

# Retinol-binding protein 4: a novel adipokine implicated in the genesis of LGA in the absence of gestational diabetes mellitus

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## Abstract

**Objective:** Adipokines (cytokines produced by adipose tissue) play a major role in the control of body weight and energy distribution. Retinol-binding protein 4 (RBP4), only recently recognized as an adipokine, has been proposed to modulate systemic insulin sensitivity. The goal of this study was to determine whether there is an association between maternal plasma RBP4 concentration and the birth of a large-for-gestational-age (LGA) newborn in women with and without gestational diabetes mellitus (GDM).

**Study design:** This cross-sectional study included pregnant women at term in the following groups: 1) normal pregnancy with an appropriate-for-gestational-age (AGA) neonate (n=64); 2) normal pregnancy with an LGA neonate (n=44); 3) GDM with an AGA neonate (n=55); and 4) GDM with an LGA neonate (n=42). Maternal plasma RBP4 concentration was determined by ELISA. Parametric and non-parametric statistics were used for analyses.

**Results:** 1) Patients with GDM, either with AGA or LGA neonates, had a higher median plasma concentration of RBP4 than normal pregnant women who delivered an AGA neonate (P=0.01 and P=0.008, respectively); 2) mothers without GDM but with LGA neonates had a higher median plasma concentration of RBP4 than those with normal pregnancy and AGA newborns (P=0.001); 3) these findings

remained significant after adjusting for maternal age, body mass index and gestational age at blood sampling.

**Conclusion:** GDM is characterized by alterations in maternal circulating RBP4 concentrations akin to those of Type 2 diabetes mellitus. RBP4 concentrations in maternal plasma may play a role in accelerated fetal growth in the absence of overt carbohydrate intolerance.

**Keywords:** Adipokine; adipose tissue; appropriate-for-gestational-age (AGA); body mass index (BMI); cytokine; insulin resistance; insulin sensitivity; metabolism; obesity; overweight; pregnancy.

## Introduction

Gestational diabetes mellitus (GDM) is defined as a carbohydrate intolerance of varying severity, with onset, or first recognition, during pregnancy [10, 24, 67, 76]. The clinical importance of GDM stems from the fact that this adverse metabolic state affects 1–10% of all pregnancies [6, 8, 12, 42], as well as from its association with maternal, fetal and neonatal complications [9, 14, 17, 21, 38, 66].

Several mechanisms of disease have been implicated in GDM including autoimmunity [48, 101], single gene mutations [22, 84], insulin resistance, and  $\beta$ -cells dysfunction [8, 11, 31, 83], supporting the notion of GDM as a syndrome. In contrast to other “obstetrical syndromes” [81], GDM has a well-recognized non-gestational counterpart, namely Type-2 diabetes mellitus (Type 2 DM). This view is supported by the observation that these two metabolic complications share common risk factors, such as advanced age [18] and ethnic origin [6]. One of the major common risk factors for GDM and Type 2 DM is obesity [36], and the term “diabesity” has been coined to highlight the strong association between excess fat accrual and carbohydrate intolerance [1]. Importantly, similar mechanisms have been implicated in GDM and Type 2 DM [7, 13], supporting the notion that these two conditions are closely related.

Adipose tissue is now recognized as a highly active organ that secretes endocrine, paracrine and autocrine proteins termed adipokines [2, 26, 90]. These bioactive molecules play an important role in the regulation of appetite, energy balance, insulin resistance, lipid metabolism and inflammation [20, 82]. The physiological importance of adipokines has led to the notion that alterations in the expression and secretion of these bioactive molecules are causatively linked to obesity-related diseases, such as diabetes [34, 44], atherosclerosis [74, 77], and dyslipidemia [23]. Recently, retinol-binding protein 4 (RBP4), a 21-kDa protein predominantly synthesized in the liver, was described as a novel adipokine

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[85, 93, 102, 103]. In addition to its well-established role as the major blood carrier of retinol, RBP4 has been implicated in the regulation of systemic insulin sensitivity [28, 70, 78, 102]. Consistent with this view, increased circulating RBP4 concentrations have been reported in several metabolic complications, such as obesity [4, 5, 28, 30, 45, 68, 95], insulin resistance [16, 28, 68, 80, 99], metabolic syndrome [4, 32, 79, 100], polycystic ovary syndrome [29, 94] and cardiovascular disease [32, 100].

Only a few studies have addressed maternal circulating RBP4 concentration in normal gestation [96] and complications of pregnancy [33, 86, 91, 97, 98], and reports regarding plasma RBP4 concentrations in patients with GDM are scarce and yielded conflicting results [15, 37, 39, 47]. Furthermore, there are no data regarding the association between maternal plasma RBP4 and either overweight/obesity or the delivery of a large-for-gestational-age (LGA) neonate. Thus, the aim of this study was to determine whether there is an association between maternal plasma RBP4 concentration, GDM, and the delivery of an LGA newborn.

## Materials and methods

A cross-sectional study was conducted by searching our clinical database and bank of biological samples, and included pregnant women at term in the following groups: 1) normal pregnant women who delivered an appropriate-for-gestational-age (AGA) newborn ( $n=64$ ); 2) normal pregnant women who delivered an LGA newborn ( $n=44$ ); 3) women with GDM who delivered an AGA newborn ( $n=55$ ); and 4) women with GDM who delivered an LGA newborn ( $n=42$ ). Women with multiple pregnancies or fetal chromosomal and/or congenital anomalies were excluded.

All participating women provided a written informed consent prior to the collection of maternal blood samples. The utilization of samples for research purposes was approved by the Institutional Review Boards of Sotero del Rio Hospital (Santiago, Chile) and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD/NIH/DHHS). Many of these samples have been employed to study the biology of inflammation, hemostasis, and growth factor concentrations in normal pregnant women, and those with pregnancy complications.

## Clinical definitions

The definition of a normal pregnancy included all of the following: (1) no medical, obstetrical or surgical complications; (2) no labor and intact membranes; (3) delivery of a term neonate ( $\geq 37$  weeks) with a birth weight above the 10<sup>th</sup> percentile; [27] and (4) a normal oral 75 g oral glucose tolerance test (OGTT) between 24 and 28 weeks of gestation [3].

Diagnosis of GDM was based on the World Health Organization (WHO) criteria of fasting plasma glucose  $\geq 126$  mg/dL ( $\geq 7.0$  mmol/L) or plasma glucose  $\geq 140$  mg/dL ( $\geq 7.8$  mmol/L) 2 h after the 75 g OGTT [3]. LGA newborn was defined as an infant with a birth weight above the 90<sup>th</sup> percentile and AGA neonate as a birth-weight between the 10<sup>th</sup> and 90<sup>th</sup> percentile [27]. First trimester body mass index (BMI) was calculated according to the following formula: weight (kg)/height (m<sup>2</sup>). A normal weight was defined as a BMI between 18.5 and 25 kg/m<sup>2</sup> and overweight/obese as a BMI  $\geq 25$  kg/m<sup>2</sup> based on the WHO criteria [2].

## Sample collection and determination of RBP4 in maternal plasma

Maternal blood samples were collected at clinical visit. The gestational age at blood sampling was  $\geq 37$  weeks for all women included in the study. Maternal blood was collected into vacutainer tubes and samples were centrifuged at 1300 g for 10 min at 4°C. The plasma obtained was stored at  $-80^{\circ}\text{C}$  until analysis. Maternal plasma concentration of RBP4 was determined by sensitive enzyme-linked immunoassays (Millipore Corporation, St. Charles, MO, USA). The RBP4 immunoassay was validated for human plasma in our laboratory, prior to the conduction of this study. Immunoassays were carried out according to the manufacturer's recommendations. The calculated inter- and intra-assay coefficients of variation for RBP4 immunoassays in our laboratory were 5% and 5.1%, respectively. The sensitivity was calculated to be 0.1 ng/mL.

## Statistical analysis

The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test for normal distribution of the data. Non-parametric methods were used to perform the statistical analysis for parameters not normally distributed, such as RBP4 concentrations. Kruskal-Wallis test with post-hoc analysis and Mann-Whitney *U*-tests were used for comparison of continuous variable among groups. Comparison of proportions was performed by Fisher's exact test. Linear regression analysis was employed to determine which factors were significantly and independently correlated with maternal plasma RBP4 concentration (after log transformation). The following parameters were included in the model: maternal age, first trimester BMI, gestational age at blood collection (as a contentious variable), and the presence of GDM or an LGA neonate (as categorical variables). A  $P < 0.05$  was considered statistically significant. Analysis was performed with SPSS, version 14 (SPSS Inc., Chicago, IL, USA).

## Results

Demographic and clinical characteristics of the study groups are presented in Table 1. Patients with GDM either with an AGA or an LGA neonate had a higher median maternal age than normal pregnant women with an AGA neonate ( $P < 0.001$  for both comparisons). The rate of overweight/obese pregnant women was significantly higher in the GDM + LGA group than in patients with a normal pregnancy and an AGA neonate (69% vs. 45.3%, respectively;  $P = 0.01$ ). RBP4 was detected in the plasma of all subjects. There was a significant difference in the median maternal plasma RBP4 concentration among the groups ( $P = 0.005$ , Kruskal-Wallis).

## Maternal plasma RBP4 concentration in women with a normal pregnancy: AGA vs. LGA

Among women with a normal pregnancy, the median maternal plasma RBP4 concentration was higher in pregnant women with an LGA neonate than in those with an AGA newborn [11,248 ng/mL, interquartile range (IQR): 8804–12,364 vs. 9094 ng/mL, IQR: 7490–10,445, respectively,  $P = 0.001$ ; Figure 1].

**Table 1** Demographic and clinical characteristics of the study population.

	Normal pregnancy		GDM	
	AGA neonate (n=64)	LGA neonate (n=44)	AGA neonate (n=55)	LGA neonate (n=42)
Maternal age (years) <sup>a,b</sup>	26 (22–29)	28 (22–32)	34 (28–39)	32 (30–38)
First trimester BMI (kg/m <sup>2</sup> )	24 (23–31)	25 (23–29)	26 (24–30)	28 (24–32)
BMI ≥ 25 <sup>c</sup>	45.3% (29)	54.5% (24)	67.2% (37)	69.0% (29)
Smoking	14.0% (9)	13.6% (6)	5.4% (3)	9.5% (4)
Gestational age at blood sampling (weeks)	39.0 (38.0–39.0)	39.5 (38.6–40.0)	38.8 (38.2–40.0)	38.5 (38.0–39.7)
Gestational age at delivery (weeks)	39.0 (38.2–39.7)	39.6 (38.7–40.1)	39.1 (38.5–40.0)	39.0 (38.1–39.8)
Birth weight (g) <sup>a,d</sup>	3330 (3157–3555)	4155 (4050–4425)	3455 (3225–3725)	4195 (4030–4405)

Values are expressed as median (interquartile range) or as percentage (number).

GDM = gestational diabetes mellitus, AGA = appropriate-for-gestational-age, LGA = large-for-gestational-age, BMI = body mass index.

<sup>a</sup>Kruskal-Wallis  $P < 0.001$ .

<sup>b</sup> $P = 0.01$  – normal pregnancy + AGA vs. GDM + AGA; normal pregnancy + AGA vs. GDM + LGA.

<sup>c</sup> $P = 0.01$  – normal pregnancy + AGA vs. GDM + LGA.

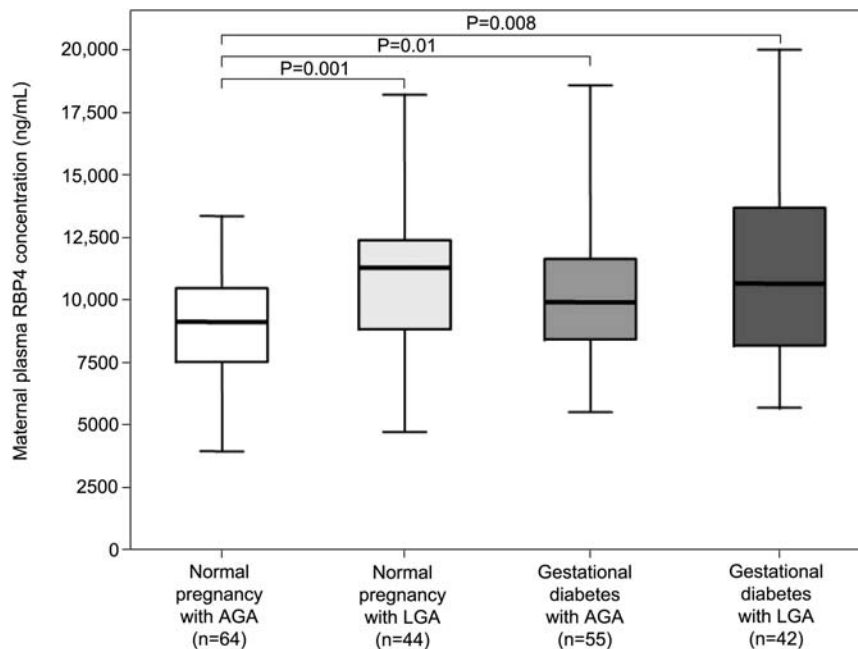
<sup>d</sup> $P < 0.001$  – normal pregnancy + AGA vs. normal pregnancy + LGA; normal pregnancy + AGA vs. GDM + LGA.

### Maternal plasma RBP4 concentration in women with a GDM: AGA vs. LGA

Among women with GDM, the median maternal plasma RBP4 concentration did not differ significantly between patients with an AGA neonate and those with an LGA newborn (9884 ng/mL, IQR: 8402–11,589 vs. 10,618 ng/mL, IQR: 8168–13,530, respectively,  $P = 0.3$ ; Figure 1).

### Maternal plasma RBP4 concentration in women with a normal pregnancy vs. patients with GDM

Among women who delivered an AGA neonate, the median maternal plasma RBP4 concentration was higher in patients with GDM than in those with a normal pregnancy ( $P = 0.01$ ; Figure 1). Among women who delivered an LGA neonate, the median maternal plasma RBP4 concentration did not



**Figure 1** Box and whisker plot of the comparison of the median maternal plasma RBP4 concentrations between women with and without GDM and/or an LGA fetus.

The median maternal plasma RBP4 concentration was higher in patients with GDM, either with an AGA or with an LGA neonate, than in those with a normal pregnancy and an AGA fetus. Similarly, pregnant women with a normal pregnancy and an LGA neonate had a higher median maternal plasma RBP4 concentration than those with a normal pregnancy and an AGA newborn.

differ significantly between patients with a normal pregnancy and those with GDM ( $P=0.9$ ; Figure 1).

### Maternal plasma RBP4 concentration in normal weight vs. overweight/obese pregnant women

Among normal weight pregnant women, the median maternal plasma RBP4 concentration did not differ significantly between the different study groups (Kruskal-Wallis,  $P=0.2$ ; Figure 2). In contrast, there was a significant difference in the median maternal RBP4 concentration among the groups when the analysis was restricted to overweight/obese patients (Kruskal-Wallis,  $P=0.015$ ; Figure 2). The median maternal plasma RBP4 concentration in overweight/obese patients with GDM, either with an AGA or an LGA neonate, was higher than in overweight/obese pregnant women with an AGA newborn in the absence of GDM ( $P=0.02$  and  $P=0.005$ , respectively; Figure 2). Similarly, the median maternal plasma RBP4 concentration in overweight/obese pregnant women with an LGA neonate was higher than in those with an AGA newborn ( $P=0.01$ ; Figure 2).

The median maternal plasma RBP4 concentrations did not differ significantly between normal weight and overweight/obese patients in normal pregnant women with an AGA neonate (normal weight: 9107 ng/mL, IQR: 7745–10,838 vs. overweight/obese: 8541 ng/mL, IQR: 6939–10,354, respectively,  $P=0.4$ ; Figure 2), in normal pregnant women with an LGA neonate (normal weight: 11,290 ng/mL, IQR: 8727–

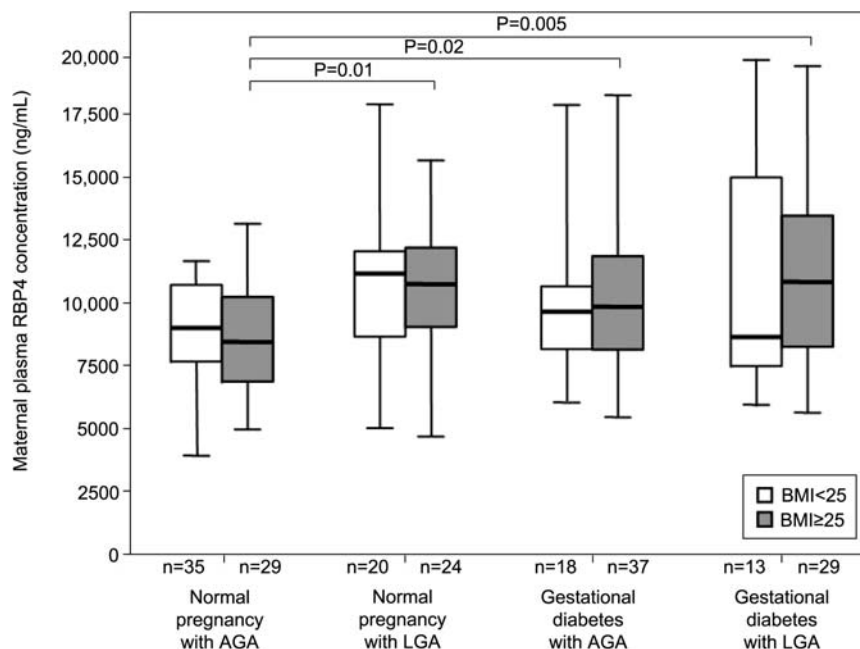
12,363 vs. overweight/obese: 10,866 ng/mL, IQR: 8979–12,364, respectively,  $P=0.9$ ; Figure 2), in patients with GDM and an AGA neonate (normal weight: 9756 ng/mL, IQR: 8325–10,691 vs. overweight/obese: 9957 ng/mL, IQR: 8238–11,986, respectively,  $P=0.4$ ; Figure 2) and in patients with GDM and an LGA neonate (normal weight: 8735 ng/mL, IQR: 7416–16,688 vs. overweight/obese: 10,963 ng/mL, IQR: 8348–13,620, respectively,  $P=0.6$ ; Figure 2).

Multiple linear regression analysis was employed to examine the relationship between GDM, delivery of an LGA neonate and maternal plasma RBP4 concentrations while adjusting for maternal age, gestational age at blood sampling and BMI (as a continuous variable). The final regression model suggested that the delivery of an LGA neonate was independently associated with high maternal plasma RBP4 concentrations ( $P<0.001$ ). Among patients who delivered an AGA neonate, the presence of GDM was also independently associated with high maternal plasma RBP4 concentrations ( $p<0.001$ ).

## Discussion

### Principal findings of the study

1) Patients with GDM, either with AGA or LGA neonates, had a higher median plasma concentration of RBP4 than



**Figure 2** Box and whisker plot of the comparison of the median maternal plasma RBP4 concentrations between normal weight and overweight/obese pregnant women, with and without GDM and/or an LGA fetus.

Among overweight/obese patients, the median maternal plasma RBP4 concentration was significantly higher in patients with GDM, either with an AGA or with LGA neonate, than in those with a normal pregnancy and an AGA neonate. Similarly, overweight/obese pregnant women with a normal pregnancy and an LGA newborn had a higher median maternal plasma RBP4 concentration than those with a normal pregnancy and an AGA neonate. In contrast, among normal weight pregnant women, there was no significant difference in the median maternal plasma RBP4 concentration between the different study groups. Within each study group, the median maternal plasma RBP4 concentration did not differ significantly between normal weight and overweight/obese pregnant women.



normal pregnant women who delivered an AGA neonate; 2) among normal pregnant women, patients with LGA neonates had a higher median plasma concentration of RBP4 than those with AGA newborns; 3) these findings remained significant after adjusting for maternal age, BMI and gestational age at blood sampling.

### **RBP4 is a novel adipokine with metabolic properties**

RBP4, the major blood carrier of retinol, is produced predominantly by the liver [93]. Recently, RBP4 was characterized as a novel adipokine, linking adipocytes glucose metabolism with systemic insulin resistance [85, 93, 102, 103]. The following findings suggest that this adipokine has a regulatory role in glucose homeostasis: 1) circulating concentrations of RBP4 are increased in knockout mice for adipocytes glucose-transporter 4 (GLUT4) [102]; 2) over expression of RBP4, or injection of recombinant RBP4 in normal mice induces insulin resistance [102]; 3) mice heterozygous or homozygous knockout of the gene encoding RBP4 have increased insulin sensitivity, as compared to wild-type mice [102]; 4) treatment with an insulin-sensitizing drug reduces the elevated concentrations of RBP4 in both adipose tissue and serum of mice [102]; 5) polymorphisms in the *RBP4* gene are associated with insulin resistance in humans [19, 69]; 6) overweight/obese patients have higher circulating RBP4 concentrations than normal weight individuals [4, 5, 28, 30, 45, 68, 95]; and 7) increased plasma concentrations of RBP4 was reported in several metabolic complications, such as insulin resistance [16, 28, 68, 80, 99], metabolic syndrome [4, 32, 79, 100], polycystic ovary syndrome [29, 94] and cardiovascular disease [32, 100].

### **RBP4 in human pregnancy**

There is only a limited number of reports regarding circulating maternal RBP4 concentration [33, 86, 91, 96–98]. Ueland et al. [96] found a significant increase in maternal fasting RBP4 concentration as a function of gestational age in normal pregnant women which was correlated with the decline in insulin sensitivity. Inuou et al. [33] and Shangguan et al. [86] reported that RBP4 is increased in patients with gestational hypertension and preeclampsia, whereas Stepan et al. [91] found no significant difference in maternal circulating RBP4 between patients with and without preeclampsia. Recently, we have reported that early-onset, but not late-onset preeclampsia, and pregnancy with a small-for-gestational-age neonate or a fetal death, are associated with a higher median maternal plasma concentration of RBP4 than normal pregnancy [98]. In addition, we reported that RBP4 is a physiologic constituent of the amniotic fluid and that the median amniotic fluid concentration of RBP4 is elevated in pregnancies complicated by intra-amniotic infection/inflammation [97]. Taken together, these data suggest that RBP4, like other adipokines, such as adiponectin, visfatin and resistin [40, 49–65, 71–73, 88], may play a role in normal human gestation, as well as in complications of pregnancy.

### **GDM is characterized by a high maternal plasma RBP4 concentration**

The findings of the present study indicate that patients with GDM, either with an AGA or an LGA neonate, have a higher median plasma RBP4 concentration than normal pregnant women with an AGA neonate. Our findings are in agreement with those of Lewandowski et al. [47], Chan et al. [15] and Kim et al. [37] who all reported higher concentrations of RBP4 in patients with GDM but in contrast to those report by Krzyzanowska et al. [39] in which serum RBP4 concentrations were lower in patients with GDM. Differences in study design can account for the discrepancy between the studies. Specifically, gestational age at blood sampling, ethnic origin, and the ELISA kit used for RBP4 determination differed between the study conducted by Krzyzanowska et al. [39] and the present report.

The findings reported herein extend the aforementioned observations by demonstrating that GDM is associated with high circulating RBP4 concentrations in patients who delivered either an AGA or an LGA neonate. In addition, our findings suggest that overweight/obese patients with GDM, but not those with normal weight, have increased maternal circulating RBP4 concentrations. The latter finding may help to reconcile the inconsistency in the literature regarding maternal circulating RBP4 concentrations in patients with GDM. Finally, this is the first study to compare RBP4 concentrations in normal pregnant women and GDM patients at term.

### **Why is GDM is associated with high maternal plasma RBP4 concentrations?**

Selective down-regulation of GLUT4 in adipocytes is almost a universal feature of insulin resistance states [87, 102]. Increased concentrations of RBP4 have been proposed as the molecular mechanism linking GLUT4 down-regulation with systemic insulin resistance [102]. Specifically, RBP4 impairs insulin signaling in muscle and induces the expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver [102]. Importantly, adipocytes GLUT4 content is decreased by ~45–60% in patients with GDM [25, 75], suggesting that similar mechanisms of disease can be applied to GDM and other insulin resistance states. The latter observation can also explain why the differences in maternal circulating RBP4 concentrations reported herein were confined to overweight/obese individuals. We recognize that the cross-sectional design of our study limited our ability to infer a causal relationship between elevated maternal circulating RBP4 concentrations and GDM. Nevertheless, it is tempting to speculate that the explanation for the high maternal RBP4 concentration is similar to that reported in Type 2 DM and other conditions characterized by insulin resistance.

### **Increased maternal plasma RBP4 concentrations is a feature of patients with an LGA neonate**

The findings of the present study which indicating that normal pregnant women with an LGA neonate had a higher median maternal plasma RBP4 concentrations than normal

pregnant women who delivered an AGA neonate are novel. Of note, delivery of an LGA neonate was independently associated with elevated maternal plasma RBP4 concentrations after adjusting for possible confounding factors. The explanation for this association is not clear. Since the data of the present study pertain only to maternal circulation, we cannot determine whether the increased RBP4 concentration in maternal circulation is a result of high maternal secretion, increased transplacental transport of RBP4 from the LGA fetus, or enhanced secretion from the larger placentas of LGA fetuses. Moreover, no conclusive data exist as to whether RBP4 can cross the human placenta, although such transport has been demonstrated in rats [89, 92]. In addition, there is paucity of information regarding cord blood RBP4 concentrations. Only two studies have addressed this question [15, 43], and only one [15] reported positive correlation between cord blood RBP4 and birthweight suggesting that LGA neonates might have higher circulating RBP4 concentrations than AGA neonates.

Another possible explanation that can account for the high maternal plasma RBP4 concentrations in patients with an LGA neonate is the presence of subtle carbohydrates intolerance in this subset of pregnant women. Minor abnormalities of glucose metabolism in patients with an LGA neonate have been reported in the absence of overt GDM [35, 41, 46, 104]. Thus, similarly to patients with GDM, it is possible that higher maternal plasma RBP4 concentrations relates to the maternal glucose metabolism rather than transfer from the fetal circulation or increase placental RBP4 secretion to the maternal circulation.

In conclusion, GDM is characterized by alterations in maternal circulating RBP4 concentrations akin to those of Type 2 DM. Pregnant women with an LGA neonate have a higher median plasma concentration of RBP4 than those of normal pregnant women with an AGA neonate, suggesting subclinical glucose metabolism in the former group. Collectively, these findings support the notion that adipokines play an important role in obesity-related complications of pregnancy.

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