was calculated to determine the association between the maternal and infant characteristics and OS biomarker concentrations. To examine the association and adjust for confounding variables, odds ratios (OR) and 95% confidence interval (CI) for OS markers and dichotomous Apgar score at 1 and 5 min after birth (≥7 or <7), preterm birth (<37 weeks of gestation) and low birth weight (<2,500 g) were calculated using logistic regression. The results are shown as unadjusted and adjusted for confounding factors.

ROC analysis was performed to determine the overall discriminatory value, sensitivity, specificity and optimal cut-off for Apgar scores and gestation binary outcome. Youden's index was used to determine the optimal cut-off level on the probability scale for distinguishing between women with normal and adverse pregnancy outcomes. Two-tailed P=0.05 was considered to indicate a statistically significant difference.

Table I. Maternal and infant characteristics of the study cohort.

Characteristic	n (%)	Mean ± SD
Maternal age, years		36.70±3.65
<35	19 (16.7)	
≥35	95 (83.3)	
Parity		2.82±1.39
Nulliparous	26 (22.8)	
Parous	88 (77.2)	
Smoking status		
Yes	19 (16.7)	
No	95 (83.3)	
Prepregnancy BMI		23.83±3.86
Underweight	2 (1.8)	18.14±0.21
Normal	76 (66.7)	21.82±1.81
Overweight	28 (24.6)	27.10±1.48
Obese	8 (7.0)	32.81±3.09
Infant gender		
Female	55 (48.2)	
Male	59 (51.8)	
Gestation at delivery, weeks		38.97±2.22
Preterm birth (<37 weeks)	13 (11.4)	34.18±3.04
Term birth	101 (88.6)	39.59±1.05
Birth weight, g		3,308.19±654.82
Low (<2,500 g)	10 (8.7)	1,693.75±596.29
Normal	104 (91.3)	3,431.19±471.25
Apgar1 and Apgar5		8.77±1.00
Low (<7)	6 (5.3)	
Normal (≥7)	108 (94.7)	

Characteristics are reported as percentages for categorical variables and the mean ± SD for continuous variables. Ordinal data (parity, Apgar1 and Apgar5) are presented as the median (range). Underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2). SD, standard deviation; IQR, interquartile range; BMI, body mass index; Apgar1, Apgar score of infant at 1 min; Apgar5, Apgar score of infant at 5 min.

Results

Population characteristics. The overall maternal and infant characteristics are summarised in Table I. The present prospective study included pregnant women between 15 and 26 weeks of gestation. Markers of OS were measured in the final cohort of 114 urine samples. The mean maternal age was 36.7±3.6 years (age range, 22-44 years). A total of 26 subjects (22.8%) were nulliparous, 23 subjects were primiparous (20.2%) and the remainder were multiparous (57%). A total of 19 women (16.7%) were active smokers. Based on the prepregnancy BMI, 28 women (24.6%) were overweight and 8 women (7%) were obese. The mean infant birth weight, birth length and gestation duration were 3,308±655 g (range, 680-4,590 g), 50.54±3.52 cm (range, 32-56 cm) and 38.97±2.22 weeks (range, 27.86-41.43 weeks), respectively. There were 59 male and 54 female infants. The mean Apgar score at 1 min was 8.77±1.0 (range, 3-9) and at 5 min was 8.93±0.05 (range, 6-10).

Urinary biomarkers of OS measured in the overall cohort of mothers in the second trimester of pregnancy are shown in Table II. Only DiY values were normally distributed. A statistically significant difference was detected in PRL levels between the smoker and non-smoker women (P=0.034). The levels of all other biomarkers did not vary based on the smoking status or on the prepregnancy BMI and infant gender.

Association of urinary biomarkers of OS with maternal and infant characteristics. Urinary OS marker levels were correlated with each other and with maternal and infant parameters (Table III). Maternal age, parity and prepregnancy BMI negatively correlated with PRL levels. Neonatal Apgar score at

Table II. Concentrations of oxidative stress markers in the second trimester of pregnancy in the maternal urine (μmol/mol Cr).

Parameter	HEL	PRL	DiY	NY
Total cases	3.18 (2.32-5.19)	26.33 (20.78-33.42)	9.11 (8.17-10.28)	0.61 (0.37-1.12)
Smoking status				
Yes	3.57 (2.84-5.22)	29.87[a] (23.89-37.65)	8.56 (7.96-10.09)	0.79 (0.35-1.26)
No	2.99 (2.23-5.19)	25.52[a] (20.15-32.72)	9.16 (8.17-10.43)	0.59 (0.37-1.10)
Prepregnancy BMI				
Underweight	6.28 (5.61-6.95)	27.19 (23.44-30.93)	11.05 (10.70-11.40)	0.49 (0.43-0.54)
Normal	3.29 (2.21-5.11)	27.02 (21.54-34.25)	9.11 (8.24-10.36)	0.62 (0.40-1.17)
Overweight	2.86 (2.43-5.31)	25.71 (20.04-32.71)	8.89 (7.89-10.08)	0.53 (0.32-0.97)
Obese	2.74 (2.53-2.95)	23.61 (19.71-28.82)	9.16 (7.40-10.05)	0.80 (0.61-1.16)
Infant gender				
Female	3.24 (2.46-5.30)	26.50 (21.53-34.51)	9.07 (7.88-10.77)	0.59 (0.37-1.07)
Male	3.09 (2.19-4.94)	25.52 (20.28-30.92)	9.15 (8.42-10.00)	0.62 (0.37-1.14)

[a]P<0.05, statistically significant difference between nominal groups (Mann-Whitney U-test). Urinary concentrations (μmol/mol Cr) as the median with interquartile range (Q1-Q3). Underweight, <18.5 kg/m^2; normal weight, 18.5-24.9 kg/m^2; overweight, 25-29.9 kg/m^2; obese, ≥30 kg/m^2; HEL, hexanoyl-lysine; PRL, propanoyl-lysine; DiY, dityrosine; NY, 3-nitrotyrosine; BMI, body mass index; Cr, creatinine.

Table III. Correlation between urinary markers of oxidative stress and maternal and infant characteristics.

Characteristic	HEL	PRL	DiY	NY
Maternal age	-0.051 (0.589)	-0.188 (0.046)	-0.119 (0.206)	0.007 (0.938)
Parity	-0.038 (0.688)	-0.200 (0.033)	-0.059 (0.536)	-0.107 (0.259)
Maternal height	-0.052 (0.579)	-0.076 (0.421)	-0.123 (0.192)	0.112 (0.237)
Prepregnancy BMI	-0.117 (0.215)	-0.193 (0.040)	-0.115 (0.221)	-0.059 (0.532)
HEL	1	0.294 (0.002)	0.249 (0.008)	-0.026 (0.783)
PRL	0.294 (0.002)	1	0.113 (0.232)	0.367 (<0.001)
DiY	0.249 (0.008)	0.113 (0.232)	1	-0.105 (0.267)
NY	-0.026 (0.783)	0.367 (<0.001)	-0.105 (0.267)	1
Gestation at delivery	0.002 (0.981)	0.002 (0.986)	-0.145 (0.124)	0.087 (0.358)
Birth weight	-0.123 (0.193)	-0.039 (0.679)	0.079 (0.406)	-0.073 (0.441)
Birth length	-0.137 (0.148)	-0.036 (0.702)	0.055 (0.563)	-0.015 (0.871)
Apgar1	-0.009 (0.928)	-0.234 (0.013)	-0.120 (0.204)	-0.250 (0.008)
Apgar5	-0.002 (0.984)	-0.158 (0.094)	-0.122 (0.197)	-0.244 (0.009)

All data shown as Spearman's coefficient (P-value). P<0.05 indicates statistically significant data. HEL, hexanoyl-lysine; PRL, propanoyl-lysine; DiY, dityrosine; NY, 3-nitrotyrosine; BMI, body mass index; Apgar1, Apgar score of infant at 1 min; Apgar5, Apgar score of infant at 5 min.

1 min after birth was negatively correlated with PRL, while Apgar score at 1 and 5 min was negatively correlated with NY.

The association between OS markers and pregnancy outcome, Apgar score at 1 and 5 min, preterm birth and low birth weight was estimated using logistic regression. A crude and adjusted logistic regression model was used to assess the likelihood of an adverse outcome as a function of OS and covariates (Table IV). The concentration of the markers was treated as a continuous variable, and the simple probabilities of Apgar score of <7, a birth weight of <2,500 g and preterm birth were estimated based on the marker levels. Increasing PRL and NY levels by one unit would on average increase the infant probability of having low Apgar score at 1 and 5 min after birth by 1.098 (95% CI, 1.024-1.178) and 2.084 (95% CI, 1.157-3.853), respectively. The significant association persisted after controlling for confounding factors. For other markers, no statistical significance was observed in the logistic regression models.

Notably, in cases of preterm birth, the statistical results indicated that for each one-unit increase in the level of DiY, there was a reduction in the probability of preterm birth in the unadjusted and adjusted model (OR=0.703; 95% CI, 0.512-0.963); thus, the DiY level exhibited negative association with preterm birth. The distribution of PRL, DiY and NY levels between groups with adverse and normal pregnancy outcomes for significant logistic regression results are presented in Fig. 1.

Table IV. Logistic regression assessing independent association between each urinary marker of oxidative stress and adverse pregnancy outcome.

Marker	Apgar1 or Apgar5 (<7; n=6)	P-value	Birth weight (<2,500 g; n=10)	P-value	Gestation at delivery (<37 weeks; n=13)	P-value
HEL						
Unadjusted	0.984 (0.690-1.402)	0.928	1.105 (0.799-1.530)	0.546	0.835 (0.683-1.021)	0.079
Adjusted	1.014 (0.638-1.611)	0.953	1.351 (0.764-2.388)	0.301	0.881 (0.701-1.107)	0.276
PRL						
Unadjusted	1.098 (1.023-1.178)	0.009[a]	0.984 (0.926-1.047)	0.614	1.018 (0.953-1.087)	0.596
Adjusted	1.113 (1.009-1.227)	0.032[a]	1.021 (0.936-1.114)	0.635	1.043 (0.962-1.132)	0.307
DIY						
Unadjusted	1.257 (0.826-1.911)	0.286	0.990 (0.690-1.422)	0.958	0.703 (0.512-0.963)	0.028[a]
Adjusted	1.623 (0.884-2.977)	0.118	0.903 (0.476-1.713)	0.755	0.632 (0.422-0.948)	0.027[a]
NY						
Unadjusted	2.078 (1.127-3.833)	0.019[a]	0.818 (0.431-1.553)	0.539	1.385 (0.542-3.538)	0.496
Adjusted	4.414 (1.107-17.599)	0.035[a]	1.235 (0.446-3.416)	0.684	1.009 (0.392-2.596)	0.985

[a]P<0.05. Data are shown as odds ratio (95% confidence interval) or as P values (based on logistic regression analysis). Data were adjusted for age (in years; continuous), parity (nulliparous or parous), gestation age at enrolment (in days), smoking status (active smoker or non-smoker), body mass index (kg/m^2; continuous) and infant gender (male or female). For Apgar1 and Apgar5, data were also adjusted for gestation at delivery (in days; continuous) and birth weight (in g; continuous). For birth weight (<2,500 g), data were also adjusted for gestation at delivery (in days; continuous). For gestation at delivery (<37 weeks), data were also adjusted for birth weight (in g; continuous). HEL, hexanoyl-lysine; PRL, propanoyl-lysine; DiY, dityrosine; NY, 3-nitrotyrosine; Apgar1, Apgar score of infant at 1 min; Apgar5, Apgar score of infant at 5 min.

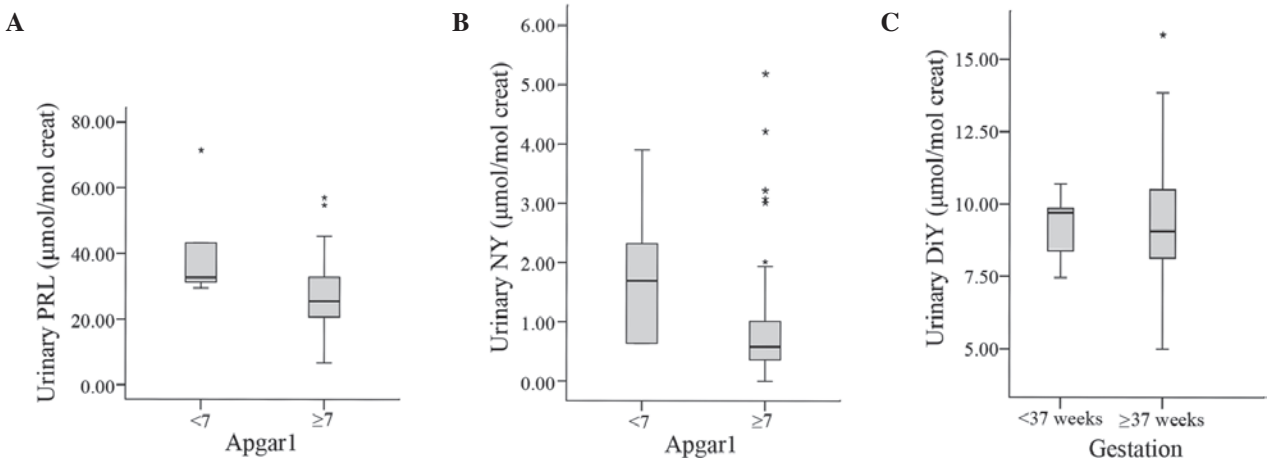

Figure 1. Box plot graphs of (A) PRL and (B) NY levels measured in the urine of mothers who delivered infants with low (<7) or normal (≥7) Apgar score. (C) Box plot graph of DiY measured in urine of mothers who delivered preterm (<37 weeks) and at term. *indicate outliers. Apgar1, Apgar score of infant at 1 min after birth; PRL, propanoyl-lysine; NY, 3-nitrotyrosine; DiY, dityrosine.

The urinary markers significantly associated with Apgar scores at 1 and 5 min after birth were analysed for their ability to predict low Apgar score, and the optimal cut-off urinary PRL and NY concentrations were determined using ROC curve analysis (Fig. 2A). The urine NY concentration had the highest area under the curve (AUC), with a value of 0.818 (95% CI, 0.734-0.884; P=0.0001) for Apgar score at 1 and 5 min after birth. In addition, the predictive cut-off for NY in urine was 0.62 μmol/mol Cr, with a sensitivity of 100% and a specificity of 57%. The positive predictive value (PPV) and negative predictive value (NPV) were 11.54 and 100%, respectively. An appropriate cut-off level for PRL in maternal urine was 29.15 μmol/mol Cr, with a sensitivity of 100% and a specificity of 62.6%, and an AUC of 0.802 (95% CI, 0.717-0.871; P<0.0001) for the prediction of a low Apgar score at 1 and 5 min. The positive predictive value reached 13.05% and the negative predictive value was 100%. Inclusion of additional confounding factors, which were used in logistic regression, did not substantially alter the positive predictive value. The results obtained for NY were as follows: AUC, 0.928; P<0.001; sensitivity, 100%; specificity, 75.5%; and PPV, 20.69. Similarly, the results obtained for PRL were as follows: AUC, 0.899; P=0.001; sensitivity, 100%; specificity, 79.4%; and PPV, 21.40. ROC curve analysis was also performed for

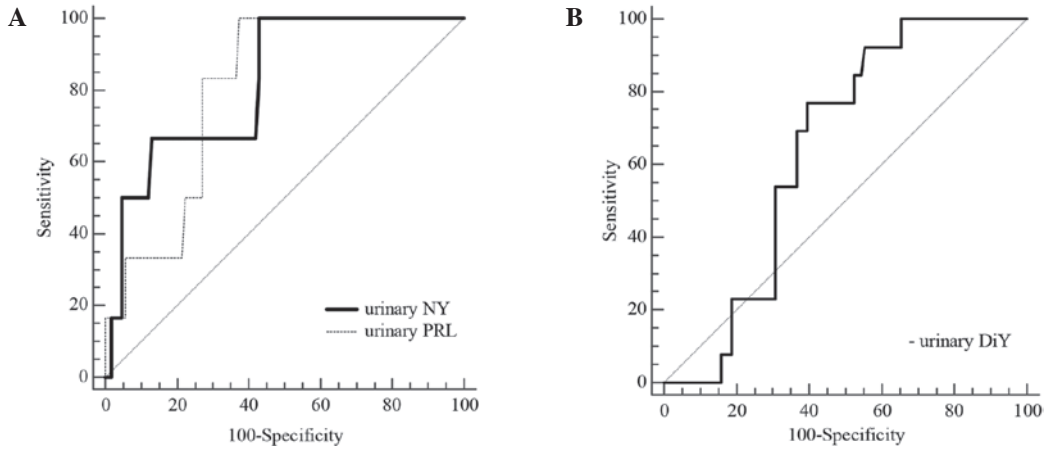

Figure 2. (A) Receiver operating characteristic curves of urinary PRL and NY in the prediction of low Apgar score of infant at 1 and 5 min after birth. (B) Receiver operating characteristic curves of urinary DiY in differentiating between term and preterm birth. Sensitivity and specificity are reported as percentages (%). PRL, propanoyl-lysine, NY, 3-nitrotyrosine; DiY, dityrosine.

the DiY level in order to evaluate its ability to identify pregnant women with a reduced risk of preterm birth (Fig. 2B). The AUC was 0.645 (95% CI, 0.550-0.732; P=0.010), the observed cut-off value was 9.33 μmol/mol Cr, the diagnostic specificity was 60.40% and the sensitivity was 76.92%. Furthermore, positive and negative predictive values reached 20 and 95.31%, respectively.

Discussion

To the best of our knowledge, the present study is the first to investigate biomarkers of the initial stages of lipid peroxidation (HEL and PRL) and of tyrosine residue oxidation (DiY and NY) in the urine of pregnant women.

HEL, PRL, DiY and NY are relatively novel biomarkers for OS monitoring, and their concentration in the general pregnant population has not been determined yet. Although 83.3% of the women included in the present study were ≥35 years old, the data may provide initial indicative values.

The levels of the measured concentrations of the markers correspond to ranges identified in previously published data, which were measured on the different groups of healthy, non-pregnant subjects (11,12,16,25,26). The concentrations of the markers vary between studies. These studies in healthy non-pregnant women reported that the urinary HEL levels were between 1.58±0.23 and 2.3±1.2 μmol/mol Cr (11,12,16), PRL levels were 21.6±10.6 μmol/mol Cr (11), DiY levels were between 8.8±0.6 and 10.1±0.4 μmol/mol Cr (16,25) and NY levels were between 0.46±0.49 and 1.4±0.4 μmol/mol Cr (16,26).

Oxidative damage may be triggered by internal and external factors. Potential confounders in the present study include factors that alter the oxidative environment and thus influence birth outcomes. Therefore, we assessed maternal characteristics and environmental risk factors.

The influence of active maternal perinatal smoking as a confounding factor on pregnancy development and outcome has been well-acknowledged. Smoking is associated with increased risk of perinatal mortality and congenital abnormalities, preterm birth and low birth weight (27-30). Tobacco smoke contains a number of oxidising species capable of producing ROS that may be associated with OS damage (31,32). Although a previous study involving non-pregnant subjects did not confirm the correlation of HEL and PRL concentrations with smoking (11), in the present study cohort of pregnant women, PRL concentrations were significantly higher in the subgroup of smokers compared with those in non-smokers. In addition, studies have also investigated the association of NY and DiY levels with smoking status in healthy subjects (32-34). Urinary levels of DiY did not differ between smokers and non-smokers, while plasma NY studies have presented conflicting results (32-34). Increased HEL concentration was detected in the tears of subject following passive exposure to cigarette smoke in healthy non-smokers compared with non-exposed subjects (35). No statistically significant association of HEL, NY and DiY levels with smoking status was identified in the present study. However, smoking status was included in the adjusted model of logistic regression for assessing biomarkers for adverse pregnancy outcome.

Increased levels of oxidative damage were expected to be associated with ageing and maternal weight, on the basis of previously reported associations (34-39). However, no correlation was detected between maternal age and the levels of HEL, DiY and NY. Notably, PRL showed a weak negative correlation with age (rho=-0.188; P=0.046). The absence of correlation of HEL and PRL levels with age has been noted previously in a healthy non-pregnant population (11). A similar difference between age groups applies to NY. The present results are consistent with the findings of the Framingham Heart Study, in which urinary concentrations of the oxidative marker 8-isoprostane were found to decrease with age, suggesting that LP is not a significant feature of normal ageing (37). Due to the selection of study cohort in the present study, the cohort age represented a relative narrow range and this may limit the application of the present results to a wider population. However, these results represent the effect of age on the markers' behaviour in the present study.

Subjects that are obese and overweight may present an increased risk of pregnancy complications. According to previous results, obesity is a state of chronic increased OS and inflammation (37); therefore, we expected to observe a positive correlation and an increased LP in the overweight and obese group. However, the present results were contrary to our

expectations, as no correlation was detected between prepregnancy BMI and HEL, DiY and NY levels. A weak negative correlation was observed between BMI and PRL (rho=-0.193; P=0.040). The linkage between increased extent of adipose tissue and decreased OS in pregnancy may be due to the hormone estradiol, which is generated and secreted by the placenta. In obese patients, estradiol is secreted by adipocytes. Estradiol has been demonstrated to exhibit an antioxidative activity, and may modulate OS by inhibiting the generation of ROS and scavenging ROS (40). The present results are consistent with this possibility.

In the case of individual markers that were correlated with each other, a positive correlation was observed between HEL and PRL, as well as between HEL and DiY, which is consistent with the findings of previous studies (11,16). However, no correlation was detected between PRL and DiY, which is inconsistent with previous results (11). The absence of a correlation between NY and the other markers has been noted previously in a healthy non-pregnant population (16); however, we detected the most marked positive correlation between PRL and NY (rho=0.367; P<0.001). Positive correlations between the selected biomarkers suggest that OS is a complex process, involving simultaneous oxidation pathways of various macromolecules. However, the biochemical pathway of the *in vivo* generation of these markers is different, and each marker can provide independent information. It is difficult to compare and simplify the results to general systemic stress, as different markers exhibit differing stability, accumulation, susceptibility to metabolism and excretion in the urine. Furthermore, this investigation of correlation serves only as an indicator of a plausible association between OS markers and does not conclusively demonstrate the existence of a causal association. Further studies are required to fully elucidate the mechanism underlying this dependence.

Urinary OS profile in the second trimester of pregnancy may be used to assess fetal exposure and probability for infant outcomes at birth. The most notable observation of the present study is that low Apgar scores at 1 and 5 min after birth are associated with high levels of PRL and NY. Apgar score is a strong routine indicator of infant survival, and is based on the evaluation of the physical condition of infants immediately after birth, including estimation of heart rate, respiratory effort, muscle tone, reflex irritability and skin colour (41). In the present study a significant negative linear correlation of urinary levels of PRL and NY with Apgar score was identified among pregnant women.

The majority of the mothers with the highest concentrations of PRL and NY in the second trimester of pregnancy gave birth to infants with a low Apgar score; however, no differences in birth weight, birth length and gestational age were observed at delivery.

Adjusted logistic regression analysis of birth outcomes revealed that high urinary PRL and NY concentrations in the second trimester of pregnancy increased the probability (OR) of a low Apgar score. By contrast, the other markers did not increase the risk of an adverse pregnancy outcome. Following adjustment of the logistic regression model, increased levels of DiY were associated with a reduced risk of preterm birth.

ROC analysis was performed on the OS markers that were significantly associated with low Apgar and preterm birth in logistic regression in order to detect possible predictors of pregnancy outcome that may be used to identify pregnancies at high risk of complications. In addition, markers for low risk estimation are valuable for avoiding unnecessary intensive monitoring and interventions.

The AUC values obtained for PRL, NY and DiY were moderate but statistically significant. The cut-off values for PRL and NY were evaluated to predict low Apgar score at 1 and 5 min after birth and the optimal value of DiY to assess probability for the normal duration of pregnancy. Despite significant AUC and high sensibility, the specificity and positive predictive value of the markers were low and insufficient for a reliable diagnostic marker. Inclusion of additional confounding factors used in logistic regression did not improve the results. The ROC curve of NY and PRL demonstrated poor positive predictive accuracy for a low Apgar score, suggesting that screening for enhanced levels of NY and PRL in the second trimester of pregnancy would be a poor approach for identifying women at risk. The presence of increased NY and PRL levels in the urine should not be interpreted as indicating an increased risk for an adverse outcome; however, levels under the cut-off value are able to indicate the absence of a low Apgar score. The negative predictive values for NY and PRL markers were 100%. The high negative predictive value reflects test performance and the low overall prevalence of low Apgar score in the study population. Unless a low Apgar score is relatively frequent, there is no substantial benefit in having a high negative predictive value. Furthermore, NY and PRL screening may represent a useful tool for excluding the risk of a poor Apgar score.

The reason why a high positive predictive value was not observed may be due to the infrequency of a low Apgar score, in addition to the heterogeneous and multifactorial causes of adverse pregnancy outcomes. The results of the present pilot study cannot be applied to laboratory and clinical practice; however, certain indicative values were provided. Individual positive predictive values for NY and PRL are too low, but in combination with other biochemical markers and maternal parameters they may provide improved predictive efficacy. Further studies for these markers and widespread screening for high levels of PRL and NY are required.

There are a number of limitations associated with the present study. The first limitation is that the study group consisted primarily of pregnant women with an elevated Down's syndrome risk, due to the increased nuchal translucency, triple test measurements and/or higher maternal age. Therefore, the possibility of a certain population selection bias cannot be excluded. Secondly, valid statistical analyses are difficult to perform on a small number of study subjects. Small data sets may possess restricted statistical accuracy and produce primarily exploratory results. The present study was a part of a larger study aimed at investigating the markers of OS in maternal urine, blood and amniotic fluid and was considered to be prospective. Therefore, the incidence of adverse pregnancy outcome in the present population was relatively low. Furthermore, the low number of cases with a low Apgar score has an effect on the accuracy of the statistical analysis performed in the study and may negatively affect the accuracy of a statistical significant association observed between PRL, NY and Apgar score. Thus, these findings cannot be generalized to a broader population.

OS is a complex process and it may be difficult to determine whether OS is a cause of complications or a consequence of internal or external interferences. Although the present study controlled for the associations with a range of potential confounders, a possibility that the observed association of OS is due to unmeasured or residual confounding factors remained. Additional confounding variables that may cause the alternation of oxidative status, such as nutritional habits, antioxidant and vitamin supplementation and genetic factors, should be included in future investigations. Similarly, food-derived substances may be a source of markers present in urine. However, these markers still have a potential value. Furthermore, the present exploratory study is unique and has generated a number of potential biomarker candidates for future investigation.

In conclusion, the present results indicated that high levels of urinary PRL and NY may be associated with low Apgar scores, while high DiY levels are associated with a reduced risk of preterm birth. Cigarette smoking was confirmed to be a confounding factor for the urinary excretion of PRL and should be factored into the investigation of PRL. This study identified statistically significant correlations among urinary markers and detected markers with a high negative predictive value. Further studies involving larger cohorts are required to elucidate the mechanism underlying the association of PRL, NY and DiY with pregnancy outcome.

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