Measurement of Cord Insulin and Insulin-Related Peptides Suggests That Girls Are More Insulin Resistant Than Boys at Birth

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OBJECTIVE — We aimed to examine sex differences in insulin and insulin propeptide concentrations at birth using validated cord blood collection.

RESEARCH DESIGN AND METHODS — We tested the impact on insulin and insulin propeptides of taking 13 cord blood samples in heparin and EDTA and then centrifuging and separating plasma after 1, 2, 24, or 48 h at room temperature (heparin) or 4°C (EDTA). Cord plasma insulin and insulin propeptides concentrations were measured in 440 babies and correlated with offspring anthropometry measured at birth.

RESULTS — Cord insulin concentrations significantly decreased (74% those at baseline by 24 h; $P = 0.01$) in the samples taken in heparin and stored at room temperature, but those taken on EDTA and refrigerated remained stable for up to 48 h. Insulin propeptides were stable in both. Cord plasma insulin and insulin propeptides measured in EDTA were related to all measures of birth size and maternal glycemia and BMI ($r > 0.11$; $P < 0.03$ for all) and were higher in those delivered via caesarean section. Girls were lighter (3,497 vs. 3,608 g; $P = 0.01$) but had higher cord insulin (46.7 vs. 41.2 pmol/l; $P = 0.031$), total proinsulin (34.1 vs. 25.8 pmol/l; $P < 0.001$), and intact proinsulin (9.5 vs. 8.3 pmol/l; $P = 0.004$) concentrations than boys; this was further confirmed when cord insulin concentrations of boys and girls were compared after pair matching for birth weight (insulin 49.7 vs. 42.1 pmol/l; $P = 0.004$).

CONCLUSIONS — When using appropriate sample collection methods, female newborns have higher insulin concentrations than male newborns, despite being smaller, suggesting intrinsic insulin resistance in girls.

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Increasing evidence suggests that girls are more insulin resistant than boys. This has been shown in fasting insulin measurements, frequently sampled intravenous glucose tolerance tests, and euglycemic clamps in children from age 5 years (1) through late childhood (2,3) and puberty and adolescence (4–6). The increase in insulin resistance is seen in Caucasian, Afro-Caribbean, and Asian-Indian races (1–4,6). Furthermore, type 2 diabetes in children is far more common in girls than boys (7–9).

That sex differences are seen early in life could reflect differences in intrinsic insulin resistance or differences in postnatal behavior. In a study of 307 subjects, Murphy et al. (1) were unable to explain sex differences at 5 years of age by looking at differences in anthropometry and physical activity. If insulin resistance is intrinsic, this would suggest that it is genetically determined and should be apparent from birth. An insulin-resistant phenotype at birth has previously been described in Indian babies (10), who have higher umbilical cord insulin concentrations and more subcutaneous fat than U.K. babies, despite being lighter. If girls are more insulin resistant than boys, one might expect to observe a similar phenotype at birth, with girls having higher cord insulin concentrations even though they are lighter than boys. Girls are consistently lighter on average than boys; however, the results of cord insulin and insulin propeptide measurements are contradictory. Higher concentrations of proinsulin and split proinsulin have been recorded in girls (11–13), but no significant sex differences in insulin were found (11–14).

One possible explanation for why sex differences in umbilical cord insulin concentrations have not previously been seen may be instability of insulin in cord blood. Lindsay et al. (13) found that insulin concentrations in cord blood fell rapidly to ~20% those at baseline with a 24-h delay before centrifugation and freezing. In contrast, they found that proinsulin remained stable in cord blood for up to 24 h. Another explanation could be that insulin, which shows pulsatile release and has a shorter half-life than proinsulin (15), may be more susceptible to fluctuations associated with delivery (12).

We aimed to establish a valid procedure for collecting cord blood for insulin assay; to use this to assess differences and interrelationships between insulin, intact proinsulin, and total proinsulin; and to examine whether there are any sex differences in insulin and insulin propeptides at birth.

RESEARCH DESIGN AND METHODS

— Ethical approval was given by the North and East Devon local research ethics committee, and informed consent was obtained from the parents of the newborns.

Study 1: Stability of insulin and insulin propeptides in cord blood

A total of 13 nondiabetic women gave consent for the collection of blood from the umbilical cord after delivery. A 20-ml sample of blood was taken from the um-

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Abbreviations: IQR, interquartile range.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

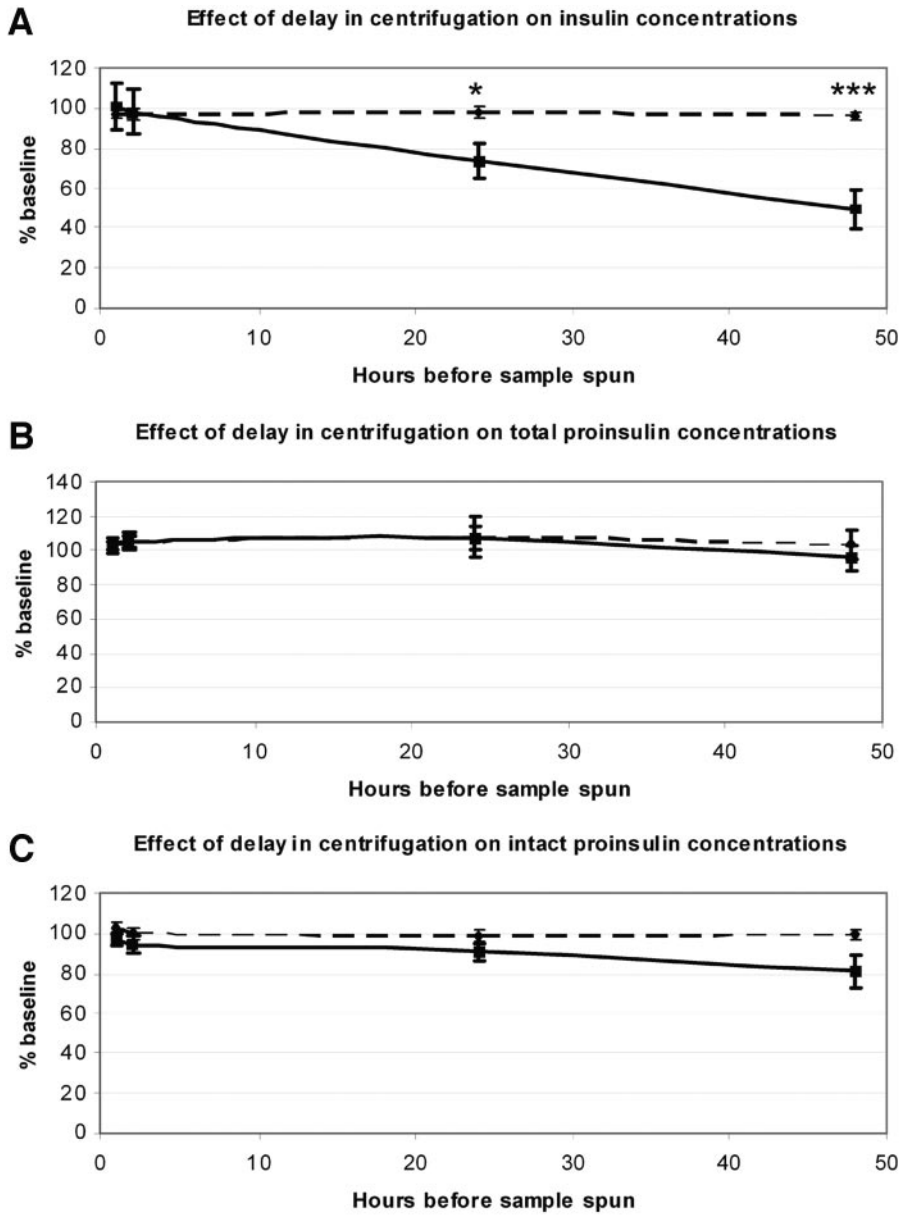

Figure 1—*Effect of delay in centrifugation and freezing of cord blood sample on concentrations of insulin* (A)*, total proinsulin* (B)*, and intact proinsulin* (C)*, depending on anticoagulant used (room temperature heparin represented by straight lines, cold EDTA represented by dotted lines). Data presented as mean percentage of baseline and 95% CIs. Significant decrease from baseline:* *P *< 0.05;* **P *< 0.001.*

bilical cord vein immediately following delivery of the placenta. This was transferred to five lithium heparin tubes and five potassium EDTA tubes (nongel tubes; Sarstedt, Leicester, U.K.). One heparin and one EDTA sample were centrifuged and the plasma separated and immediately frozen at −80°C. The remaining four heparin samples were stored at room temperature (as described by Lindsay et al. [13]), and the remaining four EDTA samples were stored at 4°C. These samples were then centrifuged and frozen after 1, 2, 24, and 48 h.

All samples were stored at −80°C for <1 month before shipping on dry ice to the Regional Endocrine Laboratories (Birmingham, U.K.), where insulin, total proinsulins, and intact proinsulin assays were performed.

Plasma insulin, total proinsulins, and intact proinsulin were measured by immunochemiluminometric assays (Molecular Light Technology, Cardiff, U.K.). The insulin assay was specific for insulin with a quoted interassay imprecision (coefficient of variation [CV] <10%) over the range 6.5–169 pmol/l and quoted cross-reactivities of 1.2% for intact proinsulin,1.6% for des 31–32 split proinsulin, and 44% for des 64–65 split proinsulin.

For the assays for total proinsulins and intact proinsulin, the quoted interassay imprecisions (CV <10%) were 3.0–257 and 8.9–390 pmol/l, respectively. Quoted cross-reactivity for insulin in the total proinsulin assay was 2% and was not detectable in the intact proinsulin assay.

All results were analyzed using nonparametric statistics because of the small numbers. The Mann-Whitney *U* test was used to assess differences between results at baseline and subsequent time points.

Study 2: Interrelationships of insulin and insulin propeptides

Recruitment and protocol. Families were recruited as part of the Exeter Family Study of Childhood Health, a large prospective study examining genetic influences on fetal and early growth (16). Maternal weight, height, BMI, and fasting glucose and insulin concentrations were measured at 28 weeks' gestation. Maternal and cord plasma glucose were assayed by the pathology laboratories at the Royal Devon and Exeter Hospital, Exeter, U.K., using a standard laboratory method (CV <2%). Maternal insulin was measured using the same assay as before. Insulin resistance in the mother was calculated using the homeostasis model assessment program for specific insulin measurement (17,18).

Detailed anthropometry was taken on the child at birth, including weight, length, head circumference, and skinfold thickness of the tricep and subscapula. A sample of cord blood (EDTA plasma) was taken following delivery of the placenta by the midwife on duty at the time of delivery and stored at 4°C until the research midwife was able to centrifuge and freeze the separated plasma at −80°C (median time 11 h). Insulin, total proinsulin, and intact proinsulin were measured using the same assays as in the stability study.

Statistics

Insulin and insulin propeptide results were log transformed to ensure normal distribution. Pearson correlations were used to assess relationships among insulin and proinsulin concentrations with birth size and maternal anthropometry and biochemistry. ANOVA was used to test for differences between modes of delivery. *t* tests were used to assess sex dif-

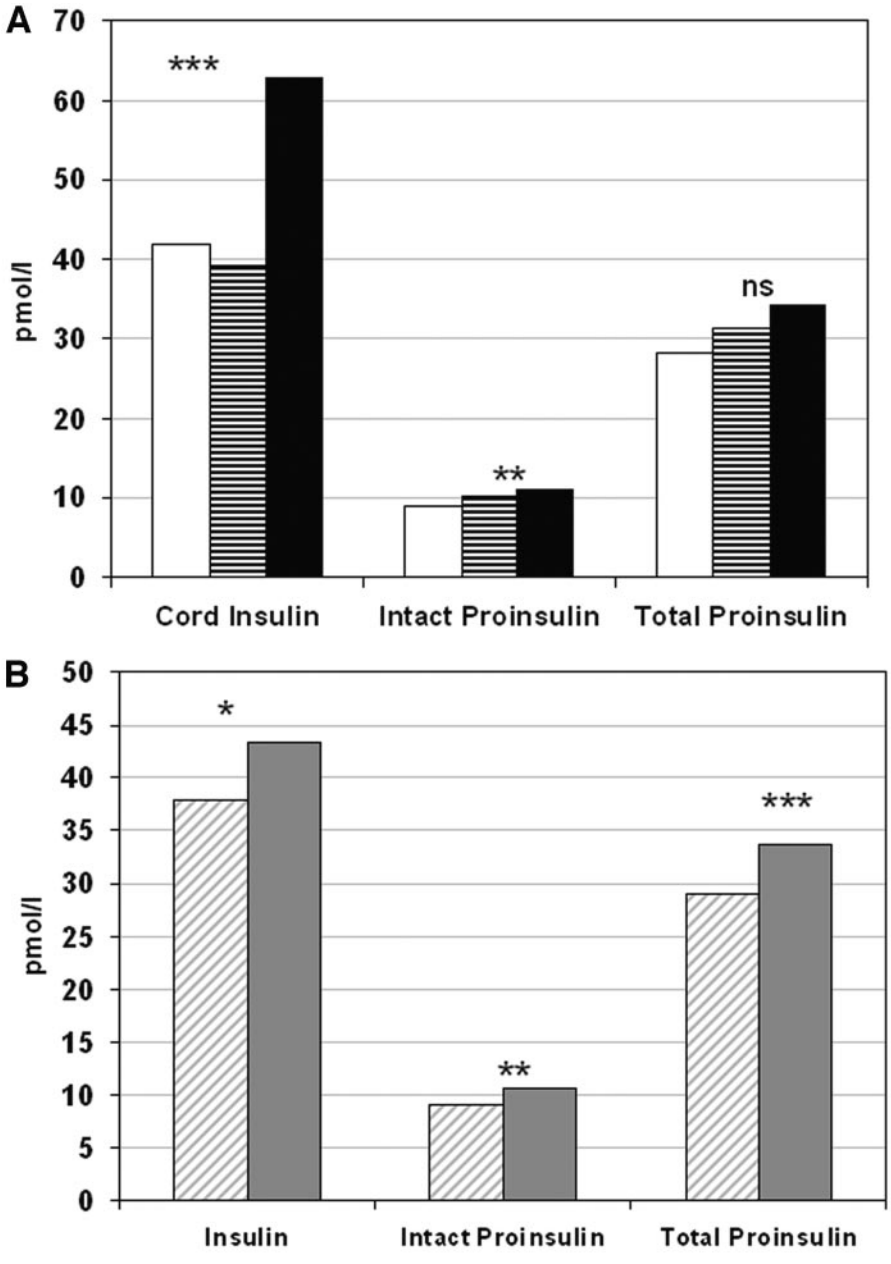

Figure 2—A: *Insulin, intact proinsulin, and total proinsulin concentrations by mode of delivery. Normal vaginal delivery* (n = *317) is represented by white bars, assisted (forceps or ventouse) delivery* (n = *60) by horizontal shaded bars, and caesarean sections* (n = *63) by black bars. Error bars represent the 95% CIs of the mean. Difference between modes of delivery assessed using ANOVA.* *P < *0.05;* **P < *0.01;* ***P < *0.001.* B*: Sex differences in geometric mean insulin, intact proinsulin, and split proinsulin. Boys* (n = *229) are represented by pale shaded bars and girls by dark gray bars. Error bars represented the 95% CIs of the mean.* *P < *0.05;* **P < *0.01;* ***P < *0.001.*

ferences. Multiple linear regression analysis was carried out to explore differences in sex while accounting for potential confounders. Finally, girls and boys were pair matched for birth weight and gestation, in a similar analysis to that carried out by Yajnik et al. (10), to further investigate sex differences in insulin and insulin propeptides.

RESULTS

Study 1: Effect of delay in centrifugation and freezing on insulin, total proinsulin, and intact proinsulin concentrations

There were no differences in concentrations of insulin, total proinsulins, or intact proinsulin between cold storage EDTA and room temperature heparin samples at baseline (medians, respectively: 52.6 vs. 51.9 pmol/l, $P = 0.78$; 30.7 vs. 34.9 pmol/l, $P = 0.96$; and 15.1 vs. 15.6 pmol/l, $P = 1.0$).

Insulin was far less stable when blood was taken on heparin and stored at room temperature, with a reduction to 74% that at baseline (interquartile range [IQR] 69–84%) by 24 h ($P = 0.012$) and to 49% (36–57%) by 48 h ($P < 0.001$). This is in contrast to samples taken on EDTA and stored at 4°C, where insulin concentrations were similar (median 96–98% those at baseline; $P > 0.6$ for all) throughout the 48-h period (Fig. 1*A*). Time delay in centrifugation and freezing had little effect on total and intact proinsulin concentrations measured in both cold storage EDTA and room temperature heparin samples (Figs. 1*B* and *C*), with most results remaining within 10% those at baseline for up to 48 h. The only deviation from this was with the intact proinsulin concentrations from the 48 h heparin samples, which decreased to 81% (IQR 75–91%) those at baseline, although this difference did not quite reach statistical significance ($P = 0.06$).

Four of the heparin samples were found to be hemolysed (one at 24 h and three of the 48-h samples). Removal of the four hemolyzed heparin samples made little difference to the results, with insulin still significantly reduced to 54.7% (IQR 38.6–66.1%) that of baseline ($P < 0.001$) in samples taken on heparin and left 48 h before centrifugation and freezing (compared with 93.5% that at baseline for total proinsulin and 83.7% for intact proinsulin).

Study 2: Interrelationships between insulin and insulin propeptides at birth

Characteristics of babies and insulin and insulin propeptide concentrations. Only subjects with all three umbilical cord insulin peptide measurements (insulin, total proinsulins, and intact proinsulin) were included in this study. Insulin and insulin propeptides were not measured in 92 samples because of hemolysis. The analysis was therefore carried out on 440 term, singleton babies. These babies had a mean ± SD birth weight of 3,554 ± 477 g and gestation of 40.1 ± 1.2 weeks. Fifty-two percent ($n = 229$) of the sample was male, and 41% of births ($n = 180$) were first-time deliveries. A total of 63 (14%) were born via caesarean section.

Those for whom all three cord peptide results were available were slightly

Table 1—*Comparison of cord insulin and insulin propeptides between boys and girls (161 pairs matched for birth weight [to nearest 100 g] and gestation [within 5 days])*

	Boys	Girls	*P*
Insulin (pmol/l)	42.1 (23.3–76.1)	49.7 (27.3–90.7)	0.004
Intact proinsulin (pmol/l)	8.6 (5.3–13.9)	10.5 (6.4–17.0)	0.001
Total proinsulin (pmol/l)	25.0 (14.5–43.0)	35.6 (18.4–68.8)	<0.001
Birth weight (g)	3535 ± 414	3534 ± 417	0.847
Gestation (weeks)	40.1 ± 1.1	40.2 ± 1.1	<0.001

Data are geometric means (SD range) or means ± SD. Significance established using paired *t* test.

heavier than those with at least one of the measurements missing (3,554 vs. 3,463 g, respectively; $P = 0.003$), but there was no difference in gestation (40.2 vs. 40.1; $P = 0.583$). Cord insulin concentrations were associated with intact and total proinsulin concentrations ($r = 0.446$ and 0.666, respectively; $P < 0.001$), and intact and total proinsulin were also strongly correlated ($r = 0.680$; $P < 0.001$). There was greater variation in total proinsulin and insulin compared with the intact proinsulin ($F = 1.6$ and 1.4, respectively; $P < 0.001$ for both). The median total proinsulin-to-insulin ratio was 0.66. Total proinsulins accounted for 40.8% of all insulin-like molecules in cord blood.

Relationships with mode of delivery. Babies delivered by caesarean section had significantly higher insulin concentrations than babies delivered by normal vaginal delivery or assisted delivery (61.9 vs. 41.7 and 39.2 pmol/l, respectively; $P < 0.001$) (Fig. 2*A*). There was also a small but significant difference in intact proinsulin concentrations between caesareans and normal vaginal deliveries (11.1 vs. 9.0 pmol/l, respectively; $P = 0.03$), but there was no difference in total proinsulin concentrations between the three modes of delivery. Those born via caesarean section also had higher birth weights than those delivered vaginally, when correcting for sex and gestation (3,663 vs. 3,505 g, respectively; $P = 0.009$), but even when adjusting for birth weight, those born via caesarean section still had higher insulin and intact proinsulin concentrations. Those born via emergency caesarean section had higher cord insulin concentrations than those born via elective caesarean sections (69.7 vs. 55.2 pmol/l, respectively), although this did not reach significance ($P = 0.11$).

Differences between insulin and insulin propeptides and their relationships with birth size and maternal factors. Insulin, total proinsulin, and intact proinsulin were significantly correlated with all measures of birth size including weight, length, head circumference, and skinfold thicknesses ($r = 0.213$–0.465; $P < 0.001$ for all). The strength of the correlations for each measure was similar for insulin, total proinsulin, and intact proinsulin, with each within the 95% CIs of one another. As expected, fasting maternal glucose at 28 weeks was significantly associated with all three insulin peptides ($r = 0.16$–0.26; $P < 0.001$ for all). Maternal age, weight, and BMI were also significantly correlated with all three peptides ($r = 0.11$–0.18; $P < 0.001$–0.03). Maternal insulin and homeostasis model assessment of insulin resistance were significantly correlated with cord insulin and total proinsulin ($r = 0.17$–0.23; $P < 0.001$ for all) but not with intact proinsulin ($r = 0.05$; $P = 0.27$ and $r = 0.06$; $P = 0.23$, respectively). Removing data from those delivered by caesarean sections made no difference to the results.

Sex differences in insulin and insulin propeptides. Cord plasma from girls had higher concentrations of insulin (46.7 vs. 41.2 pmol/l; $P = 0.031$), total proinsulin (34.1 vs. 25.8 pmol/l; $P < 0.001$), and intact proinsulin (9.5 vs. 8.3 pmol/l; $P = 0.004$) than that from boys (Fig. 2*B*). Total proinsulin-to-insulin ratio was higher in girls than boys (0.73 vs. 0.63, respectively; $P = 0.003$). Similarly, the percentage of total proinsulin out of the total insulin-like molecules was also higher (42.5 vs. 39.2%; $P = 0.003$).

Multiple linear regression analysis was carried out to assess the independent contribution of the baby's sex when accounting for other determinants of cord insulin concentrations (gestation and maternal age, BMI, glucose, and insulin). When insulin was examined, sex did not quite remain a significant independent determinant [*B* 0.046 (SE 0.025); $P = 0.064$], but it was significantly associated with total proinsulin [0.106 (0.026); $P < 0.001$] and intact proinsulin [0.052 (0.022); $P = 0.017$].

Girls were lighter (3,497 vs. 3,608 g; $P = 0.01$) and shorter (49.8 vs. 50.8 cm; $P < 0.001$) than boys but had more subcutaneous fat as measured by tricep and subscapula skinfold thicknesses (5.0 vs. 4.7 mm; $P = 0.01$ and 5.1 vs. 4.8 cm; $P = 0.01$, respectively).

Final exploration of the sex difference was carried out using pair matching, where girls and boys were matched for birth weight (to the nearest 100 g) and gestation (within 5 days). A total of 161 boy-girl pairs could be matched using these criteria. There was a slight bias in the matching because girls appeared to have slightly longer gestations (40.2 vs. 40.1 weeks; $P < 0.001$). Although this difference works out at <1 day, its significance in a paired *t* test is probably due to girls being smaller than boys; therefore, to pair match on weight, the girls would generally be of a longer gestation. When matching for birth weight and gestation, girls still had higher concentrations of all three peptides (Table 1).

The sex difference in cord insulin concentrations could reflect a difference in cord glucose concentrations. Cord glucose concentrations were negatively associated with time before centrifugation ($r = -0.483$; $P < 0.001$). However, there was no difference in this time between girls and boys (10.3 vs. 11.4 h; $P = 0.521$); therefore, a relative difference in cord glucose concentrations could be assessed. There was no difference in cord glucose concentrations between girls and boys (3.5 vs. 3.5 mmol/l; $P = 0.678$). Adjustment of cord glucoses for time before centrifugation to 0 h further confirmed that there was no sex difference (4.4 vs. 4.3 mmol/l; $P = 0.640$). There was no difference in the number of caesarean sections between boys and girls (30 vs. 33; $P = 0.45$), and exclusion of babies delivered by caesarean section from the analyses made no difference to the results.

CONCLUSIONS — We have provided data showing that newborn girls have higher insulin and proinsulin concentrations and total proinsulin-to-insulin ratios in cord blood than newborn boys despite weighing less at birth. As insulin is a principal growth factor in utero, the higher insulin coupled with reduced growth in newborn girls suggests that girls are more insulin resistant in utero and after birth.

The higher insulin and proinsulin(s) in girls compared with boys for glucose/weight indicate insulin resistance in girls. The higher proinsulins in girls compared

with boys have been noted before in neonates (11–13), and these are the more stable molecules. Changes in ratios and percentages may not have been seen if stability of insulin had not been maximized and, hence, was variable.

Our finding that insulin and insulin propeptides in cord blood are higher in girls than in boys is consistent with an intrinsic difference between the sexes, which is unlikely to be determined by environmental factors. Other research has suggested that in childhood, girls are more insulin resistant than boys with higher insulin and proinsulin concentrations and more adipose tissue from age ≥5 years, and these differences could not be explained by other known determinants of insulin concentrations (1,3,4,6,19). We think that because girls are born smaller (lighter and shorter), with greater skinfold thickness, and in the presence of higher insulin concentrations than boys but with no corresponding increase in cord glucose concentrations, they are intrinsically more insulin resistant. This is a situation similar to the difference seen between Indian and U.K. babies (10), where Indian babies are smaller with more adipose tissue and higher insulin concentrations, which is thought to reflect insulin resistance because the increase in cord insulin is not associated with augmented growth. Research has also shown that babies born to the most insulin-resistant fathers have higher cord insulin concentrations compared with babies born to the least insulin-resistant fathers when matched for birth weight, further supporting the idea that increased cord insulin without increased growth represents an insulin-resistant phenotype (20).

The higher proinsulin-to-insulin ratio and percentage of proinsulins in girls in our study could theoretically be due to either decreased clearance or differences in conversion of proinsulin to insulin (19). Higher proinsulin(s) have been found in neonates previously (11,12), and this may be part of the neonatal transition reflecting immaturity of the fetal β-cell and/or β-cell secretory granule.

Insulin regulates fetal growth of both skeletal size and soft tissue (21), and, as expected, in our data—where sample validity has been established—the insulin concentrations were significantly correlated with all measures of birth size, including weight, length, head circumference, and skinfold thicknesses, and had correlation coefficients similar to the relationships seen with total and intact proinsulin concentrations. We also found maternal glucose to be significantly associated with cord insulin concentrations, reflecting that maternal glycemia is a major determinant of fetal insulin secretion, as demonstrated by the macrosomia associated with diabetic pregnancies (22). The findings of these well-established relationships with insulin and total and intact proinsulins provide validation of our results.

Although not the primary aim of our study, our data suggest that stability is dependent on the correct combination of sample type, storage, and assay. The difference in sample stability according to sample type and assay that we have shown may be one possibility why other studies have failed to see expected relationships of cord insulin with birth weight (23,24) and length (10,12,14) and may be why sex differences in only proinsulin but not insulin concentrations have been seen (11–13) in contrast to our study. Further studies are needed to clarify the role of specimen type and storage temperature on the stability of insulin in cord blood in particular.

Even with stable sample collection, however, there are still differences in the relationships seen with insulin and proinsulin. In particular, there was a large difference in insulin concentrations between caesarean and normal vaginal deliveries, which has also been seen in other studies (12,13,25). No women in the study were given intravenous glucose because it is not the standard practice of the maternity unit, and the difference remained when adjusting for birth weight, so these factors do not explain this obeservation. Acute changes around delivery at caesarean section may be seen more clearly in insulin because of its shorter half-life than proinsulin. Therefore, in future studies of cord insulin, it may be more advisable to measure proinsulin(s) because they show less biological variability and may be more stable dependent on collection conditions.

In conclusion, by using appropriate methods of sample collection to ensure that insulin results are stable, we have shown evidence from cord insulin and proinsulin measurement to suggest that girls are intrinsically more insulin resistant than boys.

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