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First trimester concentrations of the TTR-RBP4-retinol complex components as early markers of insulin-treated gestational diabetes mellitus

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Abstract

Background: The objective of the study was to investigate the relationship between first trimester maternal serum levels of the TTR-RBP4-ROH complex components and the later insurgence of an altered glucose metabolism during pregnancy.

Methods: Retrospective case control study including 96 patients between the 12th and 14th week of gestation, 32 that developed gestational diabetes mellitus (GDM), respectively, 21 non-insulin-treated (dGDM) and 11 insulin-treated (iGDM), 20 large for gestational age fetuses (LGA) without GDM and 44 patients with normal outcome as control. Serum concentrations of RBP4 and TTR were assessed by ELISA; serum concentration of ROH by reverse-phase high performance liquid chromatography (rpHPLC). The molecular heterogeneity of TTR and RBP4 was analyzed after immunoprecipitation by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Results: iGDM patients were characterized by reduced TTR, RBP4 and ROH compared to controls (respectively, iGDM vs. controls, mean±SD: TTR 3.96±0.89 μmol/L vs. 4.68±1.21 μmol/L, RBP4 1.13±0.25 μmol/L vs. 1.33±0.38 μmol/L and ROH 1.33±0.17 μmol/L vs. 1.62±0.29 μmol/L, $p<0.05$). TTR containing Gly10 in place of Cys10 was lower in the iGDM group ($p<0.05$) compared to controls. In the final logistic regression model ROH significantly predicted the diagnosis of iGDM (OR 0.93, 95% CI 0.87–0.98, $p<0.05$).

Conclusions: First trimester maternal serum ROH, RBP4 and TTR represent potential biomarkers associated with the development of iGDM.

Keywords: first trimester; gestational diabetes mellitus (GDM); insulin; large for gestational age fetus (LGA); protein microheterogeneity; retinol (ROH); serum retinol binding protein (RBP4); transthyretin (TTR).

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Introduction

Gestational diabetes mellitus (GDM) is a pregnancy-related disease with an expected incidence of approximately 16%–18% [1]. It accounts for a relevant part of obstetrics and neonatal pathologies (e.g., fetal macrosomia, shoulder dystocia, lesions at birth, as well as neonatal hyperinsulinemia and hypoglycemia) [2], even though the pathophysiology and underlying mechanisms are yet only poorly characterized, especially in the first trimester of pregnancy [3].

Transthyretin (TTR) is a 55-kDa homotetrameric protein mainly synthesized by the liver, the choroid plexus and the syncytiotrophoblast of human placenta. In plasma, it acts as one of the carrier proteins for thyroxine (T4), along with thyroxine-binding protein (TBG) and serum albumin, even if TTR seems to play a marginal role in the T4 plasma transport [4]. Interestingly, a significant amount of TTR present in the circulation is involved in the transport of retinol (ROH) by forming a macromolecular complex with

holo retinol-binding protein 4 (RBP4, 21 kDa), mainly in a 1:1 molar ratio (molecular mass of the complex of approx. 76 kDa) [5]. The ROH molecule is known to be essential for many physiological processes, including vision, immune function, reproduction and normal embryonic and fetal development [6]. Due to the high molecular mass of the TTR-RBP4 complex, TTR binding to RBP4 prevents the glomerular filtration of the low molecular weight RBP4-ROH complex and its subsequent loss with urine [7]. RBP4 has also been indicated as a new adipokine with influence on glucose metabolism and the development of insulin resistance. Elevated concentrations of serum RBP4 are accompanied by impaired glucose uptake into skeletal muscle and increased glucose production by the liver, whereas lowered serum RBP4 concentrations greatly enhanced insulin sensitivity [8]. Nonetheless, association of serum RBP4 with insulin resistance, as well as with GDM remains debated [9, 10]. Several variants of TTR and RBP4 have been described, mainly due to post-translational modifications, but little is known about the biochemical consequence of these modifications [11, 12].

Recent studies indicate a possible role of first trimester RBP4 concentration on the later development of GDM and other pregnancy-related complications, particularly fetal growth anomalies, even though reports are yet conflicting [13, 14]. Furthermore, the relationship with its carrier protein TTR and with its transported micronutrient ROH has not been investigated yet during pregnancy. The aim of the present study was to evaluate the concentration of the components of the TTR-RBP4-retinol complex in maternal blood circulation at 11–13 weeks of gestation in women that would later develop GDM during pregnancy. Microheterogeneities of TTR and RBP4 molecular variants were also investigated.

Materials and methods

Study design and setting

This was a case-control study on maternal serum probes collected during the period between 2009 and 2011 at the University of Udine, Italy. Patients who underwent to the first trimester screening combined with the additional maternal blood examination for commercial markers of chromosomal abnormalities [pregnancy-associated plasma protein-A (PAPP-A) and free β-subunit of human chorionic gonadotropin (free β-HCG)] were considered [15]. Non-fasting serum samples were obtained during the gestational period between 11 and 13 weeks and 6 days, in occasion of the first trimester ultrasound and biochemical screening for chromosomal abnormalities. Gestational age was determined according to the last menstruation or corrected by embryonic crown-rump length (CRL) if a discrepancy >1 week with the calculated gestational age was measured. All samples were collected in dry containers and kept at 4 °C for a maximum of 24 h until samples were stored at −80 °C. An informed consent was signed before the screening program was provided and the blood sample collected for screening purposes was stored. This retrospective study was conducted in accordance with the Declaration of Helsinki and it followed the dictates of the general authorization to process personal data for scientific research purposes by the Italian Data Protection Authority. Internal Review Board approval was obtained.

Study population

A total of 32 singleton pregnant women who developed GDM were selected, including 11 with GDM requiring insulin treatment during pregnancy (iGDM) and 21 that developed gestational diabetes mellitus not requiring insulin treatment during pregnancy (dGDM). Cases were compared with 44 pregnancies having a normal outcome, characterized by an adequate for gestational age fetus (AGA) and 20 pregnancies characterized by a large for gestational age fetus (LGA). All collected samples of GDM and LGA were included, while controls were selected at random, matched with respect to gestational age of sampling.

In 2010 a new screening model according to IADPSG guidelines was introduced [16] and women who delivered in 2010 and 2011 were screened by this method. A first trimester fasting glucose ≥5.1 mmol/L and <7.0 mmol/L was considered in order to give a diagnosis of GDM (values ≥7.0 mmol/L were considered as indicative for a preexistent diabetes mellitus). If the first trimester fasting glucose was <5.1 mmol/L a 2-h oral glucose tolerance test (75 g of glucose) was performed between the 24th and 28th gestational week and GDM diagnosis was made if at least one of the measured values was over the following thresholds: fasting ≥5.1 mmol/L, 1 h ≥10.0 mmol/L, or 2 h ≥8.5 mmol/L.

Placental index (defined as the ratio between the fetal weight and the placental weight) and ethnicity (obtained stratifying the population by macro-regions and cultural backgrounds) were considered as previously described [17]. Patients were stratified according to their body mass index (BMI) in underweight (<18.5 kg/m^2), normal (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obese (>30 kg/m^2) as previously described [18].

Analytical determination of ROH, RBP4 and TTR

Concentrations of serum ROH were measured using a gradient reverse phase high-performance liquid chromatography (rpHPLC) system (Shimadzu Europe, Duisburg, Germany) after organic extraction [19]. For separation of the compounds, a reversed-phase C18 column (ReproSil 70; 5 µm, 200×3 mm; Dr. Maisch GmbH; Ammerbuch, Germany) was applied. ROH was quantified by measuring the absorption at 325 nm using an external standard purchased from Sigma (Deisenhofen, Germany). The detection limit for ROH was 2.0 ng. Coefficient of variation (CV) over time using control plasma was <4% for ROH. Concentrations of RBP4 and TTR were measured by non-commercial enzyme-linked immunosorbent assay using polyclonal rabbit anti-human antibodies (Biozol, Eching, Germany) as previously described [19]. Inter-assay CVs were 4.2% and 8.1% for RBP4 and TTR, respectively.

Analysis of molecular variants of RBP4 and TTR by mass spectrometry

For immunoprecipitation of RBP4 bound to TTR, 10 µL of serum was mixed with 10 µL Sephadex G15 (3 mg/mL in HPLC-grade water) and 5 µL of polyclonal rabbit anti-human RBP4 (Biozol) at 4 °C for 2 h. After centrifugation at 16,000×*g* for 1200 s, the supernatant was removed and the protein-antibody complex was extensively washed twice with phosphate-buffered saline (pH 7.4) and once with HPLC-grade water. After a final centrifugation, the pellet was resuspended in 10 µL HPLC-grade water. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) was performed as previously described using 2,5-dihydroxyacetophenone as matrix (100 mmol/L in 75% HPLC-grade ethanol/20 mmol/L diammonium-hydrogencitrate) [20]. The peaks obtained from MALDI-TOF mass spectra for TTR and its variants, in which Cys 10 was chemically modified, were assigned according their molecular weights as unmodified TTR (13,762 Da), S-sulfonated TTR (13,842 Da), S-cysteinylated TTR (13,881 Da), S-cysteinylglycinated TTR (13,938 Da), S-glutathionylated TTR (14,064 Da) and TTR glycine (13,719 Da). The molecular variants of RBP4 were assigned as unmodified (non-truncated) RBP4 (21,065 Da), RBP4 truncated at the C-terminus by one leucine residue (RBP-L, 20,950 Da) and RBP4 truncated at the C-terminus by two leucine residues (RBP4-LL, 20,837 Da). As the ionization efficiencies of all TTR and RBP4 variants were similar, the relative amount of TTR and RBP4 variants were determined as a percentage of unmodified TTR and unmodified RBP4, respectively.

Statistical analysis

The statistical analysis was performed using R software (version 3.0.1). It was considered significant at a p-value <0.05. The normality of data distribution was assessed with a Kolmogorov-Smirnoff test. Data were presented as mean (±standard deviation) if they were normally distributed, median and interquartile range (IQR) if they were not normally distributed, or reference values and 95% confidence interval (95% CI). The following statistical tests were also used: t-tests, Wilcoxon test, one-way ANOVA, Kruskal-Wallis test, Spearman's test, Pearson's correlation coefficient, or linear regression where appropriate in case of continuous variables. In case of categorical variables, χ^2 and Fisher exact test were applied. In addition, multivariate analysis was also performed using logistic regression. In the logistic regression analysis the diagnosis of iGDM was considered as dependent variable and all studied risk factors (maternal age, pre-pregnancy BMI, tobacco smoke, macro-region of origin, familial history of diabetes, mode of conception, parity, free-β-HCG MoM, PAPP-A MoM, and all components of the TTR-RBP4-retinol complex with and without correction for fetal CRL at the time of sampling) as independent predictors. We also analyzed the prediction accuracy of the studied factors using receiver operator characteristic (ROC) curves and area under the ROC curve (AUC) with the related 95% CI.

Results

In Table 1 we present the characteristics of our population subdivided by the studied groups. We found significant differences in maternal age, macro-region of origin, familial history of diabetes mellitus, parity, neonatal, and placental weight. Figure 1 shows that ROH, RBP4, and TTR were decreasing with the increasing of fetal CRL at the time of sampling and in particular the inverse correlation between ROH and fetal CRL was statistically significant ($r=-0.205$, $p<0.05$) (Figure 1). In addition, we found significant direct correlations between TTR and ROH or RBP4 ($r=0.462$ and $r=0.369$, $p<0.05$). We found also a significant correlation between ROH and RBP4 ($r=0.629$, $p<0.05$).

In Table 2, we show the differences of the TTR-RBP4-retinol complex components among the studied groups. We found significant lower values of ROH in iGDM than AGA and dGDM ($p<0.05$) and a non-significant lower value in iGDM than LGA ($p=0.239$). Moreover, we found a significant lower value of RBP4 in iGDM than AGA ($p<0.05$). In addition, we found a significant lower value of TTR in iGDM or LGA than AGA ($p<0.05$). In particular, TTR Gly 10 was significantly lower in iGDM than AGA ($p<0.05$).

We also performed a logistic regression analysis considering all studied risk factors to find the most predictive indicator for iGDM. In Table 3 we present the final multivariate logistic regression model. We found parity, familial history of diabetes mellitus, and log(ROH) corrected for CRL, the most predictive factors to forecast a diagnosis of iGDM (all included cases of iGDM were regularly screened and the diagnosis was made after 24th weeks' gestation). In particular, the AUC of log(ROH) to predict iGDM was 72.41% (95% CI 55.53%–89.28%) and the AUC of the most predictive multivariate logistic regression model was 87.17% (95% CI 76.07%–98.27%). In addition, after exclusion from the multivariate model of the log(ROH) the AUC result was 76.95% (95% CI 60.15%–93.75%). After multivariate adjustment for maternal age, parity, pre-pregnancy BMI, and CRL the OR for log(ROH) resulted 0.93 (95% CI 0.87–0.99) ($p<0.05$). Furthermore, none of the considered factors of TTR-RBP4-retinol complex in the multivariate logistic regression analysis was able to significantly predict dGDM or LGA.

Discussion

This is the first study considering ROH and the other components of the TTR-RBP4-ROH complex as an early markers of GDM in maternal serum in the first trimester of pregnancy. In this study we found that the components of the TTR-RBP4-ROH complex in plasma are significantly lower in women that will develop iGDM. Nonetheless, in the multivariate analysis, only a reduced plasma ROH

Table 1: Characteristics of the study population.

	AGA (n=44)	iGDM (n=11)	dGDM (n=21)	LGA (n=20)	p-Value
Maternal age, years	37.18 (±4.44)	33.55 (±4.06)	33.43 (±4.03)	32.85 (±3.47)	(1,2,3)
≤35 years	8 (18)	8 (73)	16 (76)	15 (75)	(1,2,3)
>35 years	36 (82)	3 (27)	5 (24)	5 (25)	(1,2,3)
Gestational age at sampling, weeks	11.7 (±0.55)	12.09 (±0.83)	11.95 (±0.67)	11.75 (±0.55)	NS
CRL, mm	59.11 (±6.45)	61.52 (±9.69)	62.08 (±7.68)	57.98 (±6.11)	NS
Pre-pregnancy BMI, kg/m^2	22.4 (20.7–24.6)	24.1 (22.4–25.9)	24.1 (22.4–26.1)	24.2 (21.1–27.8)	NS
<18.5 kg/m^2	4 (9)	1 (9)	0 (0)	1 (5)	NS
18.5–24.9 kg/m^2	31 (70)	7 (64)	11 (52)	12 (60)	NS
25–29.9 kg/m^2	7 (16)	2 (18)	9 (43)	5 (25)	(2)
>30 kg/m^2	2/44 (5)	1 (9)	1 (5)	2 (10)	NS
Academic degree	11 (25)	2 (18)	3 (14)	8 (40)	NS
Tobacco smoke	3 (7)	0 (0)	2 (10)	0 (0)	NS
Macro-region of origin					
West Europe	39 (89)	9 (82)	17 (81)	13 (65)	(3)
East Europe	3 (7)	0 (0)	2 (10)	5 (25)	(3[a])
Sub-Saharan Africa	2 (5)	1 (9)	0 (0)	1 (5)	NS
Other	0 (0)	1 (9)	2 (10)	1 (5)	NS
Familial history of diabetes	10 (23)	7 (64)	4 (19)	2 (10)	(1,4,5)
Mode of conception					
Spontaneous	43 (98)	11 (100)	21 (100)	20 (100)	NS
IVF/ICSI	1 (2)	0 (0)	0 (0)	0 (0)	NS
Mode of delivery					
Vaginal	35 (80)	8 (73)	13 (62)	13 (65)	NS
Cesarean section	9 (20)	3 (27)	8 (38)	7 (35)	NS
Nulliparous women	20 (45)	2 (18)	11 (52)	4 (20)	(3,6)
Gestational age at delivery, weeks	38.70 (±1.30)	38.00 (±1.00)	38.05 (±1.83)	38.8 (±0.95)	NS
Neonatal weight, g	3297.00 (±278.76)	3352.73 (±432.35)	3200.95 (±489.05)	3976.70 (±222.23)	(3,5,6)
Placental weight, g	599.35 (±108.21)	640.64 (±177.97)	588.57 (±114.47)	739.15 (±109.56)	(3,6)
Placental index	0.18 (±0.03)	0.19 (±0.04)	0.18 (±0.03)	0.19 (±0.03)	NS
Apgar score 1st min	8.3 (±1.27)	7.73 (±1.49)	8.05 (±1.20)	8.3 (±0.80)	NS
Apgar score 5th min	8.89 (±0.49)	8.64 (±0.67)	8.86 (±0.65)	9.00 (±0)	NS
Free-β-HCG MoM	0.9 (0.6–1.7)	0.7 (0.6–1.1)	0.8 (0.6–1)	1.0 (0.8–1.7)	(6)
PAPP-A MoM	0.8 (0.6–1.2)	0.6 (0.5–0.9)	0.9 (0.6–1.4)	0.8 (0.6–1.4)	NS

Differences statistically significant ($p<0.05$) between: (1) AGA and iGDM; (2) AGA and dGDM; (3) AGA and LGA; (4) iGDM and dGDM; (5) iGDM and LGA; (6) dGDM and LGA. Other differences: [a]$p=0.096$. AGA, adequate for gestational age; β-HCG, beta subunit of human chorionic gonadotropin; BMI, body mass index; dGDM, diet-treated gestational diabetes mellitus; iGDM, insulin-treated gestational diabetes mellitus; IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection; LGA, large for gestational age; NS, non-significant differences; PAPP-A, Pregnancy-Associated Plasma Protein-A. Data are reported as absolute values (percentage), median (IQR) or mean (±standard deviation) and p-values refer to t-test, χ^2 test, or Fisher's exact test.

concentration was useful to predict the development of iGDM in later pregnancy. Furthermore, among iGDM patients mass spectrometry showed a significant reduced amount of TTR-Gly 10 compared to the other molecular TTR variants.

Although randomly selected, the patients in the control group (AGA) were found to be older than the rest of the individuals in the study population. Nonetheless, a multivariate analysis of our data shows that this age factor has no influence on the conclusions of our study. We further partitioned patients into BMI categories. GDM group differs from controls with respect to BMI only in the category of overweight women. When we consider the whole BMI, the difference was no longer statistically significant, probably because of the small size of our population and/or the relative low prevalence of obese women that were included in our setting.

To date there is no reliable screening marker with GDM, available during the first trimester of pregnancy, to predict the development of GDM in later pregnancy. In our study patients that would develop GDM were compared to normal pregnancies, but also to pregnancies complicated by LGA fetuses. LGA complicated pregnancies were included in order to test whether RBP4 represents a

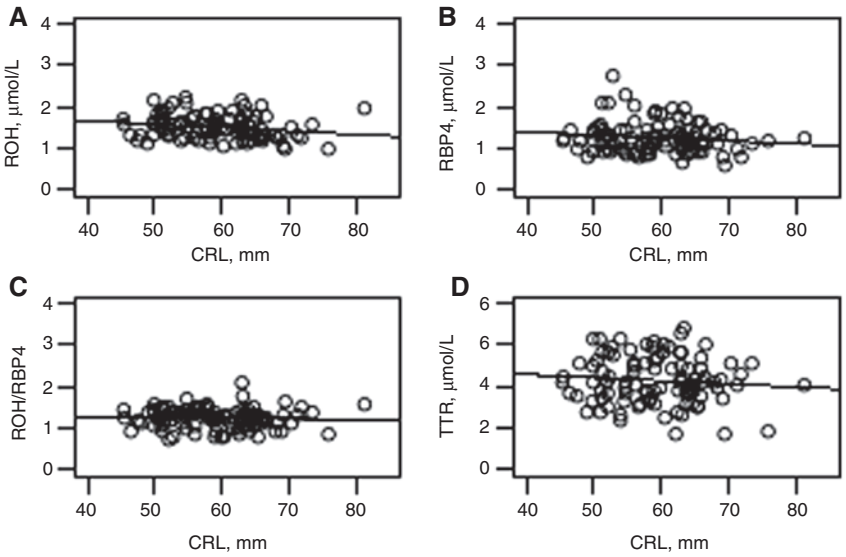

Figure 1: Correlations of studied analytes with CRL at the time of blood sampling.
(A) plot of linear regression between ROH and CRL ($p<0.05$); (B) linear regression between RBP4 and CRL ($p=0.191$); (C) linear regression between ROH/RBP4 index and CRL ($p=0.809$); (D) linear regression between TTR and CRL ($p=0.366$). The r-values in the plots refers to Pearson's correlation coefficient. CRP, crown-rump length; RBP4, retinol binding protein; ROH, retinol; TTR, transthyretin.

possible marker for LGA fetuses, particularly if not related to GDM. Indeed, even if LGA fetuses are a common complication of GDM, particularly if not in good glycemic control, some of them cannot be explained by GDM. Literature concerning a possible relationship between RBP4 and the development of LGA fetuses is scarce and conflicting [21, 22]. A recent study conducted on maternal serum at 11–13 weeks show no correlation between RBP4 and development of LGA [14] and our results confirm these first observations. Conversely, the correlation between RBP4 and the

Table 2: First trimester maternal blood concentration of RBP4, ROH, ROH/RBP4 ratio, TTR, RBP4/TTR ratio, RBP4 variants, and TTR variants values among the studied groups.

	AGA (n=44)	iGDM (n=11)	dGDM (n=21)	LGA (n=20)	p-Value
RBP4, µmol/L	1.33 (±0.38)	1.13 (±0.25)	1.20 (±0.34)	1.27 (±0.42)	(1)
ROH, µmol/L	1.62 (±0.29)	1.33 (±0.17)	1.48 (±0.26)	1.42 (±0.29)	(1,2[a],3,4)
ROH/RBP4 ratio	1.26 (±0.24)	1.21 (±0.24)	1.29 (±0.25)	1.19 (±0.27)	NS
RBP4/TTR ratio	0.27 (0.24–0.31)	0.29 (0.23–0.33)	0.27 (0.23–0.31)	0.32 (0.27–0.47)	(3,6)
TTR, µmol/L	4.68 (±1.21)	3.96 (±0.89)	4.29 (±1.03)	3.62 (±0.96)	(1,3,6)
RBP4 molecular variants[b]					
RBP4-LL	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	NS
RBP4-L	9.5 (6.8–16)	7.0 (4.0–14.0)	7.0 (4.0–13.0)	11.5 (6.8–14.2)	NS
TTR molecular variants[b]					
TTR glycine	17.0 (13.0–21.5)	13 (9.5–15.5)	14.5 (10.8–17.2)	14.0 (12.0–23.0)	(1)
S-sulfonated TTR	44.0 (34.0–66.5)	50.0 (38.5–57.0)	48.5 (39.5–81.0)	51.5 (37.8–91.2)	NS
S-cysteinylated TTR	130.5 (104.5–169.2)	118.0 (107.0–148.5)	137.5 (114.8–232.0)	156.0 (110.2–213.5)	NS
S-cysteinylglycinated TTR	44.5 (31.8–57.5)	46.0 (30.0–57.5)	41.5 (35.8–66.5)	45.0 (30.5–68.2)	NS
S-glutathionylated TTR	15.0 (9.8–19.0)	11.0 (9.0–15.5)	11.0 (9.0–16.2)	13.5 (9.0–20.8)	NS

Differences statistically significant ($p<0.05$) between: (1) AGA and iGDM; (2) AGA and dGDM; (3) AGA and LGA; (4) iGDM and dGDM; (5) iGDM and LGA; (6) dGDM and LGA. Other differences: [a]$p=0.074$; [b]Molecular variants of RBP4 or TTR expressed as percentage of unmodified RBP4 or unmodified TTR. AGA, adequate for gestational age; dGDM, diet-treated gestational diabetes mellitus; iGDM, insulin-treated gestational diabetes mellitus; LGA, large for gestational age; NS, non-significant differences; RBP4, retinol binding protein; RBP4-L and RBP4-LL, post-translational modifications resulting from the truncation of RBP4 at the C-terminus by one or two leucine residues; ROH, retinol; TTR, transthyretin; TTR molecular variants, post-translational modifications of the cysteine residue in position 10 (Cys10) in TTR. Data are reported as mean (±standard deviation) or median (interquartile range) and p-values refer respectively to t-test or Wilcoxon's test.

Table 3: Final multivariate logistic regression model to predict iGDM diagnosis (dependent variable, iGDM).

Dependent variable iGDM	OR (CI 95%)	p-Value
Nulliparity	0.22 (0.04–1.34)	0.101
Familial history of diabetes mellitus	18.02 (3.24–100.11)	<0.05
Log(ROH)[a]	0.93 (0.87–0.98)	<0.05

iGDM, insulin-treated gestational diabetes mellitus; log(ROH), logarithm of retinol concentration; OR, odds ratio. [a]Corrected for CRL value.

pathogenesis of GDM has been already debated in the past, showing conflicting results. In some studies a positive correlation between RBP4 and GDM was shown [23, 24], while in some other reports it was not [25, 26]. The only study conducted in first trimester maternal serum, already previously cited, showed no statistically significant correlation between RBP4 and GDM [14]. As stated above, we decided to differentiate in our study also between iGDM and dGDM and we found a significantly reduced concentration of RBP4 in the iGDM group compared to the control group. Nonetheless, the difference was not any more statistically significant in the multivariate logistic regression analysis and for this reason RBP4 was not included in the final model of the most predictive factors of iGDM. This could be due to the small number of patients included in the group that reduced the statistical power of the study.

We also tested the possible correlation between TTR and the insurgence of GDM later in pregnancy. TTR forms a ternary complex with RBP4-ROH and TTR plays a central role in the blood transport of both the RBP4-ROH complex, as well as the hormone thyroxine. Nonetheless, the mechanisms of passage of TTR complexed with such ligands to the human fetus through the placenta are mostly not elucidated yet. Interestingly, we found significantly reduced concentrations of TTR in iGDM, but also in patients developing LGA fetuses compared to controls and dGDM. However, differences were not any more significant in the multivariate logistic regression analysis. In the past no study investigated the relationship between TTR and GDM, while there are only few reports indicating a possible correlation between TTR and the development of pregnancy-related complications like preeclampsia, intrauterine growth restriction and HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelet count) [27–29]. Other studies report the presence of post-translational TTR modifications in several types of non-obstetric pathologic conditions, particularly under oxidative conditions [30–32]. Post-translational modifications of the cysteine residue in position 10 (Cys10) in TTR can alter the stability of the TTR tetramer [12]. In our study we investigated by MALDI-TOF mass spectrometry the post-translational modifications affecting both the transporting proteins TTR and RBP4. TTR Gly10 isoform has already been identified in the past by other authors [33]. Interestingly, we found a relative deficiency of the amount of TTR modified by chemical modification of cysteinyl side chain to form glycine. The formation of TTR Gly 10 depends on a chemical modification, but mechanism has not been clarified yet. Cys10 in TTR is located away from the sites of interaction of TTR with RBP4, and it seems unlikely to have an effect on the interaction with such TTR ligands. Also RBP4 can undergo post-translational modifications, resulting from the truncation of RBP4 at the C-terminus by one or two leucine residues, RBP4-L and RBP4-LL. The appearance of these isoforms in the serum was described in relation with an impaired kidney function [11]. In our study we found no statistically significant differences among groups.

Finally, in the present study we were able to demonstrate for the first time that low ROH plasma concentrations in the first trimester are predictive for the development of iGDM later during pregnancy. The difference was significant also after adjusting for confounding factors. In contrast, other differences in ROH concentration (reduced also in LGA and dGDM compared to controls), as well as in RBP4 and TTR concentration (iGDM compared to controls), were not significant anymore. Even if reduced, plasma ROH concentrations were found to correspond with those obtained from healthy pregnant women at delivery (1.65±0.50 μmol/L) [34], even after correction for patients' gestational age, as ROH showed to be related to the first trimester CRL values. When searching in the literature, we found no report considering the pregnancy outcome and ROH maternal serum concentration during the first trimester. Conversely, some studies report an altered concentration of ROH in the second trimester amniotic fluid [35] and in the later maternal serum of pregnancies with adverse outcome. In particular, it had been shown that maternal vitamin A status significantly correlated to fetal anthropometric characteristics (above all birth weight and birth head circumference) and outcome [36]. Considering the influence of ROH status on GDM occurrence, results are rare and controversial [23, 37, 38]. Previous studies in non-pregnant women indicated lower ROH concentrations in humans as well as animal models of insulin dependent diabetes mellitus in comparison to controls [39, 40] and also demonstrated that insulin treatment restored the reduced ROH concentration [41]. In accordance with this, recent research evidenced the importance of vitamin A in the regulation of insulin responses [42].

Also of interest, when considering the ROH/RBP4 molar ratio we found a relative excess of ROH compared to RBP4, in both cases as well as in controls. Since the hepatic secretion of RBP4 depends on the binding of ROH it is assumed that the molar ratio of both molecules should be close to 1. However, with regard to pregnancy there are conflicting results, confirming this assumption [23, 34], as well as indicating ROH-RBP4 ratio >1 [43], even if we found no report considering the ROH/RBP4 molar ratio in the first trimester of pregnancy. The only paper dealing with ROH in the first trimester found a ROH concentration linearly decreasing from the first trimester to the end of pregnancy [44].

Strengths and weaknesses of this research

To our knowledge this is the first study to characterize the TTR-RBP4-ROH complex in pregnant women. Our experimental determinations included the MS analysis of TTR and RBP4 post-translational modifications. This latter analysis aimed to identify mechanisms that might be responsible for the imbalanced serum concentration of ROH. T4 was not considered in our study, as TTR seems to play a marginal role in the T4 plasma transport [4]. A potential pitfall of this study could arise from the relative small number of patients included in the iGDM sub-group. That notwithstanding, it is worth noting that we were able to detect statistical significant differences between the ROH serum concentration compared to the control group, even after multivariate analysis. Finally, the relevance of an intriguing finding of our study, i.e., the detection of a molar ratio between ROH/RBP4 that consistently exceeds 1 in all the groups of patients, remains unclear to us. However, even the methodology used for RBP4 determination can influence the results obtained. Indeed, it has been advocated that quantitative Western blotting standardized to full-length RBP4 is the most reliable method to measure RBP4 levels, as considerable discrepancy could be found among different immunoassays, particularly in insulin-resistant subjects [45].

Conclusions

Results show a reduction in the concentration of all the components of the TTR-RBP4-ROH complex in patients developing iGDM, even though only ROH concentration was further significantly reduced in the multivariate analysis. Further research is needed in order to clarify the cause of these alterations and possible underlying mechanisms of pathology.

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