Title: Placental Determinants of Fetal Growth: Identification of Key Factors in the Insulin-Like Growth Factor and Cytokine Systems using Artificial Neural Networks.

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Author's response to reviews: see over
Dear Editor,

please find the following amendments to the manuscript as suggested by the reviewers which we thank for their helpful comments. We appreciate having given us the opportunity to revise the manuscript. We are attaching a revised version of the manuscript, with changes in bold, and the following cover letter which addresses specifically all points raised by the reviewers.

Answers to Reviewer L. Ibanez:

Reviewer's report:
“The Study Population should be better described. For example, it is not clear why the authors chose AGA newborns delivered by cesarean section. In addition, all FGR newborns were preterm. I wonder whether the present results can be also applied to: 1) full term FGR newborns; 2) newborns delivered vaginally. The authors should provide birthweight SDS.
-In line with the above mentioned, the authors should discuss the relevance of the present results in FGR term pregnancies”.

Reply:
Thank you for this comment. We have now described better the study population in the subjects section in patients and methods. In particular, we have specified how we selected the FGR and AGA newborns. At variance, with previous studies this is not a retrospective study and thus the distinction was not between AGA and SGA, based on birth weight and/or birth length only.
We identified fetal growth restriction by US during pregnancy, all with an onset before 32 weeks GA, thus the classical distinction of -2 SD with respect to the referral was not used. That the FGR were premature is not surprising as almost all cases of “real FGR” require birth before term by CS. Thus, controls were also born from CS to avoid confounding factors modifying assays in placental lysates. Data comparing FGR and AGA comparable for gestational age and mode of delivery were previously published (Street et al. Eur J Endocrinol. 2006, 155: 567-574).
As specified in the “database section” in patients and methods this new study (Page 7, database section) re-analysed the data, with TNF-alfa in addition, with a completely new method with the aim to analyse this known set of clinical and biochemical data in FGR and normal newborns to establish if a mathematical model existed and was capable of identifying conditions of FGR and appropriateness for gestational age, identifying the variables, among those analysed, which had a mathematically consistent biological relevance to fetal growth, and their relationships (introduction). The mean gestational age is different from the previous study as some cases had to be excluded as ANNS require the database to be 100% complete for all variables. Moreover, ANNS study non-linear relationships between variables, and work independent of gestational age, sex, birth weight, etc. Moreover, the system works “blind” of the conditions AGA and FGR. Thus, results are applicable to all newborns in all conditions.

Finally, the published Italian reference data for birth weight do not allow the calculation of SDS (50° percentile values and SDs for each week of GA are not available) which is not required anyway for this kind of analysis.

The relevance of the findings consists in identifying the peptides within the IGF system and the proinflammatory cytokines studied which have a key role or rather a significant biological relevance to the growth of the fetus and, thus, are the most affected in FGR. This has been evidenced in the discussion, 2nd paragraph: “….This meant that those variables contained specific information on the occurrence of FGR. In particular, IGF-II, IGFBP-2 and IL-6 concentrations in placental lysates were the most important determinants of fetal growth”, and in the conclusions: “These analyses confirmed the importance of the biochemical variables, IL-6, IGF-II and IGFBP-2 protein concentrations in placental lysates, and offered a new insight into placental markers of fetal growth within the IGF and cytokine systems, confirmed they had relationships and offered a critical assessment of studies performed to date. It remains to be elucidated whether the factors identified at the placental site are part of a pathological process or in connection with regulatory mechanisms induced by disturbances in the placenta resulting from previous pathological changes. The data provided useful information for the directions of future research, and the overall evidence suggested that further research in humans should focus on these biochemical data, at variance with the studies which have focused mainly on IGF-I and IGFBP-1”. The understanding of this biological system is
relevant to the development of future therapeutical interventions possibly aiming at reducing IL-6 and IGFBP-2 concentrations preserving IGF bioactivity in both placenta and fetus. This concept has now been added to the abstract and conclusions (in bold)).

**Answers to Reviewer C. Levy-Marchal**

*Reviewer’s report:*

“ This study explored the association between the IGF’s and cytokine systems and fetal growth with novel non-linear approaches (supervised neural networks and semantic connectivity maps). Data indicate that both IGF'S and IL-6 protein concentration in placental lysates are able to predict fetal growth restriction.

I am myself aware of the concept of biological supervised networks but I am not familiar with the methodology implies and I would suggest that most the readers would claim the same.

I have therefore a few major methodological questions. When we refer to standardized regular statistical tests, we all know the limitation and we know how to calculate the sample size to conclude on the data. Here, the population size is very limited (20 and 28) and there is no comment how this sample size may affect the observation “.

*Reply:*

Thank you for this observation.

An important obstacle in approaching in a conventional manner the mathematical basis of a relatively rare condition like FGR, is related with the difficulty to find an homogeneous sample population large enough to be analysed for a wide number of clinical and laboratory variables. Artificial neural networks, at variance with the classical statistical tests, can manage complexity even with relatively small samples and the subsequent unbalanced ratio between variables and records. In this connection, it is important to note that adaptive learning algorithms of inference, based on the principle of a functional estimation like artificial neural networks, overcomes the problem of dimensionality.
The internal validation of the prediction accuracy is one of the most important problems in neural networks analysis. In fact, the restriction of training procedures to just a part of the dataset, generally to half of it, causes the potential loss of power to recognize hidden patterns. In this study the issue of optimization of the training and testing procedure was addressed with the use of the evolutionary algorithm training and testing, which ensured that the two halves of the dataset contained the same amount of relevant information. Thus, the best division of the whole dataset into a training and a testing set was reached after a finite number of generations.

In this study we succeeded in collecting almost 50 medical cases with an accurate diagnosis of FGR or AGA. Although the FGR newborns analyzed here represent a little cohort, it is well representative from an epidemiological point of view.

Furthermore, it is extremely difficult to collect placental samples at birth from newborns of whom one has precise follow-up data during pregnancy and thus has the entire history of FGR. The distinction of FGR and AGA is not based simply on birth weight but the criteria was the following “All pregnancies were dated correctly by ultrasound during the first trimester of gestation”. “The FGR pregnancies were diagnosed by ultrasound according to the following criteria: abdominal circumference <10th centile and shift of fetal growth with a reduction of abdominal circumference with respect to the measure taken within the 20th week of gestation. The diagnosis of FGR was made within the 32nd week of gestation and was ascribed to a probable placental cause after excluding other causes as infections, chromosomal abnormalities, genetic syndromes, maternal malnutrition, substance abuse, gross placental abnormalities and multiple fetuses” as specified in the subjects section in Patients and Methods.

This is very different from collecting data retrospectively based on birth weight and/or length only, and explains why the sample size is smaller. Moreover, results and conclusions in previous studies on placentas came from as many as 14 samples (Laviola L et al. Endocrinology 146: 1498, 2005).

**Reviewer's report:**

“The authors aim at comparing placentas from AGA newborns and those from Fetal Growth Restriction. However, this small group encompasses not only FGR but also prematures and this is a very different issue between maturation and fetal
growth restriction. The degree of fetal growth restriction is not indicated.

What was the rationale to select the IGF’s system and some cytokines and no other hormone involved in fetal growth, such as insulin, cortisol and other ...?

Reply:
Prior to this study we analysed physiological variations from week 35 to 40 of gestation, studying two groups of controls (Street et al. Interleukin-6 and insulin-like growth factor system relationships and differences in the human placenta and fetus from the 35th week of gestation. Growth Horm IGF Res. 2006 Oct-Dec;16(5-6):365-72. Epub 2006 Nov 13).

Based on these results we compared FGR cases with controls of comparable gestational age, all born after 32 weeks of gestational age using traditional statistical analysis as “usually” appropriate (Street et al., Interleukin (IL)-6 and IGF-IGFBP relationships in placenta and cord Blood: possibile determinants of fetal growth restriction (IUGR). *Eur J Endocrinol.* 2006, 155: 567-574).

This new study, as specified (Page 7, database section) re-analysed the data, with TNF-alfa in addition, with a completely new method with the aim to analyse this known set of clinical and biochemical data in FGR and normal newborns to establish if a mathematical model existed and was capable of identifying conditions of FGR and appropriateness for gestational age, identifying the variables, among those analysed, which had a mathematically consistent biological relevance to fetal growth, and their relationships (introduction). ANNS study non-linear relationships between variables, and are used thus independent of gestational age, sex, birth weight, ... . Moreover, the system works “blind” of the conditions AGA and FGR.

The reasons for studying pro-inflammatory cytokines and IGF system is the result of several studies from previous years (Street M.E. et al. Interleukin-1β (IL-1β) and IL-6 modulate insulin-like growth factor binding protein (IGFBP) secretin in colon cancer epithelial (caco-2) cells. *J Endocrinol* 2003; 179 (3): 405-415; Street ME et al. The insulin-like (IGF)-IGF binding protein (IGFBP) system is modulated by interleukin-1β and interleukin-6 in chronic inflammatory bowel disease.*Hormone Research* 2004; 61 (4): 159-164; 53.

The reasons for studying these relationships in FGR are specified as follows:

“Most cases of FGR are still of unknown origin and therapeutic interventions to date are unsatisfactory. The IGF-IGFBP system is crucial for fetal growth, as experiments in knockout mice have shown. Cytokines are also thought to play an important role in regulating placenta formation and growth although they are poorly studied. The placenta produces pro-inflammatory cytokines as IL-6, and TNF-α and human decidua cells, in vitro, secrete IL-6, which increases markedly after stimulation with IL-1α, IL-1β and TNF-α. In recent years cytokine and IGF-IGFBP interactions and relationships have been shown. These relationships are cell and tissue-specific, and could occur also within the placenta”.

These data have been specified in both the introduction and briefly in the abstract.

Cortisol, insulin, adiponectin, resistin and insulin signal transduction are part of further studies. We have just submitted a paper on markers of insulin sensitivity in the placenta of IUGR versus AGA newborns; other data are yet unpublished and were not available at the moment of the study using ANNS which aimed at answering one simple question concerning the IGF system, IL-6 and TNF-α.

Looking forward to hearing soon from you,
Kind Regards

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