

## **Mutations in the glucokinase gene of the fetus result in reduced placental weight**

Beverley M. Shields PhD<sup>1\*</sup>, Gill Spyer MD, MRCP<sup>1\*</sup>, Annabelle S. Slingerland MD MPH<sup>1</sup>,  
Bridget A. Knight PhD<sup>1,2</sup>, Sian Ellard PhD, MRCPATH<sup>2</sup>, Penelope M. Clark PhD, FRCPath<sup>3</sup>,  
Sylvie Hauguel-de Mouzon PhD<sup>4</sup>, Andrew T. Hattersley DM, FRCP<sup>1</sup>

*\*These authors contributed equally to this paper*

1. Peninsula Medical School, Exeter, UK
2. Royal Devon and Exeter Hospital, Exeter, UK
3. The Regional Endocrine Laboratory,  
University Hospital Birmingham NHS Trust, Birmingham, UK
4. Case Western Reserve University, Cleveland, Ohio

**Running Title:** Fetal insulin and placental growth

### **Corresponding Author:**

Professor Andrew T. Hattersley  
Diabetes Research  
Peninsula Medical School,  
Barrack Road,  
Exeter  
EX2 5DW  
UK  
Andrew.Hattersley@pms.ac.uk

Received for publication 5 September 2007 and accepted in revised form 2 January 2008.

## **ABSTRACT**

*Objective:* In human pregnancy, placental weight is strongly associated with birth weight. It is uncertain whether there is regulation of the placenta by the fetus or vice versa. We aimed to test the hypothesis that placental growth is mediated, either directly or indirectly, by fetal insulin.

*Research Design and Methods:* Birth weight and placental weight were measured in 43 offspring of 21 parents with mutations in the glucokinase (*GCK*) gene (25 had inherited the mutation, 18 had not), which results in reduced fetal insulin secretion. Birth weight, placental weight, umbilical cord insulin and maternal glucose and insulin concentrations were measured in 573 nondiabetic, healthy, term pregnancies.

*Results:* *GCK* mutation carriers, as well as being lighter, also had smaller placentas (610g v 720g,  $p=0.042$ ). This difference was also seen in 17 discordant sibling pairs (600g v 720g,  $p=0.003$ ). *GCK* mRNA was not detected in the placenta by RT PCR. In the normal pregnancies, placental weight was strongly correlated with birth weight ( $r=0.61$ ,  $p<0.001$ ). Cord insulin concentrations were directly related to placental weight ( $r=0.28$ ), and birth weight ( $r=0.36$ ) ( $p<0.001$  for both).

*Conclusions:* These results suggest insulin, directly or indirectly, plays a role in placental growth, especially as a mutation in the glucokinase gene, which is known to only alter fetal insulin secretion, results in altered placental weight. This is consistent with the preferential localization of the insulin receptors in the fetal endothelium of the placenta in the last trimester of pregnancy.

The placenta plays a vital role in transporting nutrients from the mother to the fetus. Glucose, amino acids and other substrates cross the placenta via a combination of active transport and facilitated diffusion, maintained by a plasma concentration gradient (1-3), enabling continuous nutrient supply to the fetus. The nutrients have a direct effect on growth and glucose has an indirect effect by stimulating fetal insulin which is an important regulator of fetal growth (4, 5).

In late gestation, placental and fetal size are closely correlated (6). Fetal insulin could represent a possible link between placental and fetal growth. Recently, significant correlations between measurement of umbilical cord insulin and placental weight have been observed (7, 8). It is known that insulin is a major determinant of fetal growth and it has been proposed that fetal insulin regulates placental growth as well as fetal growth (1). Insulin receptors are found on the maternal side of the placenta (syncytiotrophoblast) in the first trimester of pregnancy, but by term, the vast majority of insulin receptors are found on the fetal side of the placenta (in the endothelium) suggesting they bind to fetal insulin rather than maternal insulin in the last trimester of pregnancy (9, 10). Insulin stimulates mitogenesis in placental cells (11), however, whether the insulin action cascade is initiated by the binding of maternal or fetal insulin to the placental receptors is not known. Placental weights are increased in diabetic pregnancies, a condition known to be associated with fetal hyperinsulinaemia (12-14) although this could reflect a direct effect of the maternal hyperglycaemic environment on the placenta rather than being mediated by fetal insulin.

Observations in monogenic disease where a gene is mutated can give insights into normal physiology in humans. One example is mutations in the gene encoding glucokinase

that result in life long fasting hyperglycaemia due to altered sensing of glucose by the pancreatic beta-cell. These mutations, when present in a pregnant mother, result in an increase in offspring birth weight of 600g, reflecting the impact of maternal hyperglycaemia in pregnancy increasing insulin mediated growth in the fetus (15). When the same mutation is present in the fetus it results in a reduction in offspring birth weight by 540g as the mutation reduces fetal insulin secretion and hence reduces insulin mediated growth (15). These effects are additive so a mother who has an offspring who inherits the same mutation will be of normal weight (15). As glucokinase specifically phosphorylates glucose to glucose-6-phosphate, the large weight changes in the fetus must reflect altered response to maternal glucose levels. Knock-out animal studies have confirmed that the changes associated with reduced glucokinase activity on birth weight are mediated through fetal insulin (16).

We aimed to test the hypothesis that placental growth is mediated, either directly or indirectly, by fetal insulin by, examining placental weight in offspring with and without glucokinase mutations. Offspring who inherit the mutation have reduced fetal insulin secretion as a result of their glucokinase mutation and therefore it would be possible to assess if placental growth is lower in these pregnancies.

To ensure that any effect on placental weight reflects changes in fetal insulin requires that that glucokinase is not expressed in the placenta. Previous studies have not detected glucokinase in rodent placenta, but this would not discount expression in the human. Therefore, we performed reverse transcription PCR to rule out low-level expression in the human placenta.

Correlations of placental weight with cord insulin were also studied in a non-diabetic

birth cohort and subjects from this cohort provided a reference population for the glucokinase study.

## **RESEARCH DESIGN AND METHODS**

***Placental weight in glucokinase pregnancies.*** Subjects were identified from the UK MODY referral database, stored at the Molecular Genetics Laboratory at the Royal Devon and Exeter Hospital (Exeter, UK), an international referral centre for identifying glucokinase gene mutations. Women with pregnancies involving heterozygous mutations in the glucokinase gene were asked to provide details of each pregnancy resulting in a live birth and permission was obtained to retrospectively review their obstetric case notes for each pregnancy.

Details of birthweight, gestational age at birth, and placental weight were obtained retrospectively from hospital obstetric records. Unfortunately, recording of the placental weight in hospital notes was not consistently performed in UK hospitals and only available in 43 (33%) of births.

Genotype of the offspring was obtained using direct sequencing from a mouth swab, if the child was aged under 16, or from a blood sample. Where possible, samples were taken by the investigators. If this was impractical, mouth swabs were performed by the parents of the children and sent back to Exeter via return post; blood samples were taken by nurses at the patients' local health centres. Patients were not informed of genotyping results.

For this study, we present data on 43 live singleton births born to 21 parents with a mutation in the glucokinase gene (19 mothers). 18 offspring were unaffected (16 born to glucokinase mothers) and 25 (23 born to glucokinase mothers) were heterozygous for the familial *GCK* mutation. A subset of this data included 17 sibling pairs discordant for the glucokinase mutation.

***Methods for GCK PCR.*** Biopsies from 8 placentas were obtained at the time of elective C-section delivery ( $38.9 \pm 0.4$  weeks) of uncomplicated pregnancies. Full villous thickness specimen (approximately  $1 \text{ cm}^3$ ) were washed in sterile PBS saline, blotted to remove excess blood and immediately snap-frozen in liquid nitrogen. For control specimens, liver specimen obtained at the time of hepatic surgery in adult men for cirrhosis was obtained after pathology examination. Total RNA was prepared from whole tissue samples using CsCl gradient. Placental samples were electrophoresed individually to verify RNA integrity, pooled and reversed transcribed using standard method (Stratagene, La Jolla, CA). Real time PCR analysis was performed using a fluorescence temperature cyler (Lightcycler, Roche Molecular Diagnosis, IN) with annealing temperature  $62^\circ\text{C}$  for 30 cycles. PCR primers were forward: 5'-CAT GAA GAG GCC AGT GTG AAG-3' and reverse: 5'-GAT GCC CTT GGT CCA GTT GAG-3'.

***Normal reference population.*** Families were recruited as part of the Exeter Family Study of Childhood Health (EFSOCH) (17), a prospective study investigating genetic influences on fetal and early childhood growth. Parents were approached at the time of the ante-natal booking visit and invited to take part if they were Caucasian and living in the EX1-EX4 post code region of Exeter. Multiple pregnancies and women with diabetes were excluded.

Informed consent was obtained from the parents of the newborns and the study was approved by the North Devon Local Research Ethics Committee.

The full protocol is described elsewhere (17). In brief, mothers were seen by the Research Midwife at 28 weeks gestation and a fasting blood sample was taken for glucose and insulin measurement. Umbilical cord blood was obtained from the placental end of the cord after the placenta was delivered, and centrifuged as soon as possible (18). Plasma

aliquots were stored at  $-80$  degrees Celsius. Cord plasma insulin and fasting insulin in both parents were measured using an immunochemiluminometric assay (ICMA) (Molecular Light Technology/Invitron, Monmouth). The assay is specific for insulin and the interassay coefficients of variation were less than 9.0% over the concentration range reported.

Babies were weighed within 24 hours of delivery using Tanita electric scales (to nearest 0.1kg). Placental weight was measured using Brabantia scales (to nearest 25g). Placental ratio was calculated by placental weight (g) divided by birth weight (g) multiplied by 100 and expressed as a percentage. We present data on 573 families with placental weight and cord insulin results available.

**Statistics.** Due to the smaller numbers in the GCK group, non-parametric statistics were used, so data are presented as medians and inter-quartile ranges, and differences between those with and without mutations in the glucokinase gene were assessed using the Mann-Whitney U test and Wilcoxon test (for sibling pair data). Associations in this group were assessed using Spearman's correlations. For the EFSOCH group, all data were tested for normality. Insulin results were log transformed due to a skewed distribution. Data are presented as means and standard deviations, or standard errors for means adjusted for sex and gestation, by analysis of variance. Pearson correlation coefficients were used to assess associations in the EFSOCH data. Bonferroni adjustments to p values were made for multiple comparisons. Adjustments for gestational age and sex were carried out using ANOVA and partial correlations. For consistency, comparisons between the GCK and EFSOCH groups were carried out using non-parametric statistics.

## RESULTS

### ***Placental weight in glucokinase pregnancy.***

Table 1 shows the characteristics of 43 offspring born to 21 parents heterozygous for a mutation in the glucokinase gene, 25 of which had inherited the mutation (GCK NM), 18 had not (GCK NN). The median gestational age was 38 weeks predominantly due to medical intervention in birth for those that were classified as high risk due to maternal hyperglycaemia/diabetes. Placental weights were similar for male and female babies (691g v 697g,  $p=0.91$ , respectively). Babies who did not inherit the mutation (GCK NN) were significantly heavier at birth than offspring that did inherit the mutation (GCK NM) (3.82 v 3.36 kg,  $p=0.007$ ). GCK NN babies had larger placental weights than GCK NM babies (720 v 610g,  $p=0.042$ ). There was no difference in placental ratio between the two groups ( $p=0.63$ ). Insulin treatment was given in 11/23 diabetic pregnancies resulting in affected (NM) babies and 6/16 diabetic pregnancies with non affected (NN) babies. There was no difference in placental weight between those treated with insulin and those not (median 680g v 680g, respectively,  $p=0.69$ ).

Further analysis was carried out on a subgroup of these babies consisting of 17 discordant sibling pairs from 11 nuclear families. Babies who had inherited the mutation had significantly lower placental weights than their unaffected siblings in 15 out of 17 cases ( $p=0.02$ , by chi-squared) (median (IQR): 600g (475-652) v 720g (650-810), respectively,  $p=0.003$ ) (Fig 1) and lower placental ratios (median (IQR): 18.9% (16.3-20.3) v 21.6% (17.4-23.4), respectively), although this did not quite reach significance ( $p=0.051$ ).

***Expression of GCK in placenta.*** mRNA for glucokinase was not detected in placental villous tissue obtained in term pregnancy but was easily detected in the positive control (liver).

**Normal Birth Cohort.** In 573 normal pregnancies from the EFSOCH study placental weight was highly correlated with birth weight ( $r=0.61$ ,  $p<0.001$ ) and cord insulin concentration ( $r=0.28$ ,  $p<0.001$ ). There was also a weaker but significant correlation between placental weight and maternal insulin ( $r=0.13$ ,  $p=0.021$ ). As expected, maternal glucose and maternal insulin were significantly correlated with cord insulin ( $r=0.26$  and  $r=0.20$ , respectively,  $p<0.001$ ), and maternal glucose and cord insulin were significantly associated with birthweight ( $r=0.27$  and  $r=0.36$ , respectively,  $p<0.001$ ).

We examined a subgroup of 118 births with gestational age of 38 weeks, to serve as a reference population for the GCK group (table 1). Median (IQR) placental weight was 600 (500 - 700)g, which was lower than the placental weights of the babies that had not inherited the GCK mutation ( $p<0.001$ ), which is what may be expected with the large proportion of hyperglycemic/diabetic pregnancies. However, this was similar to the placental weights of babies that had not inherited the mutation ( $p=0.26$ ) (see figure 2).

## CONCLUSION

Our study of placental weight in the offspring with and without heterozygous mutations in the glucokinase gene supports the hypothesis that fetal insulin may directly or indirectly regulate placental growth.

We found significant correlations of placental weight with umbilical cord insulin concentrations and birthweight, confirming relationships seen in previous studies (7, 8) and in agreement with data from diabetic pregnancies (12-14). The glucokinase study gives us more insight into the direction of the causal relationship. In the glucokinase pregnancies, the weight of the placenta, like birth weight, depends on the mutation status of the fetus. We have shown that the babies

that inherit the mutation (GCK NM) have a lighter placenta than the fetus without the mutation (GCK NN). This was demonstrated most clearly in the discordant sibling analysis (Fig 1), but a significant difference was also seen when comparing all cases with all controls. This shows that the presence of the mutation results in a smaller placenta. This is similar to the previously observed effect of inheriting a *GCK* mutation on offspring birthweight (19)

Glucokinase is the pancreatic glucose sensor and mutations in the gene have been shown to cause beta-cell dysfunction resulting from altered glucose sensing with a reduction of the insulin secretion rate for a given glucose (19). Although cord insulin values were not measured in the *GCK* births there is animal data suggesting that the fetus with a heterozygous knockout and reduced glucokinase activity has reduced insulin secretion and hence reduced insulin-mediated growth effects (16).

We propose that the differences in placental size between GCK NM and GCK NN babies is mediated either directly or indirectly through reduced fetal insulin secretion. Glucokinase was shown not to be expressed in the rat placenta using northern blot analysis (23) and we showed using a highly sensitive rtPCR method that no mRNA from the *GCK* gene can be detected in term placenta. Absence of placental glucokinase expression was further supported by the absence of signal in microarray analysis of 15 term placentas (data not shown). This means the impact of the presence of a glucokinase mutation must be mediated through the change in the fetal *GCK* activity. This could be through changes to fetal insulin secretion having a direct impact on placental growth. In keeping with this, in the third trimester of pregnancy, the insulin receptors are more abundant in the vascular endothelial cells facing the fetal systemic circulation than on the maternal side (12). Alternatively,

decreased fetal insulin concentrations could have an indirect effect on placental mitogenesis with reduced secretion of insulin reducing fetal growth and a factor or a cascade of signals other than those elicited by fetal insulin resulting in the reduction in placental size.

There were a number of limitations in these studies. Firstly, maturity-onset diabetes of the young is relatively rare, therefore, we only had small numbers in the glucokinase group. However, we have replicated results from previous small studies that have found relationships between birth weight and glucokinase mutations (15, 20) and the results from both studies presented here are internally consistent. As insulin was not measured in the glucokinase patients we have had to make assumptions about fetal insulin based on surrogate markers of fetal birthweight and animal studies. We can not exclude a co-incidental problem in the placental vasculature but this is very unlikely,

especially as fetal *GCK* is not detected in the placenta and the placenta of the fetuses with and without the mutation were both exposed to a similar degree of maternal glycaemia, and is consistent with the insulin receptors being present predominantly in the fetal endothelium in the last trimester of pregnancy.

In conclusion, we have data from normal and monogenic pregnancies that supports the hypothesis that fetal insulin can regulate placental weight.

#### **ACKNOWLEDGEMENTS**

This study was funded by South West NHS Research and Development, Exeter NHS Research and Development and the Darlington Trust. ATH is a Wellcome Trust Research Leave fellow. BK holds a NHS Research and Development studentship. The support of University Hospital Birmingham Charities is gratefully acknowledged (PMC).

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**TABLE 1.** a) Characteristics of Offspring of Mothers with mutations in the Glucokinase gene. GCK (NM) are offspring who inherited the mutation, GCK (NN) are offspring who did not inherit the mutation. b) Characteristics of 118 babies in EFSOCH born at 38 weeks gestation as a comparison group. P values refer to the differences between GCK (NN) and GCK (NM) groups.

	<b>GCK (NN)</b>	<b>GCK(NM)</b>	<b>p</b>	<b>EFSOCH</b>
n	18	25		118
Placental Weight (g)	720 (650-834)	610 (540-788)	0.04	600 (500-700)
Placental Ratio (%)	21.4 (17.0-22.9)	20.7 (18.7-24.4)	0.63	18.5 (16.4, 20.6)
Birth weight (Kg)	3.82 (3.25-4.32)	3.36 (2.59-3.71)	0.007	3.20 (2.94, 3.53)
Male n (%)	10 (56%)	13 (52%)	0.82	65 (55%)
Gestation (wks)	38.0 (37.0-40.0)	38.0 (38.0-40.5)	0.36	38

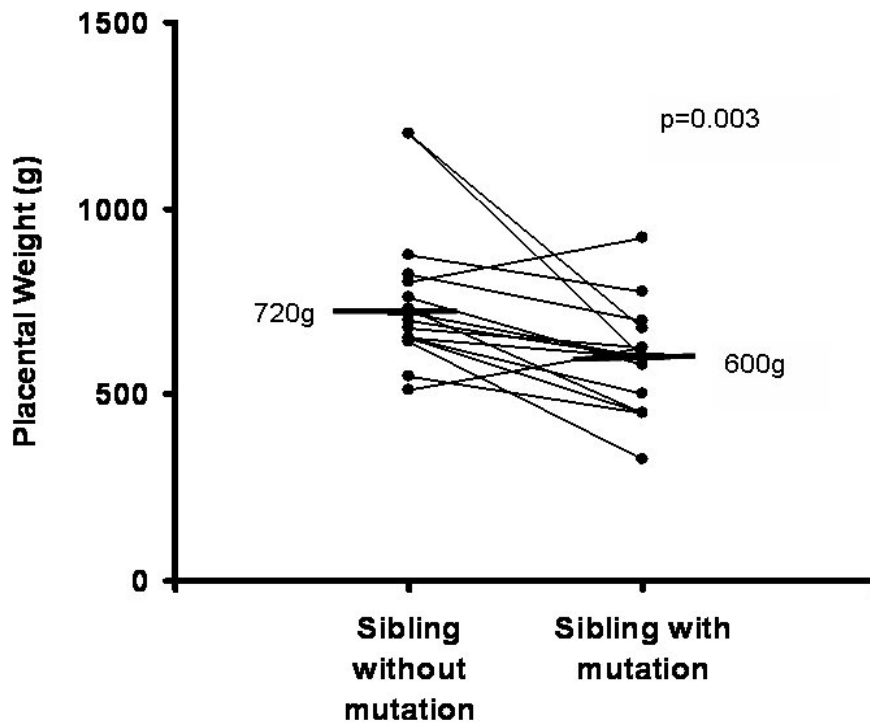
Data are presented as median and inter-quartile range unless otherwise stated.

## **FIGURE LEGENDS**

**Figure 1.** Placental weights of 17 sibling pairs discordant for a mutation in the glucokinase gene. The median of each group is represented by a horizontal line. Birth weight was lower in the sibling with the mutation in 15 of the 17 sibling pairs ( $p=0.02$ ).

**Figure 2.** Bar chart showing median placental weight for babies heterozygous for a mutation in the glucokinase gene (GCK NM), babies without a mutation in the glucokinase gene born to glucokinase parents (GCK NN), and Control babies born at 38 weeks gestation from the EFSOCH study. \*\*\*  $p<0.001$ , \*  $p < 0.05$

FIGURE 1



**FIGURE 2**

