

# Transcriptomic analysis of human placenta in intrauterine growth restriction

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**BACKGROUND:** Intrauterine growth restriction (IUGR) is a frequent complication of pregnancy defined as a restriction of fetal growth. The objective of this work was to improve the knowledge on the pathophysiology of IUGR using a genome-wide method of expression analysis.

**METHODS:** We analyzed differentially expressed genes in pooled placental tissues from vascular IUGR (four pools of three placentas) and normal pregnancies (four pools of three placentas) using a long nucleotide microarray platform (Nimblegen). We first did a global bioinformatics analysis based only on *P* value without any a priori. We secondly focused on “target” genes among the most modified ones. Finally, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on an extended panel of tissue samples (*n* = 62) on selected “target”.

**RESULTS:** We identified 636 modified genes among which 206 were upregulated (1.5 and higher; *P* < 0.05). Groups of patients were classified unambiguously. Genes involved in mitochondrial function and oxidative phosphorylation were decreased affecting three out of five complexes of the respiratory chain of the mitochondria, and thus energy production and metabolism. Among the most induced genes, we identified *LEP*, *IGFBP1*, and *RBP4*.

**CONCLUSION:** Complementary studies on the role and function of *LEP*, *IGFBP1*, and *RBP4* in IUGR pathophysiology and also in fetal programming remain necessary.

Intrauterine growth restriction (IUGR) is a frequent complication of pregnancy with a prevalence of 8% in the general population. It is defined as a restriction of fetal growth during pregnancy, “a foetus that doesn’t reach its growth potential”. It can lead to a birth weight and/or length below the 10th percentile for a given gestational age in new-borns thus considered as “Small for Gestational Age” (1,2). IUGR represents a major public health problem, being one of the main causes of prematurity, perinatal mortality, neurological, and respiratory morbidities (3–6). It is also suspected to be a determining factor in the development of cardiovascular diseases, obesity, and type 2 diabetes later in adulthood (7–9).

Fetal growth is inseparable from placental growth and requires a nutrient supply both continuous and adapted to each pregnancy period, as documented by animal model analysis (10). Several microarray studies have analyzed the transcriptome of pre-eclamptic (associated or not to IUGR) placentas to identify genes that could be involved in placental dysfunction. In general, similar categories of differentially expressed genes were reported including genes involved in vascular regulation, inflammation, cell proliferation, apoptosis, differentiation, and cellular metabolism. However, in some cases, the results appeared controversial. These differences may be due to the type of IUGR, the sampling of placentas, gestational age, ethnicity, the mode of delivery, microarray platforms, and the filtering and statistical analysis. Recently, two meta-analyses of placental gene expression patterns in pre-eclampsia case/control studies have been reported, identifying consistently modified genes and biological pathways such as cell responses to inflammation, hypoxia, DNA damage, and proliferation (11,12). Up to recently, none of such analyses had been reported focusing on IUGR. The objective of this work was to compare the global expression profile of human placentas obtained after Caesarean section from pregnancies close to term complicated or not by vascular IUGR.

## RESULTS

The average term of the IUGR population was 33.8 wk of gestation vs. 38.7 in the control group, with an average weight of 1,418 g vs. 3,320 g and a sex ratio of 1:1 in the IUGR population and 5:1 in the control group (Table 1). The index of RNA integrity (RIN) obtained for each pool ranged between 7.2 and 8.1.

### Global Analysis Based on *P* Value

We have decided to first present a global analysis based only on *P* value without any a priori with Array Mining software (School of Computer Science, Nottingham University, UK). The 500 most significant genes according to their increasing *P* value are presented in greater detail in Supplementary Table S1 online. Boxplots for expression values of the four top-ranked

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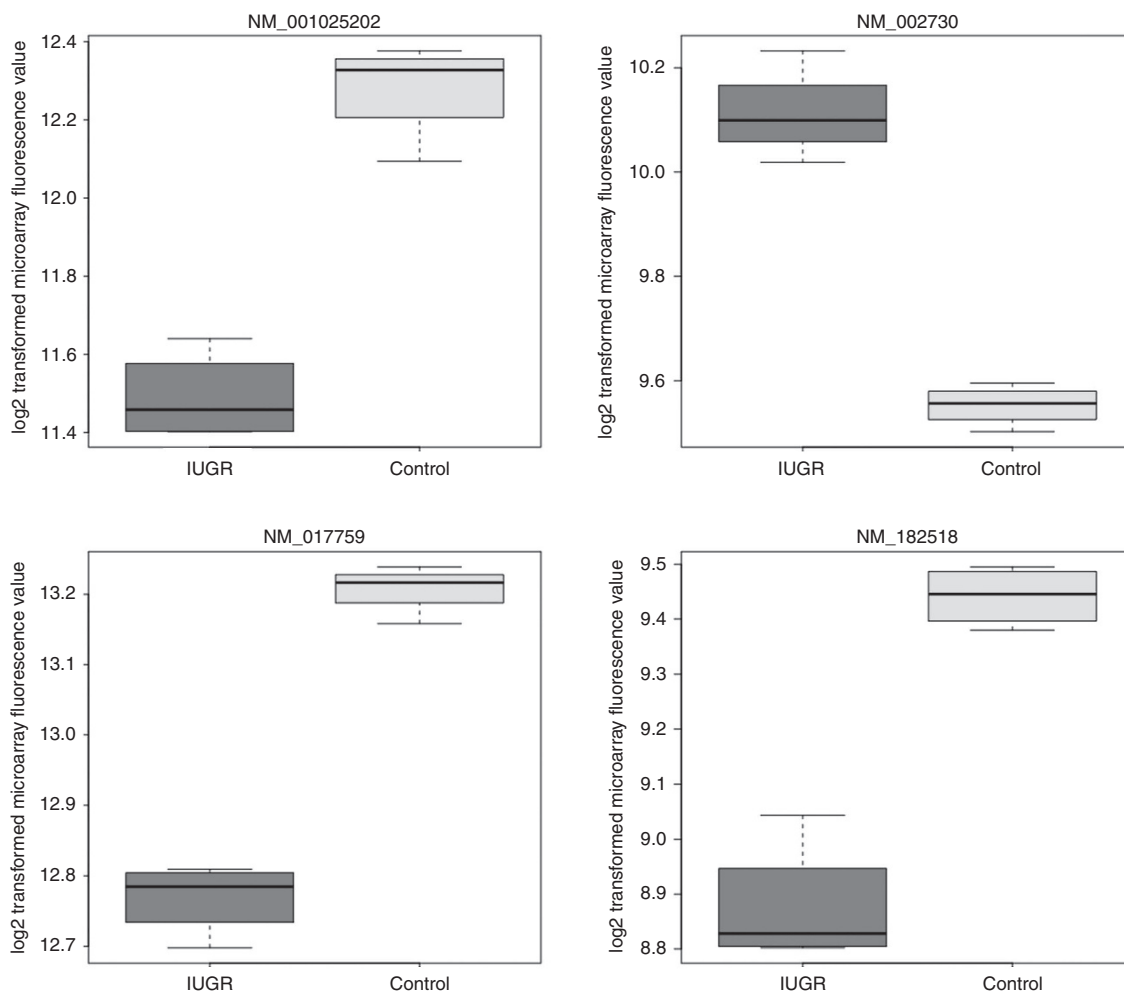
genes (*STAG3L2*, *PRKACA*, *INO80D*, *SZT2* respectively) were done with a very good segregation between IUGR and control placenta samples (Figure 1). A heatmap (nonsupervised clustering) classifying the 500 genes selected through increasing

significance classified the groups of patients unambiguously as presented in **Supplementary Figure S1** online and in **Figure 2** for the 100 most significant ones. In terms of gene ontology, starting from the 500 most modified genes, significant enriched

**Table 1.** Baseline characteristics of the mothers and newborns from whom placental samples were obtained for the microarray analysis

Group	Pool; id number	Mother's age (y)	Mother's BMI (kg/m <sup>2</sup> )	GA (wk)	Birth weight (g)	Sex ratio (male:female)
IUGR	1; 419231A01	29.7 (18; 36)	31.5 (24; 39)	33.6 (31.4; 37.3)	1,420 (1,000; 1,880)	1:2
IUGR	2; 419231A02	33.7 (30; 38)	20.5 (17.5; 25)	33.5 (32.4; 35.4)	1,347 (1,145; 1,580)	1:2
IUGR	3; 419231A03	28.7 (24; 32)	27.8 (18; 33.5)	33.5 (33; 34.3)	1,370 (1,155; 1,670)	2:1
IUGR	7; 414993A03	29.3 (29; 30)	28.5 (22; 35)	34.4 (33.3; 36)	1,535 (1,210; 1,930)	2:1
Total IUGR		30.3 (18; 38)	27.1 (17.5; 39)	33.8 (31.4; 37.3)	1,418 (1,000; 1,930)	1:1
Control	4; 419231A04	37.6 (33.4; 42.6)	21.4 (18.4; 23.7)	38.4 (38.3; 38.4)	3,343 (2,970; 3,540)	2:1
Control	5; 414993A01	37.3 (35.7; 39)	21.8 (20; 25.1)	38.1 (37; 38.9)	3,136 (2,760; 3,580)	3:0
Control	6; 414993A02	34.3 (34; 34.6)	20 (18.4; 22)	38.8 (38.6; 39)	3,145 (2,800; 3,490)	3:0
Control	8; 414993A04	37.9 (34; 40)	19.4 (18; 20.2)	39.6 (38; 39.9)	3,480 (3,340; 3,580)	2:1
Total control		37.6 (33.4; 40)	20.9 (18.4; 25.1)	38.7 (37; 39.9)	3,320 (2,760; 3,580)	5:1
<i>P</i> ( <i>t</i> -test)		<0.01	0.03	<0.01	<0.01	

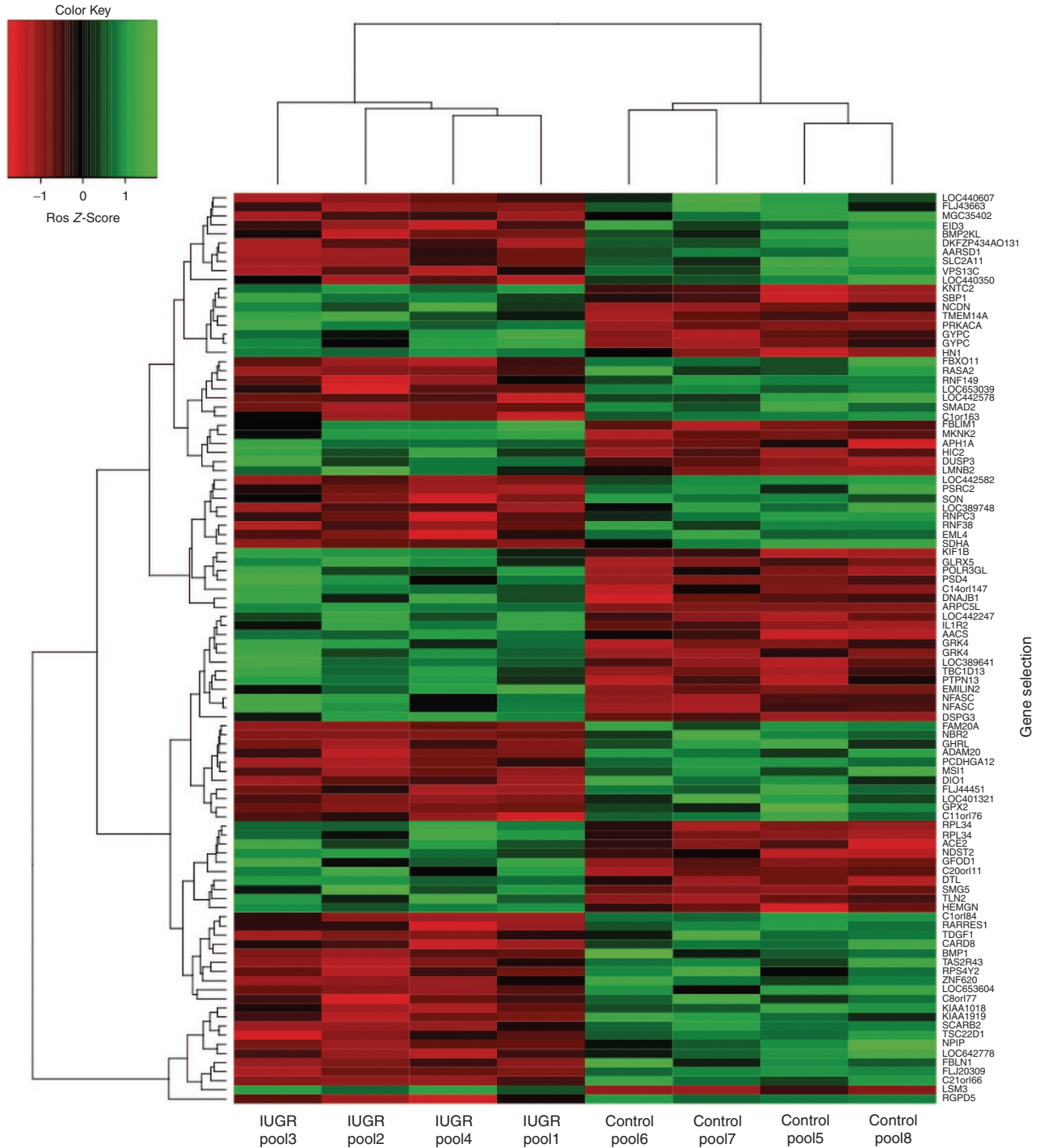
Data presented as average (min; max).



**Figure 1.** Boxplots top ranked genes ( $n = 4$ ) selected according to their *P* value, with a very good segregation between intrauterine growth restriction and control placenta samples. Y-axis represents log<sub>2</sub> transformed microarray fluorescence value.

KEGG pathways could be identified, several between them closely related to lipid metabolism (fatty acid metabolism, PPAR pathway, adipocytokine signaling pathway) (Table 2). Boxplots of enriched pathways ( $n = 4$ ) were done with a very good segregation between IUGR and control placenta samples (Figure 3). A complementary analysis by DAVID, focusing on repressed genes identified 227 downregulated transcripts and the KEGG analysis reveals downregulation of a series of genes

involved in cell division, and MAP kinase signaling pathways (anaphase promoting complex (APC/C),  $\beta$ -transducin repeat ( $\beta$ -TRCP), cyclin B1, PKC $\alpha$ ). However, the decreased expression of these genes is mild ( $-20$  to  $-30\%$ ). Similarly, genes involved in mitochondrial function and oxidative phosphorylation were decreased (NADH dehydrogenase, cytochrome C oxidase Va, ubiquinol-cytochrome C reductase), thus able to affect three out of five complexes of the respiratory chain of the



**Figure 2.** Heatmap representation of the 100 most significant genes selected through their increasing  $P$  value. Each measurement represents the normalized ratio of fluorescence from the hybridized experimental material to a common internal reference (intrauterine growth restriction and control).

**Table 2.** Pathways analysis: according to significant enriched KEGG pathways and according to gene ontology (Array Mining Software)

Pathway name (KEGG)	PC (meta-gene vs. outcome)	Q-values	Median fold change
04742 Taste transduction	-0.81	0.28	1.14
00310 Lysine degradation	0.87	0.28	1.19
05130 Pathogenic <i>Escherichia coli</i> infection—Enterohaemorrhagic <i>E. coli</i>	0.81	0.57	1.05
05131 Pathogenic <i>Escherichia coli</i> infection—Enteropathogenic <i>E. coli</i>	0.81	0.57	1.05
04920 Adipocytokine signaling pathway	0.7	0.59	1.22
00071 Fatty acid metabolism	0.8	0.59	1.11
00512 O-Glycan biosynthesis	0.77	0.66	1.14
04120 Ubiquitin mediated proteolysis	0.81	0.66	1.03
00562 Inositol phosphate metabolism	0.73	0.66	1.16
03320 PPAR signaling pathway	0.78	0.71	1.14
Pathway name (gene ontology)	PC (meta-gene vs. outcome)	Q-values	median fold change
GO:0044433 cytoplasmic vesicle part	0.85	0.79	1.1
GO:0006814 sodium ion transport	-0.91	0.79	15.31
GO:0010564 regulation of cell cycle process	0.83	0.79	15.31
GO:0001558 regulation of cell growth	0.74	0.79	1.13
GO:0048015 phosphoinositide-mediated signaling	0.71	0.79	15.31
GO:0019748 secondary metabolic process	0.7	0.79	3.45
GO:0031402 sodium ion binding	-0.82	0.79	15.31
GO:0051258 protein polymerization	0.77	0.79	1.08
GO:0000075 cell cycle checkpoint	0.79	0.79	1.39
GO:0031968 organelle outer membrane	0.78	0.79	1.08

Q-values, the minimum false discovery rate at which the test may be called significant.  
PC, Pearson correlation.

mitochondria, and affect energy production and metabolism. The downregulation, while significant in terms of statistics is mild (-25 to -30%), which may nevertheless have a biological effect.

The same analysis on the part of significantly induced genes in the list of 500 identified, allowed to identify 157 transcripts, among which the most important category grouped genes involved in mRNA metabolism, processing, and transport. Among these 157 genes, 11 are involved in RNA binding with induction ratios from 1.17 to 2.28 (Table 3).

### Target Approach Based on the Ratio of Expression

The array analysis allowed detecting 23,456 transcripts, among which only 636 were differentially expressed at a threshold of 1.5 ( $P < 0.05$ ) in placentas with IUGR, representing 2.7% of the whole transcripts tested, among which 206 were induced and 430 repressed.

The 500 most significant genes according to their  $P$  value are different from the one selected only on the ratio of expression. Indeed, 3,667 transcripts among the 23,456 detected have a  $P$  value below 0.05. Nevertheless, we found a good correlation between individual genes and pathways when we focused on the first 2,000 most significant genes according to their  $P$  value or to their ratio of expression.

Among the most induced genes in IUGR, we identified *LEP* (leptin) (3.5-fold), *IGFBP1* (insulin-like growth factor binding protein 1) (3.4-fold) and *SERPIN A3* (2-fold) (Table 4).

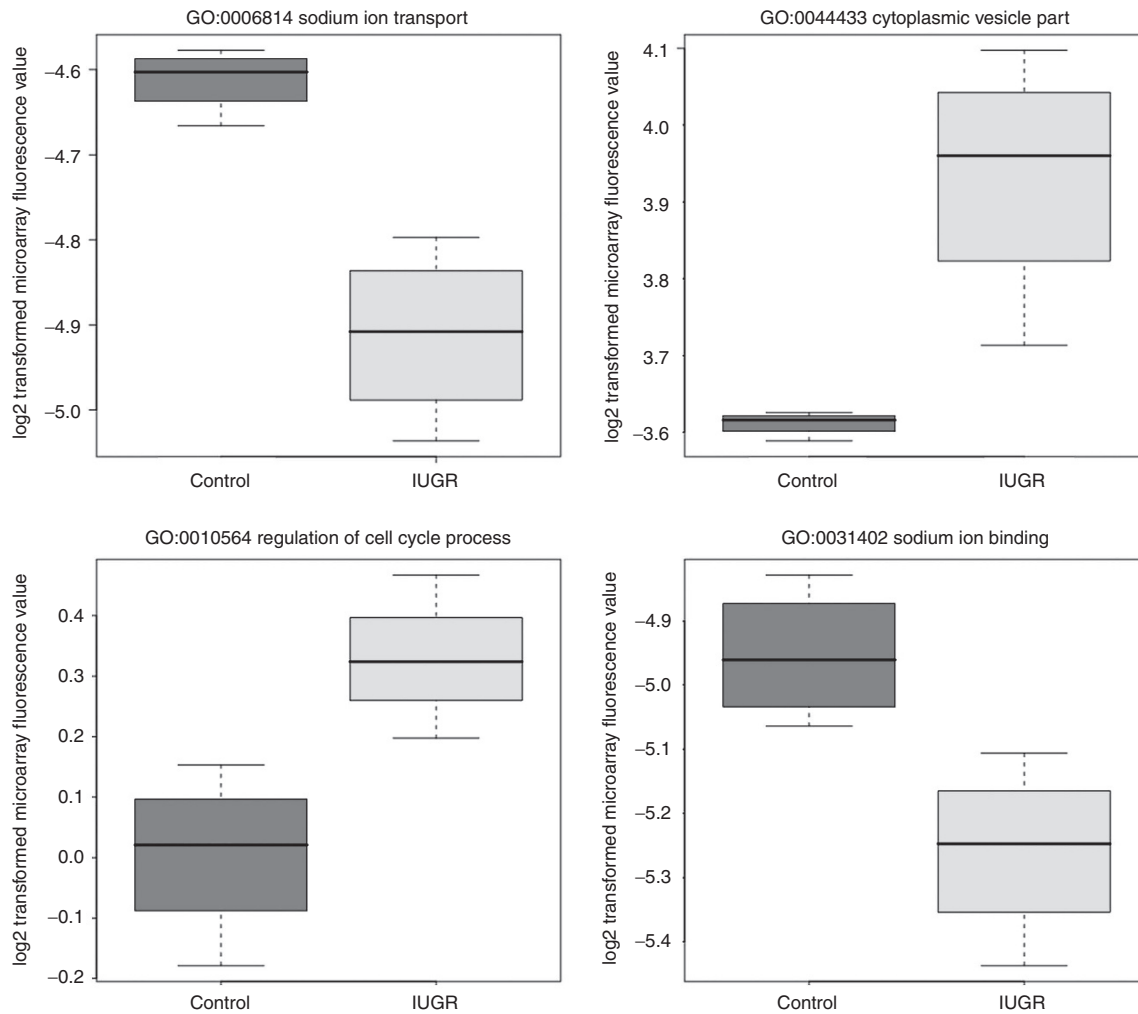
The bioinformatic analysis of signaling pathways by clustering on the most modified genes in IUGR revealed gene clusters involved in growth control, response to insulin, hormonal regulation with a cluster encompassing *IGFBP1-LEP-RBP4* (retinol-binding protein 4)—ceruloplasmin-chemokine-complement factor H related 1-fibrinogen  $\gamma$ -chain (enrichment score 1.31).

The promoters of the most modified genes ( $n = 40$  induced and  $n = 40$  repressed genes) were compared to a panel of 40 random nonmodified genes with the Genomatix software. It highlighted a significant increase ( $P < 0.01$ ) in the frequency of potential binding sites for known transcription factors including CTCF (CCCTC-binding factor), which belongs to the Zinc Finger protein family.

### Validation of Microarray Data

qRT-PCR on an extended panel of normal placentas ( $n = 21$ ), placentas with IUGR ( $n = 26$ ), with pre-eclampsia ( $n = 9$ ), with pre-eclampsia and IUGR ( $n = 6$ ) successfully validated the microarray data for a selection of deregulated genes: *LEP*, *IGFBP1*, and *RBP4* (Table 5; Figure 4). The expression level of each gene was also analyzed according to the sex of the fetus. We confirmed a highly significant increase in the expression of these three genes in IUGR placentas. We compared the present data with that obtained from the recent study by Nishizawa et al. (13), with a particular interest in the most modified genes in our data ( $n = 636$ ) (publicly available on GEO profiles <http://www.ncbi.nlm.nih.gov/gate2.inist.fr/geoprofiles>). The two data sets were found to be strongly correlated ( $r = 0.41$ ;  $n = 353$ ;  $P < 0.001$ ) and a list of 13 common genes were shown to be similarly and significantly affected (Table 6).

Surprisingly, among the 13 commonly modified genes, 5 genes were located on the Y chromosome and significantly repressed. Among the genes from the Y chromosome that were significantly different in IUGR placentas (ratio  $< 0.67$  and  $> 1.5$ ) in our study, 12 genes were repressed while only one was induced, a very significant bias toward repression ( $P < 9 \times 10^{-17}$ ). We had the same observation when we focused our statistical analysis only on the pool with the same sex-ratio 2:1 male:female (IUGR pool 3 and 7; control pool 4 and 8).



**Figure 3.** Boxplots of enriched pathways ( $n = 4$ ) were done with a very good segregation between intrauterine growth restriction and control placenta samples. Y-axis represents  $\log_2$  transformed microarray fluorescence value.

**Table 3.** Significantly induced genes involved in RNA binding in the list of the 500 most modified genes according to  $P$  value

Genbank_Accession	Gene name	Induction ratio
NM_004640	HLA-B associated transcript 1	1.33
NM_017619	RNA-binding region (RNP1, RRM) containing 3	1.43
NM_032195	SON DNA binding protein	1.80
NM_022037	TIA1 cytotoxic granule-associated RNA binding protein	1.46
NM_001033506	Cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kDa	1.87
NM_001889	Crystallin, zeta (quinone reductase)	1.49
NM_003902	Far upstream element binding protein 1	1.51
NM_002024	Fragile X mental retardation 1	1.17
NM_002442	Musashi homolog 1 (Drosophila)	1.38
NM_001039567	Ribosomal protein S4, Y-linked 2	2.28
NM_007185	Trinucleotide repeat containing 4	1.59

In addition, there was a very strong correlation between the repression ratio ( $<0.67$ ) and the position of the genes along the Y chromosome ( $r = 0.75$ ;  $n = 12$ ;  $P = 0.002$ ).

## DISCUSSION

We analyzed differentially expressed genes in pooled placental tissues from vascular IUGR and normal pregnancies (four pools of 3 placentas each) using a microarray method. We identified 636 modified genes among which 206 were upregulated.

One limitation of our study was the difference in gestational ages, even if the IUGR new-borns population is a mild preterm one. But, matching gestational control placentas would have been premature ones and thus exposed to RNA variations due to other causes of premature births (such as preterm labor, preterm rupture of membranes, chorioamnionitis and so on). Moreover, the nonspecific transcriptional variations that could occur between placentas from different gestational ages, is mainly described in extreme gestational ages (13). We also only used placentas collected after Caesarean sections to avoid the putative alterations in gene expression induced by labor. The inclusion of vascular IUGR placentas permits the study

**Table 4.** Most modified genes according to ratio of expression

Ten most induced genes			Ten most repressed genes		
Gene name	Genbank_Accession	Induction ratio	Gene name	Genbank_Accession	Induction ratio
<i>S100A14</i>	NM_020672	4.31	<i>C8orf79</i>	NM_182598	0.45
<i>LEP</i>	NM_000230	3.48	<i>C3orf65</i>	NM_001039788	0.45
<i>IGFBP1</i>	NM_001013029	3.41	<i>ANP32D</i>	NM_012404	0.45
<i>RBP4</i>	NM_006744	3.34	<i>BEX1</i>	NM_018476	0.45
<i>BTNL9</i>	NM_152547	3.20	<i>RPS4Y2</i>	NM_001039567	0.44
<i>IGFBP1</i>	NM_000596	2.97	<i>TARP</i>	NM_001003799	0.43
<i>FGG</i>	NM_021870	2.93	<i>FBXO32</i>	NM_148177	0.42
<i>VCY</i>	NM_004679	2.83	<i>OR51B5</i>	NM_001005567	0.39
<i>TREM1</i>	NM_018643	2.68	<i>PPIAL4</i>	NM_178230	0.37
<i>KRTHA4</i>	NM_021013	2.66	<i>OR51B4</i>	NM_033179	0.28

Ratios associated with a probability based on a Student's test,  $P < 0.05$ .

**Table 5.** Baseline characteristics of the mothers and newborns from whom placental samples were obtained for reverse transcription quantitative polymerase chain reaction

Total (n = 62)	Gestational age (weeks)	Birth weight (g)	Sex ratio (male:female)
Control (n = 21)	38.9 (38; 40.3)	3,506 (3,030; 4,430)	9:12
IUGR (n = 26)	34.4 (28.8; 38)	1,487 (700; 2,330)	13:13
PE (n = 9)	32 (29.7; 34.3)	1,557 (1,070; 1,960)	3:6
IUGR + PE (n = 6)	33.2 (28.7; 38.4)	1,485 (830; 2,400)	2:4

Data presented as average (min; max).  
PE, pre-eclampsia.

of a more reliable and homogeneous population. While pooling of samples for microarray experiments might be considered as a limitation of this study, we successfully validated the most significant data on individual placentas and on a larger panel of samples from all groups. Furthermore, we were able to find some common transcriptional changes with two similar recent reports (14,15), which studied populations of similar gestational age and using the similar method of placenta collection after Caesarean section. The small number of significantly modified genes, 252 found by Nishizawa *et al.* (14) and 241 found by Struwe *et al.* (15), may be due to the disease by itself that is heterogeneous in nature. The heterogeneity in cell types within the placenta can also affect the signal-to-noise ratio in the identification of modified genes (16). We have only suggestive elements and no strong evidence of downregulation of Y-linked genes in IUGR placenta and the sex ratio to 1:5 male:female in the control group may constitute a limitation in our observation.

### Signaling Pathways

In IUGR, pathways encompassing genes involved in cell cycle are enriched. By all means, there is an impact on these cascades, whatever the sense. It is also possible that the effect is overall very small when opposite mechanisms attempt to compensate the IUGR effects in terms of shortage of nutrients for instance.

Genes involved in mitochondrial function and oxidative phosphorylation were decreased affecting three out of

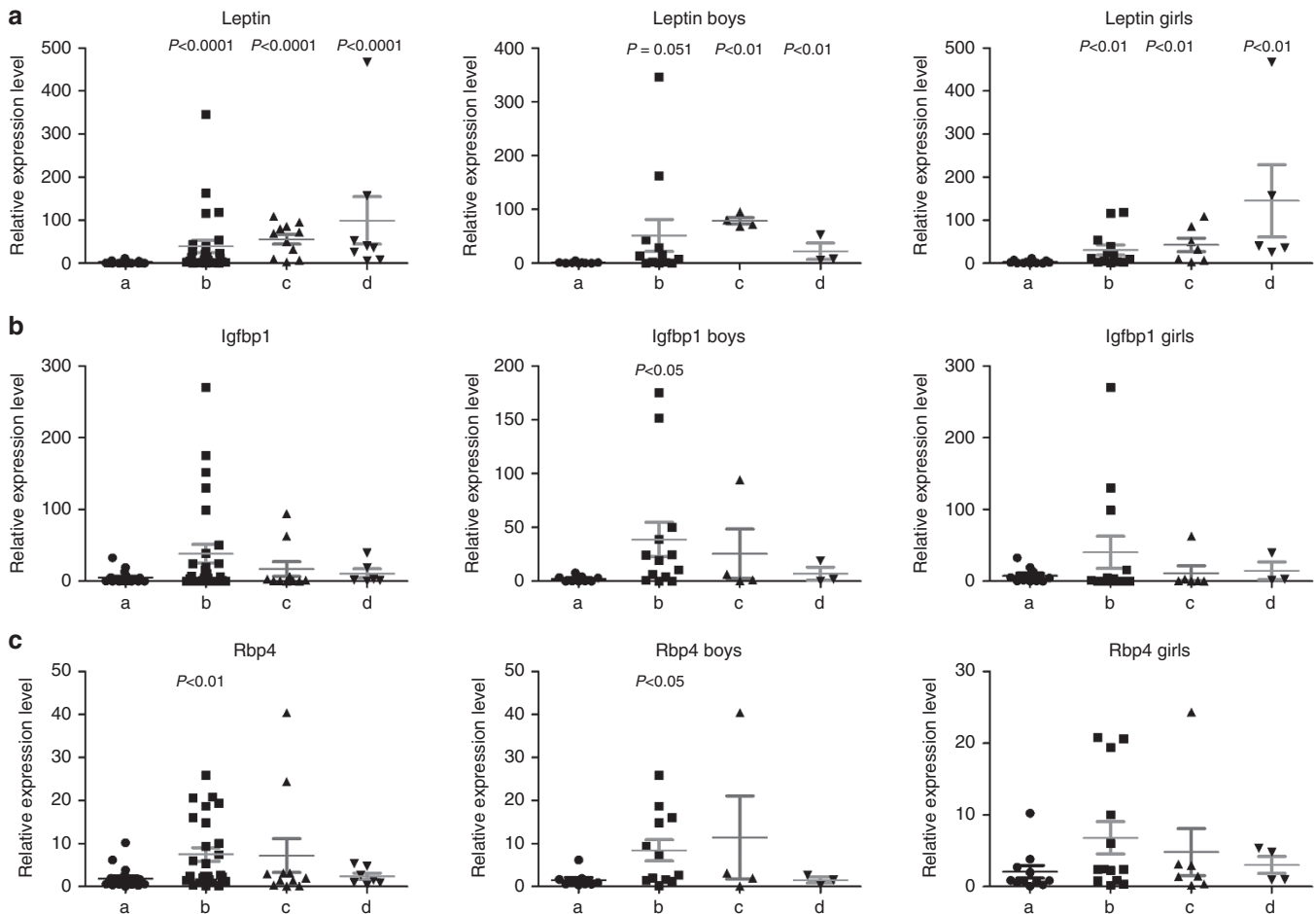
five complexes of the respiratory chain of the mitochondria, and thus energy production and metabolism. Recent studies done on placentas from pregnancies with IUGR and/or pre-eclampsia suggested modifications on placental mitochondrial content and function that could be important in the genesis of pre-eclampsia or IUGR (17,18). Lower mRNA levels of complex II, III, and IV were found in IUGR cytotrophoblast cells but no differences at the protein level (18). Finally, cytotrophoblast respiratory chain complexes activity was significantly increased in placentas of IUGR fetuses (18). Nevertheless, increased placental oxygen consumption might represent a limiting step in fetal growth restriction, preventing adequate oxygen delivery to the fetus.

RNA metabolism alterations in IUGR were already seen in the placenta in one of our previous works in the rat model where IUGR was triggered by maternal protein restriction during pregnancy (10). It was especially apparent in induced gene clusters, where SWI/SNIF, subunits of the DNA polymerase and splice factors were duly modified, and may suggest a general involvement in adaption to IUGR either experimentally induced in animal models or in humans. While the data about this aspect are relatively scarce, it would be an interesting trail to follow to understand the consequences of IUGR. In some rare human syndromes including growth retardation among the symptoms (Taybi-Linder syndrome), mutations in elements of the RNA splicing complex (U4atac snRNA) were indeed discovered (19).

### Target Genes

Among the genes most highly induced by IUGR, we observed inductions in *LEP* (3.5-fold), also found by Nishizawa and Struwe, *IGFBP1* (3.4-fold) and *RBP4* (3.3-fold) as also found by Struwe.

*Placental leptin* is produced by syncytiotrophoblasts and has both autocrine and paracrine actions. It is exported to both maternal and fetal circulations, affecting maternal (fertility and energetic metabolism), placental (angiogenesis, growth, and immune tolerance), and fetal functions (implantation, development, and growth) (20–22). A deregulation of leptin



**Figure 4.** Expression of the *LEP* (a), *IGFBP1* (b), and *RBP4* (c) genes in normal and pathological placentas and stratified according to the sex of the fetus by real-time quantitative RT-PCR. Expression levels were normalized to succinate dehydrogenase subunit A and TATA box binding protein as housekeeping genes. Data are presented as mean  $\pm$  SEM and the expression in pathological placentas (intrauterine growth restriction (IUGR) (b, black squares) ( $n = 26$ ); pre-eclampsia (PE) (c, black triangles) ( $n = 9$ ); IUGR + PE (d, black upside-down triangles) ( $n = 6$ )) were presented as ratios relative to the control group (a, black circles) ( $n = 21$ ).

**Table 6.** List of genes similarly and significantly affected in previous transcriptomic study

Gene name	Genbank_Accession	Induction ratio	Induction ratio (14)
<i>LEP</i>	NM_000230	3.48	2.78
<i>CXCL9</i>	NM_002416	2.40	1.81
<i>HTRA4</i>	NM_153692	1.94	1.81
<i>FSTL3</i>	NM_005860	1.93	1.67
<i>NNAT</i>	NM_005386	1.78	1.78
<i>HIST1H1T</i>	NM_005323	0.65	0.63
<i>PCDH11X</i>	NM_032967	0.63	0.62
<i>GABRE</i>	NM_004961	0.63	0.58
<i>RPS4Y1</i>	NM_001008	0.61	0.61
<i>USP9Y</i>	NM_004654	0.61	0.55
<i>DDX3Y</i>	NM_004660	0.55	0.51
<i>EIF1AY</i>	NM_004681	0.54	0.66
<i>UTY</i>	NM_182660	0.51	0.58

Ratios associated with a probability based on a Student's test,  $P < 0.05$ .

metabolism and/or function in the placenta is suspected in some pregnancy pathologies including recurrent fetal loss, gestational diabetes, IUGR, and pre-eclampsia, and is associated with growth defects and metabolic anomalies (23–25). In addition, resistance to leptin is a physiopathological mechanism implicated in obesity (26).

*IGFBP1* has a high affinity for both insulin-like growth factor (IGF)1 and IGF2, which are important fetal and placental growth factors (27). An increase in *IGFBP1* expression could inhibit IGF function in the fetoplacental unit. The placental expression of *IGFBP1* is negatively correlated to both placental weight and birth weight (18). In addition, *IGFBP1* acts as an antiapoptotic factor in fetal liver cells (28). In the placenta, it could protect the fetoplacental unit from an excess of apoptosis secondary to placental insufficiency. Moreover, *IGFBP1* has recently been shown to be a marker of insulin resistance and obesity (29).

The *RBP4* gene, coding for a retinoic acid transporter, has also been identified in other studies as modified in IUGR. It has been shown that the umbilical serum concentration of *RBP4* was correlated with the child's birth weight (30). The circulating level of *RBP4* has been shown to be increased in a

rat model IUGR and later development of obesity. This level is normalized by physical exercise (31). A high level of RBP4 is also associated with an increased incidence of glucose intolerance, obesity, and arterial hypertension (32,33).

*LEP*, *IGFBP1*, and *RBP4* overexpression therefore provide a good rationale for both the development of IUGR and also the potential progression toward a metabolic syndrome in adults that were growth restricted *in utero*.

### Conclusion

*LEP*, *IGFBP1*, and *RBP4* are good candidates to play a role in the origin of IUGR as well as to predict the potential development toward a metabolic syndrome in adults that were growth restricted *in utero* (IUGR). Complementary studies on the role and function of these genes in IUGR pathophysiology and also in fetal programming of cardiovascular pathologies, obesity, and glucose intolerance in adulthood remain necessary.

### METHODS

#### Ethical Issues and Patients

All placentas were collected from two maternity wards (Hôpital Cochin and Institut de Puériculture, Paris, France). This study was approved by the Ethics Committee and CCPPRB (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) of Paris Cochin. All patients gave their written consent for the use of their placenta and blood samples. Placentas of 30 or more weeks of gestation were obtained from Caesarean sections before onset of labor. For the microarray analysis, patients were classified in two groups: isolated vascular IUGR ( $n = 12$ ) and control group ( $n = 12$ ). Vascular IUGR was defined by a reduction of fetal growth during gestation, with a notch observed by Echo-Doppler in at least one uterine artery and with Doppler abnormalities on umbilical Doppler and/or cerebral Doppler and/or ductus venosus, and with a birth weight below the 10th percentile according to Audipog growth curves (34). Vascular IUGR was confirmed by the anatomopathological analysis of the placenta after birth. Women who exhibited any of the following criteria were excluded from the study: diabetes, pre-eclampsia, chromosomal and fetal malformations, maternal infections, aspirin treatment, and additions (such as tobacco or drug usage). In addition, an extended and separate panel of control, vascular IUGR and/or pre-eclamptic placentas was used for further validation studies ( $n = 62$ ). The inclusion criteria used for pre-eclampsia were systolic pressure above 140 mmHg, diastolic pressure above 90 mmHg and proteinuria above 0.3 g per day. Women who underwent Caesarean section without disease during pregnancy formed the control group.

#### Placental Biopsy Collection

All IUGR and control placental biopsies were obtained following Caesarean sections to avoid any effects of labor upon gene expression. To avoid RNA degradation, only placental tissues dissected less than 30 min after delivery were included. After removal of maternal deciduas and amniotic membranes, sections of 1 cm<sup>3</sup> of placental villi were dissected from four different cotyledons between the basal and chorionic plates, as previously described (35,36). After vigorous washing with saline solution (0.9%) to remove maternal blood, tissues were immediately immersed in Trizol (5 ml/g, Invitrogen, Carlsbad, CA) and stored at  $-80^{\circ}\text{C}$  until use. Placenta samples obtained from different areas of a single placenta were pooled before RNA extraction.

#### RNA Preparation, cDNA Synthesis, and Microarray Hybridizations

RNA isolation of 12 control and 12 IUGR placenta samples was performed according to standard protocols using Trizol reagent and samples were quantitatively and qualitatively controlled (37). Three individual placenta samples within a group were then pooled, to produce four pools of vascular IUGR and four pools of normal

pregnancy for the control group. The eight pools were then evaluated for purity, integrity, and quantification by the Agilent Bioanalyser 2100<sup>R</sup>. One microgram total RNA from each pool was reverse transcribed, labeled, and then hybridized to NimbleGen Human HG18 60mer 4x72K arrays representing 23,456 human transcripts. Image analysis was performed with the NimbleScan software (Roche NimbleGen, Madison, WI), and feature intensities were exported as pair files. ArrayStar 3.0<sup>R</sup> software (DNASTAR, Madison, WI) was used for probe summarization and normalization (RMA algorithm, quantile normalization), statistical analysis of differentially expressed genes (Student's *t*-test with Benjamini-Hochberg false discovery rate correction), and gene ontology analysis. The entire microarray data set is available at the EMBL-EBI ArrayExpress site under the reference E-MTAB-1956.

#### Real-Time RT-PCR

Validation of the microarray data was done by quantitative real-time PCR (qRT-PCR) for a selection of deregulated genes of interest on individual placentas from a separate cohort of normal placentas ( $n = 21$ ), placentas with IUGR ( $n = 26$ ), with pre-eclampsia ( $n = 9$ ), with pre-eclampsia and IUGR ( $n = 6$ ) was used. cDNA synthesis was performed using the MMLV cDNA or the SuperScript II synthesis kits (Invitrogen) from 2  $\mu\text{g}$  of RNA, previously treated with DNase I. Primers were designed using the PRIMER3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). The list, sequences, and conditions of use of the primers are in **Supplementary Table S2** online. Real-time PCR was performed using the LightCycler 480, the corresponding LC480 SYBRGreen Master kit and 96-well plates (Roche, Madison, WI). Conditions were as follows: 95  $^{\circ}\text{C}$  for 5 min, and 40 cycles of three temperature steps (95  $^{\circ}\text{C}$  for 10 s, 55  $^{\circ}\text{C}$  for 10 s, and 72  $^{\circ}\text{C}$  for 10 s). Finally, samples were submitted to a progressive temperature ramping resulting in a melting curve, validating the specificity of the amplification. Amplification products were also checked by agarose gel electrophoresis and sequencing. The threshold cycle numbers ( $C_t$ ) were collected using the LightCycler 480 software (Roche) in the exponential phase of the amplification. The  $C_t$ 's was normalized by the  $C_t$  values obtained for the succinate dehydrogenase subunit A and TATA box binding protein, which have been shown previously to be stable and highly expressed in the human placenta (38). Succinate dehydrogenase subunit A and TATA box binding protein, which were used for normalization did not differ between the experimental groups in our study. Experiments were conducted in triplicates. As the data were normally distributed, we did ANOVA with a *post hoc* test (Student-Newman-Keuls).

#### Microarray Analysis

Global analysis was first made according to *P* value. Nonsupervised clustering analyses were performed using the Array Mining software (School of Computer Science, Nottingham University, UK) (39) and DAVID database (Database for Annotation and Visualization and Integrated Discovery, Laboratory of Immunopathogenesis and Bioinformatics, Frederick, MD). Heatmaps were generated from the most significantly modified genes based on the *P* value using Array Mining software. Pathway analysis was carried out using Array Mining software and DAVID database. Secondly, the tables of genes modified more than 1.5-fold with a  $P < 0.05$  were submitted as text files with the level of induction/repression. This made it possible to generate pathways with a  $P < 0.01$  threshold for significance in the gene clustering. Promoters of the forty most induced and repressed genes compared to a panel of 40 nonmodified genes were obtained using the Genomatix software and database (Munich, Germany), selecting the gold promoters where possible and silver ones alternatively. Predicted transcription factor binding sites were identified and counted. A statistical analysis was performed by comparing the induced and repressed genes to the control group using a Student *t*-test corrected for multiple testing.

#### Statistical Analysis

The induction ratios were calculated from the difference between the raw data of fluorescence  $\log_2$  transformed. Correlation tests were performed with these ratios using Microsoft Office Excel and GraphPrism. Values of  $P < 0.05$  were considered significant.



## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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