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ORIGINAL ARTICLE

Gender dimorphic increase in RBP-4 and NGAL in children born after IVF: an epigenetic phenomenon?

Sophia D. Sakka*, Alexandra Margeli†, Dimitrios Loutradis‡, George P. Chrousos*, Ioannis Papassotiriou† and Christina Kanaka-Gantenbein*

*Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics, Athens University Medical School, "Aghia Sophia" Children's Hospital, Athens, Greece, †Department of Clinical Biochemistry, "Aghia Sophia" Children's Hospital, Athens, Greece, ‡Division of In Vitro Fertilization, First Department of Obstetrics and Gynaecology, Athens University Medical School, Athens, Greece

ABSTRACT

Background *In vitro* fertilisation (IVF) has been widely used during the last decades. Recent studies demonstrated some alterations in IVF children's metabolic profile compared with controls. The recently reported lipocalins retinol-binding protein 4 (RBP-4) and neutrophil gelatinase-associated lipocalin (NGAL), as well as visfatin, which are associated with glucose intolerance and could help in the early detection of metabolic abnormalities, have not been studied in IVF children as yet. We studied the lipocalins RBP-4 and NGAL as well as visfatin in children born after IVF.

Subjects and methods A total of 100 children born after IVF (47 boys) and 60 controls born after normal conception (30 boys), aged 4–14 year, were studied cross-sectionally. All children had a physical examination, their fasting glucose, insulin, lipid profile, RBP-4, NGAL, and visfatin were determined and their homoeostasis model assessment (HOMA) index was calculated.

Results Children born after IVF had significantly higher RBP-4 ($P = 0{\cdot}009$) and NGAL ($P = 0{\cdot}028$) levels than controls. When divided by gender, RBP-4 remained higher in IVF girls ($P = 0{\cdot}002$), whereas NGAL was higher in IVF boys ($P = 0{\cdot}021$). Linear regression analysis had revealed that the differences are attributed to the IVF procedure *per se*.

Conclusions In our study, IVF children had significantly higher RBP-4 and NGAL levels than controls, suggesting early metabolic derangements that could be attributed to an epigenetic phenomenon. These results are in accordance with our earlier findings of higher blood pressure and triglycerides in IVF children than controls. Further prospective studies in IVF children will determine the natural course of their metabolic profile.

Keywords Adipokines, insulin sensitivity, IVF offspring, NGAL, RBP-4.

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Introduction

Assisted reproductive techniques (ART), namely *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), are widely used in the treatment of human infertility. ART carry an increased risk of multiple pregnancies, preterm delivery, low birth weight and congenital malformations. Also, various studies have revealed a possibly increased incidence of genomic imprinting disorders, such as the Beckwith-Wiedemann and Angelman syndromes among children conceived after ART [1,2]. Despite IVF having been practiced for almost 30 years, there are still many aspects to be studied on the metabolic profile of these children. Miles *et al.* [3] found that IVF children had a more favourable lipid profile, with higher high-density lipoprotein (HDL) and lower triglyceride levels than spontaneously conceived controls, while other studies demonstrated higher systolic and diastolic blood pressure (BP) and higher triglyceride and/or glucose levels in IVF children than controls [4]. Furthermore, Ceelen *et al.* [5] showed a higher percentage of peripheral body fat in IVF children than controls. We have also reported in our previous study increased systolic pressure and triglyceride levels in children born after classic IVF in comparison with controls [6].

Adipose tissue is a metabolically active organ releasing and responding to signals that modulate appetite, insulin

sensitivity, energy expenditure, inflammation, and immunity, such as leptin, adiponectin and interleukin-6 [7,8]. Recently, RBP-4 has been identified as an adipocyte-secreted molecule that induces insulin resistance, by upregulating hepatic gluconeogenesis and inhibiting insulin signalling in skeletal muscle, contributing thus to the pathogenesis of diabetes type 2 [9]. It is elevated in the serum before the development of frank diabetes and appears to identify insulin resistance and associated cardiovascular risk factors in children and adults [10,11]. Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL), a secretory glycoprotein that belongs to the same protein family as RBP-4, is also a known inflammatory biomarker positively correlated with body mass index (BMI) and other variables of the metabolic syndrome (MS) [12]. It was recently found to promote insulin resistance, similarly to RBP-4 [13]. Visfatin is a novel adipokine, which was initially described as being highly expressed in visceral fat, and possessing insulin-mimetic bio-activity, including promotion of glucose disposal and more recently as a regulator of insulin secretion [14]. A number of further studies demonstrated that visfatin levels in humans correlate with obesity, visceral fat mass, diabetes type 2 and the presence of the metabolic syndrome. Recent studies, however, did not confirm an association of visfatin and visceral adipose tissue or parameters of insulin sensitivity in humans and rodents [8].

We have recently reported normal levels of the adipokines leptin and adiponectin, as well as of the low-grade inflammation markers high sensitivity C-reactive protein (hs-CRP) and high sensitivity interleukin-6 (hsIL-6) in IVF children compared to BMI- and age-matched controls [6]. In this study, we evaluated retinol-binding protein-4 (RBP-4), neutrophil gelatinase-associated lipocalin (NGAL) and visfatin in IVF children in comparison with spontaneously conceived controls to clarify whether they can be used as markers for the prediction of insulin resistance and cardiovascular risk in the IVF population in addition to traditional risk factors.

Materials and methods

Subjects

One hundred IVF-conceived children and 60 naturally conceived (NC) ones were included in the study. *IVF children.* About 100 caucasian children from the Section of IVF of the First Department of Obstetrics and Gynaecology of the University of Athens [47 boys (15 pubertal- 32 prepubertal), 53 girls (21 pubertal- 32 prepubertal)] aged 8·9 ± 2·9 years were recruited for the study. All subjects were conceived after traditional IVF; children conceived after ICSI were excluded. The IVF children were recruited consecutively from families that had IVF and were willing to participate to the study. Among the families that we contacted, those willing to participate reached a percentage of about 95%. There were no differences among participating and nonparticipating families, regarding age, sex, birth weight, gestational age, SGA-AGA and family history. *NC controls:* 60 caucasian, age- (P = 0·951), sex- (P = 0·746) and pubertal stage- (P = 0·618) matched subjects randomly selected among healthy children who came to 'Aghia Sophia' Children's Hospital for their annual check-up and were willing to participate, with the only restriction of not having been conceived with IVF, served as controls [30 boys (nine pubertal- 21 prepubertal), 30 girls (15 pubertal- 15 prepubertal)] aged 8·9 ± 3·0 years.

All children were healthy, not receiving any medication.

History records

A detailed medical history was obtained with information taken from the files of the Section on IVF of the First Department of Obstetrics and Gynecology, the children's health books and their parents for both IVF children and controls. Data recorded were the presence of maternal gestational diabetes mellitus (GDM), arterial hypertension (AH) of the mother during pregnancy, maternal age and BMI at conception, gestational age, birth weight, birth length, as well as the child's personal and family medical history. Children were classified as small for gestational age (SGA) or appropriate for gestational age (AGA) if their birth weight was < 10th or between 10th and 90th percentile, respectively, according to the individual percentile calculator of www.gestation.net, which took into account birth weight, gestational age, parity, ethnic group, sex of the child and the height and weight of the mother. The local ethical committee of the 'Aghia Sophia' Children's Hospital approved the study and children were included only after informed written consent was obtained from their parents or legal guardians.

Physical examination

All children were examined by the same-trained physician. Physical examination included height, weight, head circumference, waist to hip ratio (W/H), BMI, pubertal status and systolic (SBP) and diastolic blood pressure (DBP) measurements. BMI Standard Deviation Scores (SDS) were used for the statistical analysis based on the Greek National Growth Charts [15], arterial BP-SDS were used for the statistical analysis based on Pediatrics 2004-4th report on the diagnosis, evaluation, and treatment of high BP in children and adolescents [16] according to the children's age and height using the mean of three measurements. Girls with Breast Tanner stage I and boys with testicular size < 4 mL were considered prepubertal, while girls with Breast Tanner stage ≥ II and boys with testicular size ≥ 4 mL were considered pubertal.

Blood chemistry and hormones assays

After an overnight fast, venous blood was withdrawn from all participants at 8 am and circulating glucose, insulin, total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein-A1 (Apo-A1), apolipoprotein-B (Apo-B) and lipoprotein (a) [Lp(a)], as well as RBP-4, NGAL, visfatin and thyroid-stimulating hormone (TSH) levels were measured.

Determination of serum glucose, total cholesterol, triglycerides, HDL and LDL levels were performed using the Siemens Advia 1800 Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Apo A-I, Apo-B and Lp(a) concentrations were determined by latex-particle-enhanced immunonephelometric assays on the BN ProSpec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany), whereas serum insulin and TSH concentrations were determined with the automated chemiluminescence Siemens ACS180 System Analyzer (Siemens Healthcare Diagnostics, Liederbach, Germany). The intra- and interassay coefficients of variation of the determination of all biochemical variables did not exceed 5%.

Serum RBP4 levels were determined using a solid-phase ELISA assay (R&D Systems, Minneapolis, MN, USA). Fifty assays were evaluated, and the minimum detectable dose (MDD) of RBP4 ranged from 0·053–0·628 ng/mL. The mean MDD was 0·224 ng/mL. The intra- and interassay CVs ranged from 5·7% to 8·1% and from 5·8% to 8·6%, respectively, according to the manufacturer. Serum NGAL levels were determined by a solid-phase ELISA technique (R&D Systems). Forty assays were evaluated and the minimum detectable dose (MDD) of NGAL ranged from 0·003–0·040 ng/mL. The mean MDD was 0·012 ng/mL. The intra-assay and interassay CV's ranged between 3·1% and 4·4% and between 5·6% and 7·9%, respectively, according to the manufacturer. Serum visfatin-C-terminal levels were determined by the human visfatin COOH-terminal ELISA kit (Phoenix Pharmaceuticals, Belmont, CA, USA), minimum detectable concentration was 1·85 ng/ml. The intra-assay and interassay CVs were 4% and 10·5%, respectively.

Fasting glucose to insulin ratio (FGIR) and homoeostasis model assessment (HOMA), as markers of insulin resistance, were calculated according to the original formulae [17,18].

Metabolic syndrome criteria

The criteria we used to diagnose the metabolic syndrome were modified from those of the Adult Treatment Panel III (ATP III) [19] according to Weiss *et al.* [20]. The children were classified as having the metabolic syndrome if they met three or more of the following criteria for age, sex and ethnic group:

1 BMI > 95th percentile [15,21]
2 Triglycerides > 95th percentile [22]
3 HDL < 5th percentile [22]
4 Blood pressure > 95th percentile [16]
5 FGIR < 7 [17]

Statistical analyses

Continuous variables are presented as mean (standard deviation, SD) or median (interquartile range), while quantitative variables are presented with absolute and relative frequencies. Variables were first tested for normality using the Kolmogorov–Smirnov criterion. Log-transformations were conducted when the normality assumption was not satisfied. For the comparisons of means, the Student's *t*-test was used and for the comparison of proportions the Fisher's exact tests were applied. Mann–Whitney *U*-tests were employed when the normality assumption was not satisfied after log-transformation. Pearson's correlation coefficient was applied to explore the association of RBP-4, lipocalin-2 and visfatin with various parameters. To explore the effect of IVF and SGA on RBP-4, visfatin and NGAL levels linear regression analyses were conducted adjusting for sex, age and BMI. regression coefficients (β) with their standard errors (SE) were computed from the results of regression analyses. Possible interactions of the independent variables were also tested in the regression models.

Statistical significance was set at 0·05, and analyses were conducted using SPSS statistical software (version 13.0, SPSS Inc., Chicago, IL, USA).

Ethical aspects

The local ethical committee of the 'Aghia Sophia' Children's Hospital approved the study, and children were included only after informed written consent was obtained from their parents or legal guardians.

Results

Perinatal and physical examination data and traditional insulin resistance indices

The physical examination and medical history characteristics of IVF children and controls are summarised in Table 1. There was no significant difference between the two groups studied in chronologic age, gender, pubertal stage, waist to hip ratio (W/H) and BMI. IVF children, however, had a significantly smaller gestational age, and lower birth weight and length compared with SC ones ($P < 0·001$). Moreover, the IVF group had more SGA infants and twins and triplets than the control group ($P = 0·004$ and $P < 0·001$, respectively). The mothers of IVF children were significantly older at conception ($P < 0·001$), had a significantly higher BMI ($P = 0·034$) and gestational diabetes mellitus ($P = 0·003$). However, there were no significant differences in the incidence of maternal arterial hypertension during pregnancy between the two groups.

Table 1 Perinatal and anthropometric characteristics and laboratory parameters of (IVF) children and age-matched controls.

	IVF *N* (%) Mean (SD)	Control *N* (%) Mean (SD)	*P* value χ^2 test
Sex			
Boy	47 (47·0)	30 (50·0)	0·746
Prepubertal	32 (68·1)	21 (70·0)	0·618
Pubertal	15 (31·9)	9 (30·0)	
Girl	53 (53·0)	30 (50·0)	
Prepubertal	21 (39·6)	15 (50·0)	
Pubertal	32 (60·4)	15 (50·0)	
Birth weight (g)	2434·3 (638·3)	3195·3 (571·1)	< 0·001*
Birth length (g)	48·0 (3·6)	51·2 (2·9)	< 0·001*
W/H	**0·9 (0·1)**	**0·8 (0·1)**	**0·501**
BMI-SDS	0·1 (1·1)	0·0 (1·0)	0·487
Group for GA			
AGA	66 (66·0)	52 (86·6)	0·004
SGA	34 (34·0)	8 (13·4)	
Twins			
Singletons	56 (56·0)	56 (93·3)	< 0·001
Twins	37 (37·0)	4 (6·7)	
Triples	7 (7·0)	0·0 (0·0)	
Gestational age (week)	35·7 (2·7)	38·1 (2·2)	< 0·001†
Preterm delivery (< 37 weeks)			
No	47 (47·0)	50 (83·3)	< 0·001
Yes	53 (53·0)	10 (16·7)	
Age of mother at conception (years)	36·6 (5·7)	30·5 (5·7)	< 0·001*
BMI of mother	24·1 (3·9)	22·7 (3·7)	0·034*
Gestational DM			
No	80 (80·0)	58 (96·7)	0·003
Yes	20 (20·0)	2 (3·3)	
Blood Pressure of mother			
Normal BP	91 (91·0)	59 (98·3)	0·092‡
AH of pregnancy	9 (9·0)	1 (1·7)	
FGIR**	15·2 (10·7)	15·8 (13·5)	0·988
HOMA**	1·8 (1·4)	1·7 (1·1)	0·736*
Glucose (mg/dL)	83·8 (9·0)	83·7 (8·5)	0·754*
Insulin** (mU/L)	8·7 (6·4)	7·9 (4·6)	0·743*

*Student's *t*-test.
†Mann–Whitney *U*-test.
‡Fisher's exact test.
**The test has been performed using the neperian logarithm of the parameter.
SD, standard deviation; GA, gestational age; AGA, adequate for gestational age; SGA, small for gestational age; W/H, waist to hip ratio; BMI, body mass index; SDS, standard deviation score; DM, diabetes mellitus; BP, blood pressure; AH, arterial hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA, homoeostasis model assessment; TGL, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FGIR, Fasting glucose to insulin ratio; IVF, *in vitro* fertilisation

Glucose, insulin, HOMA and FGIR values were not significantly different between IVF children and controls. All children had normal fasting glucose levels.

Circulating RBP-4, NGAL and visfatin concentrations

Table 2 describes the comparison of RBP-4, NGAL and visfatin, between IVF-children and controls in the entire group and in boys and girls separately. When both genders were examined together, RBP-4 and NGAL were significantly increased in the IVF group compared with controls ($P = 0·009$ and 0·028, respectively). In boys, RBP-4 and visfatin showed no significant difference between groups, while NGAL was significantly increased in the IVF group compared with controls ($P = 0·021$). In girls, RBP-4 was significantly higher in the IVF group compared with controls ($P = 0·002$).

When multiple linear regression analysis was conducted with dependent variable the RBP-4 (Table 3), it was found that IVF and not SGA had a significant effect on RBP-4 levels. Specifically, IVF children had significantly greater levels of RBP-4. Also, a significant interaction of sex with IVF was found ($\beta = -3·88$, SE = 1·84, $P = 0·037$) indicating that the difference of RBP-4 between IVF and controls was significantly greater in girls compared with boys. Multiple analysis for visfatin showed no significant effect for either IVF or SGA. Furthermore, regression analysis indicated that IVF was independently associated with NGAL levels and IVF children had significantly greater levels of NGAL compared with controls. Additionally, NGAL levels were not significantly different between SGA and AGA children. No significant interaction of IVF with SGA was found in any of the aforementioned analyses.

Table 2 Comparison of RBP-4, NGAL and visfatin between IVF-children and controls in the entire group and in boys and girls separately

	Whole group			Boys			Girls		
	IVF (*N* = 100) Mean (SD)	Controls (*N* = 60) Mean (SD)	*P**	IVF (*N* = 47) Mean (SD)	Controls (*N* = 30) Mean (SD)	P†	IVF (*N* = 53) Mean (SD)	Controls (*N* = 30) Mean (SD)	*P*†
RBP-4 (mg/L)	28·4 (5·7)	26·0 (5·7)	**0·009**	28·1 (5·5)	27·4 (6·3)	0·620	28·7 (5·9)	24·5 (4·7)	**0·002**
Visfatin (ng/mL)	21·8 (9·2)	24·0 (6·8)	0·103	23·0 (10·7)	25·7 (8·1)	0·240	20·6 (7·5)	22·3 (4·8)	0·282
NGAL (ng/mL)	58·0 (14·1)	53·5 (10·6)	**0·028**	59·1 (14·1)	52·9 (8·2)	**0·021**	57·1 (14·1)	54·1 (12·7)	0·353

*Adjusted for sex, age and BMI.
†Adjusted for age and BMI.
IVF, *in vitro* fertilisation; NGAL, neutrophil gelatinase-associated lipocalin; RBP4, retinol-binding protein 4.

Table 3 Regression coefficients (β) and standard errors (SE) derived from multiple linear regression models with dependent variables the RBP-4, visfatin and NGAL

	β*	SE	*P*
RBP-4			
Group for GA (SGA vs. AGA)	−0·04	1·04	0·970
Group (IVF vs. controls)	2·49	0·95	**0·009**
Visfatin			
Group for GA (SGA vs. AGA)	−1·60	1·55	0·303
Group (IVF vs. controls)	−2·02	1·40	0·152
NGAL			
Group for GA (SGA vs. AGA)	2·86	2·34	0·223
Group (IVF vs. controls)	4·14	2·10	**0·049**

*Adjusted for sex, age and BMI.
GA, gestational age; IVF, *In vitro* fertilisation; NGAL, neutrophil gelatinase-associated lipocalin; RBP4, retinol-binding protein 4.

When children were separated into pubertal and prepubertal ones, the differences found remained present in the pubertal group, that is, RBP-4 and NGAL levels were significantly higher in pubertal girls ($P = 0{\cdot}001$) and in pubertal boys ($P = 0{\cdot}035$), respectively, while there were no significant differences in prepubertal children (Fig. 1). Furthermore, by using maternal BMI, age at conception and the presence of GDM as confounding factors in the multivariate analysis, we found that the differences in RBP-4 and NGAL between the two groups were still significant (data not shown).

Correlations

Evaluation of the correlations of RBP-4, NGAL and visfatin with various metabolic parameters, that is, HOMA, FGIR, HDL, triglycerides, LDL, total cholesterol, SBP-SDS, DBP-SDS, BMI, waist-to-hip ratio, ApoA1, ApoB, Lp(a), ApoA1/ApoB and cortisol in IVF children and controls, adjusted for gender and age, revealed that in IVF children, RBP-4 and NGAL were positively correlated with HOMA ($P = 0{\cdot}003$ and $P = 0{\cdot}014$, respectively) and negatively correlated with FGIR ($P = 0{\cdot}007$ and $P = 0{\cdot}004$, respectively). Also RBP-4 was positively ($P = 0{\cdot}005$) and visfatin negatively correlated ($P = 0{\cdot}034$) with SBP-SDS, while NGAL was positively correlated with DBP-SDS and BMI-SDS ($P = 0{\cdot}031$). In the control group, RBP-4 was also negatively correlated with FGIR ($P = 0{\cdot}020$) and positively with triglycerides ($P = 0{\cdot}011$) and BMI-SDS (0·031), while NGAL was positively correlated with HOMA ($P = 0{\cdot}020$). When all these correlations were also adjusted for BMI the only significant correlation remaining was a positive correlation between RBP-4 and SBP-SDS in IVF children ($P = 0{\cdot}007$), and between RBP-4 and triglycerides in the controls ($P = 0{\cdot}027$) (Fig. 2).

An evaluation of the correlation of RBP-4 with TSH values, adjusted for sex, age and BMI, in both IVF children and controls, revealed no significant correlations ($r = -0{\cdot}06$, $P = 0{\cdot}558$ and $r = 0{\cdot}191$, $P = 0{\cdot}162$, respectively).

Metabolic syndrome

In Table 4a, RBP4, NGAL and visfatin were compared between children with and without the metabolic syndrome in IVF children and controls, separately, but there was no significant difference between groups. Table 4b shows the correlations between RBP4, visfatin, NGAL and the total number of metabolic syndrome criteria for IVF children and controls, separately. RBP-4 was positively correlated with the total number of criteria for the metabolic syndrome only in IVF children ($P = 0{\cdot}036$), while NGAL was positively

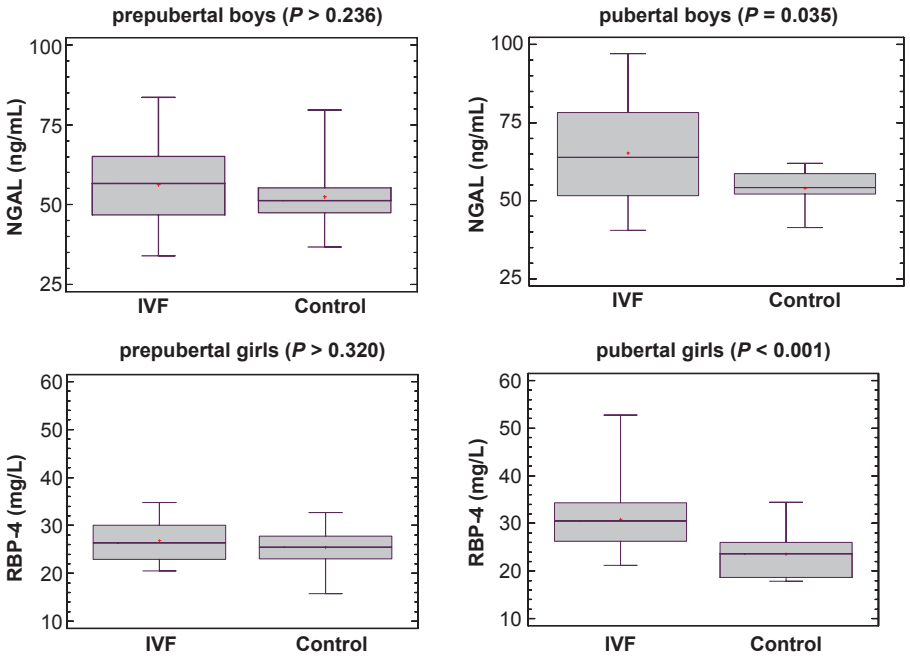

Figure 1 Comparison of neutrophil gelatinase-associated lipocalin (NGAL) in prepubertal and pubertal boys and of retinol-binding protein 4 (RBP-4) in prepubertal and pubertal girls between *in vitro* fertilisation (IVF) children and controls.

correlated with the total number of criteria in IVF children as well as in controls ($P = 0{\cdot}028$ and $P = 0{\cdot}013$, respectively).

Discussion

In this study, new markers of insulin resistance, such as RBP-4 and NGAL, were higher in IVF children than controls. All children had normal fasting glucose concentrations, a finding that excluded any severe glucose intolerance at this period. Moreover, we found similar results regarding the smaller birth weight and length, gestational age, multiple births and preterm delivery and a higher prevalence of SGA in our IVF cohort, independently of the children being singletons, twins or triplets, in accordance with previous studies [1,2].

The increases in RBP-4 and NGAL levels in the entire group of IVF children compared with controls demonstrated gender dimorphism. Therefore, when separated by gender, only IVF girls showed higher RBP-4 levels and only IVF boys showed higher NGAL levels than controls. Sexual dimorphism of RBP-4 and NGAL is confirming previous results [12,23] and this might be attributed to a sexual dimorphic pattern of environmental epigenetic programming, as very recently reported [24]. Moreover, in the regression analysis, RBP4 showed a significant interaction of sex with the IVF procedure, with greater differences between the two groups in girls compared with boys. It is noteworthy that higher RBP4 levels have been reported in women with PCOS than controls [25]. Taken into consideration that IVF girls have already been reported to be at increased risk to present premature adrenarche, which is a for-runner of PCOS, it is evident why IVF girls are found to have higher RBP4 levels than naturally conceived controls [2]. It remains to follow these girls into adulthood to elucidate whether they will develop PCOS.

In vitro fertilisation (IVF) offspring are at a higher risk of preterm delivery and intrauterine growth restriction (IUGR), frequently born SGA [1]. SGA children are generally more prone to develop the MS [26], in agreement with Barker's theory of foetal origin of adult disease [27]. Furthermore, adipocytokines have been recently demonstrated to be involved in intrauterine growth and may play an important role in linking poor foetal growth to the subsequent development of adult diseases [28]. Therefore, to eliminate SGA as a confounding factor, multiple linear regression analysis was conducted and it was shown that IVF-procedure *per se* and not SGA was responsible for the elevation of RBP4 and NGAL in IVF children vs. controls.

Moreover, as an increased incidence of diabetes type 2, obesity and cardiovascular alterations has been associated with *in utero* exposure to increased glucose concentrations [28], maternal GDM [29], BMI and age at conception were used as

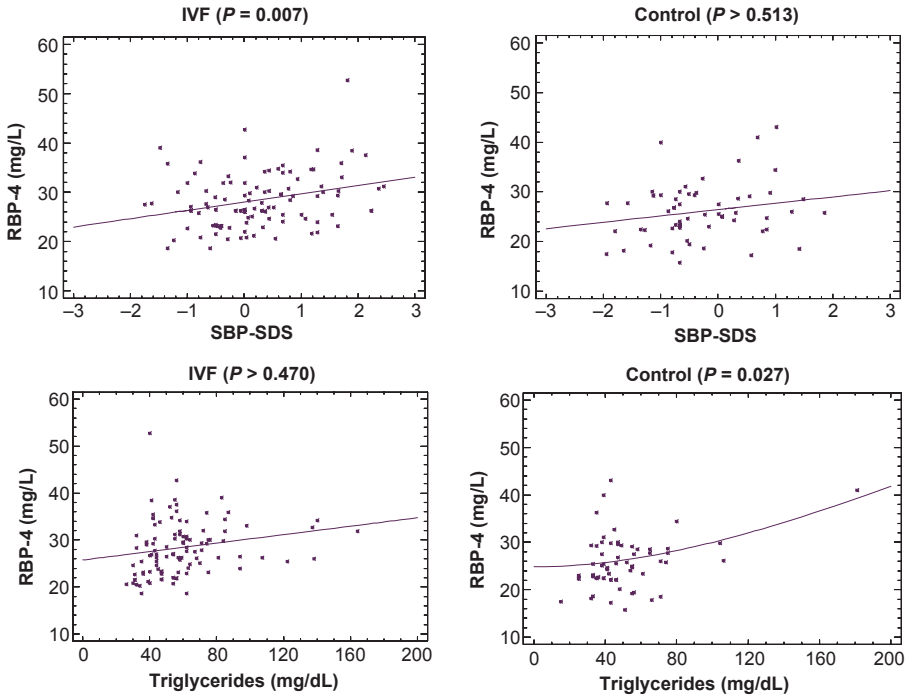

Figure 2 Correlation between retinol-binding protein 4 (RBP-4) and SBP-SDS and triglycerides adjusted for body mass index (BMI) in *in vitro* fertilisation (IVF) children and controls.

confounding factors in the multivariate analysis of RBP-4 and NGAL elevations. However, the differences between the two groups were still significant, which excludes these factors as responsible for the increased levels of RBP-4 and NGAL observed in IVF children.

Another aspect that seems to affect RBP-4 and NGAL levels is puberty, a state of inherent insulin resistance. When subdivided to prepubertal and pubertal children, the differences in RBP-4 and NGAL remained only in pubertal girls and boys, respectively. In a previous study, RBP4 concentrations were also higher in pubertal than in prepubertal children, implying a role of sex steroids in the control of this adipokine's secretion [30]. It is evident that as IVF children grow and enter puberty their metabolic profile, in terms of nontraditional cardiovascular risk indices, becomes less favourable.

One possible explanation for these differences in IVF children would be the increased peripheral adipose tissue mass and decreased percentage of peripheral lean tissue observed among IVF children as compared with controls [5]. Lee *et al.* [31] determined that serum RBP-4 levels were independently associated with visceral fat and LDL-cholesterol levels, suggesting that irrespectively of body weight, visceral obesity is an independent predictor of serum RBP-4 levels. Moreover, visceral fat accumulation has shown a strong influence on the conventional cardiovascular risk factors, including hypertension, dyslipidaemia and diabetes type 2 [32], some of which have also been found to be increased in IVF children [4–6]. Therefore, RBP-4 may represent a link between visceral obesity and metabolic alterations in IVF children.

As no difference in insulin resistance markers, such as HOMA and FGIR, was observed between IVF children and controls, a plausible explanation for the higher levels of RBP-4 and NGAL among IVF children is the possible epigenetic alterations that might occur during pre-implantation manipulations and might cause alterations in gene expression. Genetic imprinting diseases, such as the Beckwith-Wiedemann and Angelman syndromes, have been associated with ART, mainly ICSI, leading to broader implications of periconceptual manipulations on future health of these children [2,33,34]. Furthermore, Katari *et al.* [35] found DNA methylation and gene expression differences in IVF children compared with spontaneously conceived children, several of which genes have been implicated in metabolic disorders, such as obesity and type 2 diabetes. It should be further examined whether the expression of GLUT4 glucose transporter can be epigenetically modified and selectively decreased in adipocytes, resulting in a diminished glucose influx through GLUT4 and a respective increase in RBP-4 and insulin resistance [9,10].

Table 4 Comparison of retinol-binding protein 4 (RBP4), NGAL and visfatin between children with and without the metabolic syndrome (a) and their correlation with the total number of Metabolic syndrome criteria (b) in IVF children and controls, separately

(a)	**IVF**			**Controls**		
	Metabolic syndrome			**Metabolic syndrome**		
	No Mean (SD)	**Yes** Mean (SD)	***P****	**No** Mean (SD)	**Yes** Mean (SD)	***P****
RBP-4 (mg/L)	28·2 (5·8)	32·0 (2·5)	0·180	25·8 (5·6)	28·6 (7·3)	0·511
Visfatin (ng/mL)	21·9 (9·4)	20·4 (6·8)	0·497	23·9 (6·8)	26·1 (8·1)	0·589
NGAL (ng/mL)	57·5 (14·0)	65·2 (15·5)	0·473	53·3 (11·0)	55·5 (4·4)	0·640

(b)	**Total criteria**	
	IVF	**Controls**
RBP-4		
r	0·21	0·23
P	**0·036**	0·081
Visfatin		
r	−0·05	−0·12
P	0·616	0·353
NGAL		
r	0·22	0·32
P	**0·028**	**0·013**

*Adjusted for sex and age (Analysis of Covariance-ANCOVA).
IVF, *In vitro* fertilisation; RBP4, retinol-binding protein 4; NGAL, neutrophil gelatinase-associated lipocalin.

Therefore, more research needs to be conducted in this group of children because they are exposed to peri-conceptual manipulations that could influence their future health -outcome.

Previous studies have shown that RBP-4 and NGAL concentrations are positively correlated with several adiposity variables, including BMI and waist cirumference, with insulin resistance indices, systolic and diastolic blood pressure and lipid profile [10–12,23]. Our study also showed a positive correlation of RBP-4 and NGAL with the HOMA index and a negative correlation with FGIR, which disappeared after adjustment for BMI. We had previously demonstrated that in children and adolescents, irrespective of BMI, there was no significant correlation between RBP4 concentrations and the HOMA index, fasting insulin or fasting glucose concentration [36]. When all correlations were adjusted for BMI, the only significant correlation remaining was a positive correlation between RBP-4 and SBP-SDS in IVF children, and between RBP-4 and triglycerides in controls, which are both factors of the MS, also confirming the assumption that RBP-4 may be a marker of metabolic abnormalities [23]. These results are in accordance with our earlier findings of higher triglycerides and blood pressure in IVF children than controls [5].

As RBP-4 was recently found to be associated with subclinical hypothyroidism and correlated with TSH levels [37] and because we have also demonstrated an increased incidence of euthyroid hyperthyrotropinaemia in IVF children, reminiscent of epigenetic modification of the TSH set point [38], we correlated RBP-4 with TSH levels in IVF children and controls, but no significant correlation was observed. Therefore, the increase in RBP-4 cannot be attributed to the higher levels of TSH in IVF children, but to the IVF procedure *per se*.

This study demonstrated no association of RBP-4, NGAL or visfatin with the MS, although some previous studies showed such an association [39,40]. However, there was a positive correlation of RBP-4 and NGAL with the number of MS criteria, in IVF children, suggesting that, although these children have not yet developed the full MS, RBP-4 and NGAL may show a trend towards more severe future metabolic abnormalities.

Despite our initial assumption, visfatin showed no differences between IVF children and controls and no association with MS. Haider *et al.* [41] reported that the release of visfatin by adipocytes was dependent on the duration and magnitude of glucose elevations. In accordance with our findings, they failed to detect a relation between visfatin and some parameters of the MS. There are also a number of inconsistencies among the different studies on visfatin, and the role of this adipokine in obesity and insulin resistance still remains unclear. Therefore, it might be still early for a childhood population to show any alterations in visfatin levels.

In conclusion, we have demonstrated a sexually dimorphic increase in the lipocalins' RBP-4 and NGAL in IVF children, especially after the onset of puberty, which further highlights the need of a careful longitudinal follow-up of IVF children to fully assess their cardiovascular risk in later life. As serum RBP4 correlated with components of the MS, such as SBP and triglycerides, and both RBP-4 and NGAL correlated with insulin resistance indices, only before adjustment for BMI, and because they are noninvasive and accessible markers of insulin resistance, diabetes type 2 and cardiovascular disease, they might be considered as potential novel early markers for the assessment of metabolic abnormalities at follow-up of IVF population. The ability to assess a person's risk of impaired glucose tolerance and diabetes type 2, before the onset of the disease, would help implement preventive lifestyle interventions, which have a better cost-benefit ratio before overt glucose intolerance and diabetes type-2 are established [30].

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Conflict of interests

The authors declare that they have no financial disclosures and reported no potential conflicts of interest.

Address

Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics, Athens University Medical School, "Aghia Sophia" Children's Hospital, Athens 11527, Greece (S. D. Sakka, G. P. Chrousos, C. Kanaka-Gantebein); Department of Clinical Biochemistry, "Aghia Sophia" Children's Hospital, Athens 11527, Greece (A. Margeli, I. Papassotiriou); Division of In Vitro Fertilization, First Department of Obstetrics and Gynaecology, Athens University Medical School, Athens 11527, Greece (D. Loutradis).

Correspondence to: Dr Ioannis Papassotiriou, Department of Clinical Biochemistry, "Aghia Sophia" Children's Hospital, Athens 11527, Greece. Tel/Fax.: +302132013171; e-mails: biochem@paidon-agiasofia.gr; ipapassotiriou@gmail.com

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