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New aids for the non-invasive prenatal diagnosis of achondroplasia: dysmorphic features, charts of fetal size and molecular confirmation using cell-free fetal DNA in maternal plasma

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ABSTRACT

Objectives *To improve the prenatal diagnosis of achondroplasia by constructing charts of fetal size, defining frequency of sonographic features and exploring the role of non-invasive molecular diagnosis based on cell-free fetal deoxyribonucleic acid (DNA) in maternal plasma.*

Methods *Data on fetuses with a confirmed diagnosis of achondroplasia were obtained from our databases, records reviewed, sonographic features and measurements determined and charts of fetal size constructed using the LMS (lambda-mu-sigma) method and compared with charts used in normal pregnancies. Cases referred to our regional genetics laboratory for molecular diagnosis using cell-free fetal DNA were identified and results reviewed.*

Results *Twenty-six cases were scanned in our unit. Fetal size charts showed that femur length was usually on or below the 3rd centile by 25 weeks' gestation, and always below the 3rd by 30 weeks. Head circumference was above the 50th centile, increasing to above the 95th when compared with normal for the majority of fetuses. The abdominal circumference was also increased but to a lesser extent. Commonly reported sonographic features were bowing of the femora, frontal bossing, short fingers, a small chest and polyhydramnios. Analysis of cell-free fetal DNA in six pregnancies confirmed the presence of the c.1138G > A mutation in the FGRF3 gene in four cases with achondroplasia, but not the two subsequently found to be growth restricted.*

Conclusions *These data should improve the accuracy of diagnosis of achondroplasia based on sonographic findings, and have implications for targeted molecular confirmation that can reliably and safely be carried out using cell-free fetal DNA.*

INTRODUCTION

Achondroplasia is the most common nonlethal skeletal dysplasia, with an incidence of around 5–15 per 100 000 births. It results from a mutation in the fibroblast growth factor receptor-three (*FGFR-3*) gene located on 4p16.3[1,2]. Ninety-eight percent of cases of achondroplasia are caused by a c.1138G > A mutation (p.Gly380Arg) in the transmembrane domain of the *FGFR-3* gene, although other disease-causing mutations have also been reported[3,4]. The majority of cases are sporadic and result from a *de novo* paternal mutation[5]. Prenatal sonographic diagnosis often fails as limb length is preserved until around 22 weeks' gestation, after the time of the routine fetal anomaly scan[6–10]. Presentation of *de novo* cases often occurs in the third trimester when the fetus is scanned for some other reason and short limbs and other features, such as trident hand and frontal bossing[6], may be evident. Even then, misdiagnosis is common[11]. Other features, such as a small chest[11,12] and bowing of the femora[11] have only occasionally been reported. The aim of this study was to construct size charts for fetuses with achondroplasia and to define clearly

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ORIGINAL PAPER

the frequency of other sonographic features associated with this condition in order to aid prenatal diagnosis and facilitate targeted molecular analysis for definitive diagnosis. This seems particularly pertinent in view of the recent advances in molecular diagnosis based on cell-free fetal deoxyribonucleic acid (DNA), which permits genetic diagnosis of this condition using a maternal blood sample[13].

METHODS

The databases in our fetal medicine units from 1992 to 2009 were searched to find data on all fetuses that presented prenatally with short limbs and where achondroplasia was the etiology. Only those cases with a positive diagnosis based on the presence of a mutation in the *FGFR-3* gene or where postnatal radiology confirmed the diagnosis were included. The prenatal records were examined to determine all fetal measurements made and to ascertain what other sonographic features were present. Charts of fetal size were constructed for head circumference (HC), abdominal circumference (AC) and femur length (FL) using the LMS method[14], which models the age-changing distribution of the measurement in terms of the median and coefficient of variation after applying a suitable Box–Cox power transformation. Here the medians were fitted as smooth curves, while the coefficients of variation all decreased linearly with increasing gestation. The distributions of all three measurements were skewed to the left, requiring Box–Cox transformations of power 2.5, 2.4 and 4.4 for FL, AC and HC, respectively. The data derived were also compared with charts recommended for routine assessment of fetal size in the UK[15–17], both as centile plots and *Z*-score plots for FL, HC and AC relative to these normal ranges.

All cases referred to the North East Thames Regional Genetics Unit for analysis of cell-free fetal DNA in maternal plasma to screen for mutations in the *FGFR-3* gene were identified by review of departmental records. Results of cell-free fetal DNA testing and pregnancy outcome were ascertained. Details of sample preparation and cell-free fetal DNA analysis are included in a supplementary file accessible online (Appendix S1).

RESULTS

Twenty-six cases had been scanned in our units. In two cases one parent was affected, and the rest arose *de novo*. In 21 of the 26 cases at least one measurement of FL, AC and HC were available in both second and third trimesters. In the remaining five, only third-trimester measurements were available. Measurements of other long bones and feet were available only in the third trimester for the majority of cases. Charts were constructed using all available measurements, treating them as independent. The medians plotted against gestation were slightly curved for FL (Figure 1a) from around 25 weeks' gestation, when they began to fall away from the normal centiles (Figure 1d). The shape of the HC (Figure 1b) and AC (Figure 1c) charts both followed the normal pattern (Figure 1e and f), although both tended to be greater in achondroplastic fetuses. *Z*-score plots comparing cases with the normal ranges[15–17] show that before 25 weeks the FL lies within the normal range for all except three cases (Figure 1g). By contrast, *Z*-plots confirm that the HC and AC tend to be greater than normal for all gestations (Figure 1h and i), particularly for the HC, with many measurements above the 95th centile. Measurements of the humerus in the third trimester were all below the 3rd centile with those of the radius and ulna lying on or below the 3rd centile, confirming the rhizomelic nature of long-bone shortening in the arms. All measurements of tibia and fibula taken after 25 weeks' gestation lay below the 3rd centile.

Detailed reports describing other ultrasound findings were available for 17 cases (Table 1). Mild bowing of the femora (Figure 2a,b) was seen in 29%, short fingers (Figure 2c,d) in 100% where hands could be examined, frontal bossing (Figure 2e–h) in 92%, relative macrocephaly was observed in 69%, a small chest (Figure 2i–k) in 35% and increased amniotic fluid volume in 65%. Skeletal dysplasia was suspected in 16 cases and a definitive prenatal diagnosis made in 14. In seven, the diagnosis was confirmed following invasive testing and molecular analysis, which showed a mutation in the *FGFR-3* gene; in five the diagnosis was based solely on the ultrasound findings, and in two analysis of cell-free fetal DNA extracted from maternal blood confirmed the presence of the mutation.

Review of molecular genetics unit records revealed six cases where maternal blood had been sent for cell-free fetal DNA analysis to screen for mutations in the *FGFR-3* gene following identification of short limbs on ultrasound at 29 to 35 weeks' gestation. Two of these cases were part of the cohort described above; the remaining cases were referred from other units. In four cases the c.1138G > A mutation was identified (Figure 3) and the diagnosis of achondroplasia was confirmed radiologically after birth. No mutation was identified in the remaining

Table 1 Frequency of sonographic findings and accuracy of diagnosis in 17 cases of achondroplasia

	Feature present		*Not examined/no comment*
Feature	*Yes* (n)	*No* (n)	(n)
Bowed femora	5	12	—
Frontal bossing	11	1	5
Short fingers	9	0	8
Relative macrocephaly	9	4	4
Small chest	6	11	—
Amniotic fluid volume ≥ 95th centile	11	6	—
Skeletal dysplasia suspected	16	1	—
Accurate diagnosis made	14*	3	—

*Six by amniocentesis; five by ultrasonography alone; two by non-invasive prenatal diagnosis using cell-free fetal DNA; one by fetal blood sampling.

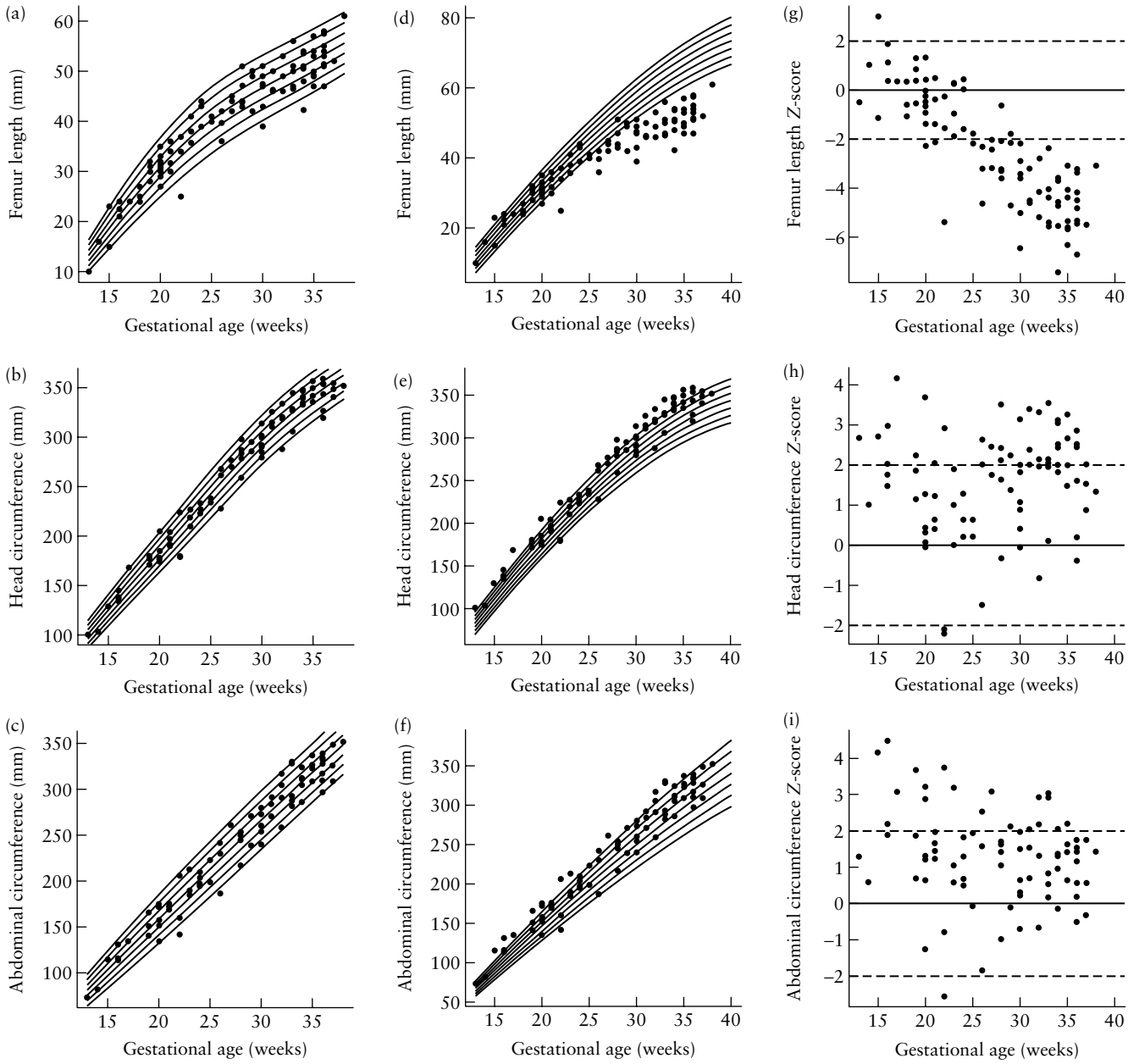

Figure 1 Fetal size charts for femur length (a), head circumference (b) and abdominal circumference (c) against gestational age in fetuses with achondroplasia. Note the curvature in the femur-length chart as growth declines in the third trimester. Comparison with normal fetuses is demonstrated by overlaying measurements from affected fetuses on charts of fetuses of normal size (d–f, respectively) (derived from Chitty *et al.*[15–17]) and by plots of *Z*-scores showing the deviation from the normal range (g–i, respectively).

two cases, both of which were delivered prematurely because of intrauterine growth restriction (IUGR).

DISCUSSION

We have confirmed that obvious limb shortening in achondroplasia is only apparent in most cases in the early third trimester (Figure 1d), when the differential diagnosis includes other skeletal dysplasias, Down syndrome, fetal growth restriction and constitutional short stature (Table 2). We have shown that relative macrocephaly is a feature across gestation (Figure 1e). A new observation is that the AC also tends to be increased, although to a lesser degree than the HC, across gestation (Figure 1f). Further modeling of the data to construct likelihood ratios for FL, AC and HC[18] may aid earlier diagnosis as the trends in relative increase in HC and AC begin earlier in pregnancy (Figure 1e,f).

The postnatal features of achondroplasia include short long bones, frontal bossing, short fingers or trident hand and bowing of the femora. All can be detected antenatally as demonstrated in this series and can further raise the suspicion of this condition. A small chest has occasionally been reported prenatally in fetuses with achondroplasia[11], although it was seen in 6 out of 17 of our cases (35%) where a detailed description of ultrasound findings was

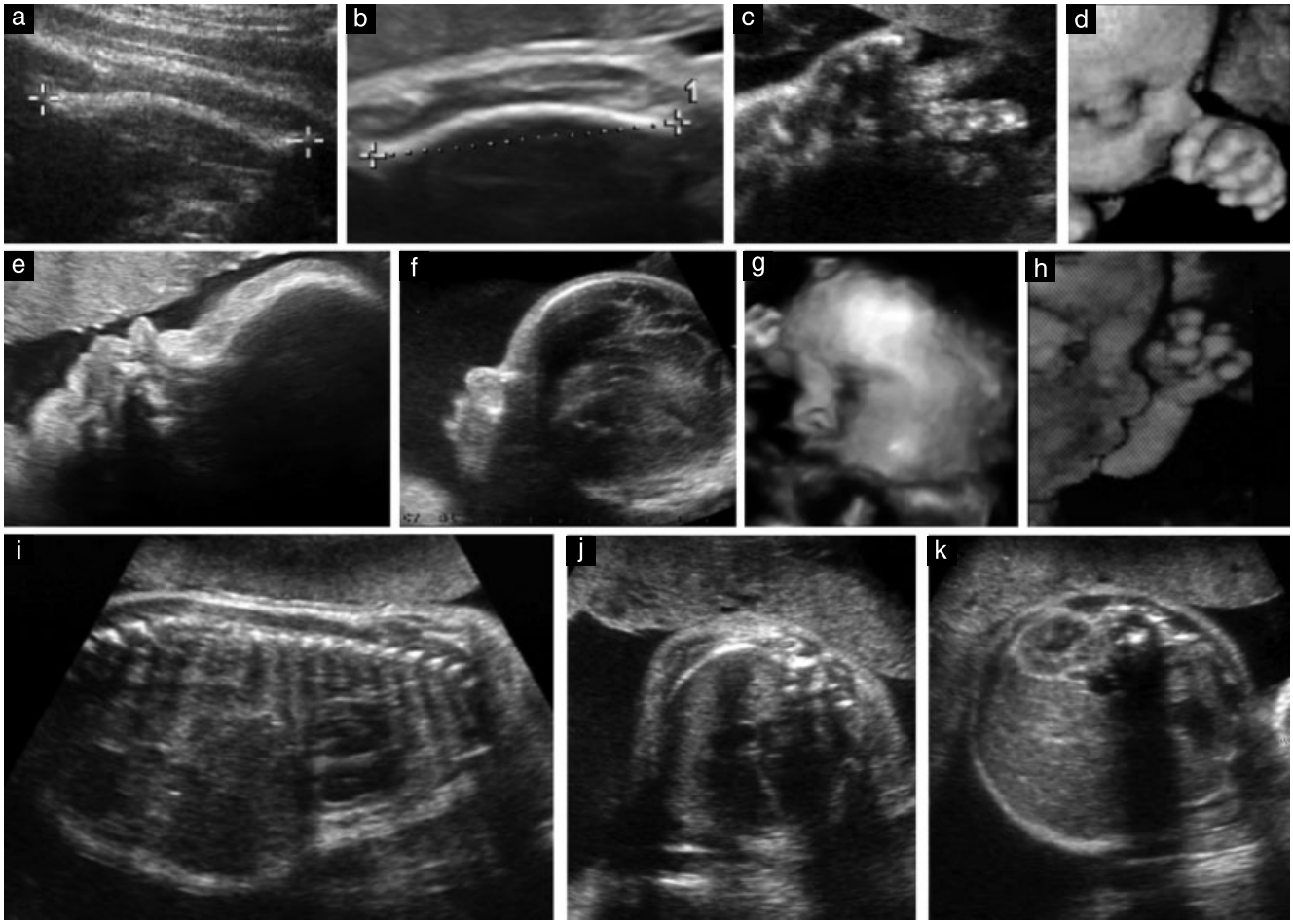

Figure 2 Sonographic features of achondroplasia detectable prenatally, including mild femoral bowing (a,b), short fingers (trident hand) shown in two-dimensional (2D) (c) and three-dimensional (3D) views (d), frontal bossing shown in 2D views of the profile (e,f) and in 3D views (g,h) and small chest shown in the parasagittal plane (i) and in a comparison between axial sections of the chest (j) and abdomen (k). The increased amniotic fluid volume is evident in (f).

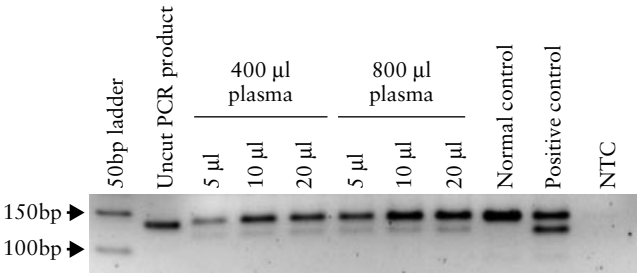

Figure 3 Polymerase chain reaction (PCR) showing amplification of the 132 base pair (bp) region of exon 8 containing the mutation causative for achondroplasia using 5, 10 or 20 µL of deoxyribonucleic acid (DNA) extracted from 400 µL or 800 µL of plasma, as well as on genomic DNA from a normal and a positive control. In the unaffected DNA sample, restriction digest of the PCR product with BsrG1 does not cut the DNA, giving rise to a single 132 bp fragment, whereas if the mutation is present a BsrG1 restriction site is created, and digestion produces fragments of 132, 112 and 20 bp, which results in two bands, rather than the single band seen in the control sample. In all six dilutions of the maternal plasma sample the faint second band can be seen, indicating the presence of the mutation in the maternal plasma sample.

available. Around 10% of neonates with achondroplasia can have significant respiratory difficulties, some of which may be manifest at birth[19] while others can occur later in infancy or childhood, when they usually present as sleep apnea. Although in one study early respiratory function testing in a group of symptomatic infants demonstrated normal lung volumes[20], studies in adults have demonstrated decreased lung volumes and compliance, which may contribute to the respiratory difficulties[21]. It is interesting to note that although a small chest was noted in six of our cases, none of these suffered obvious respiratory difficulties at birth. A small chest in the third trimester has previously been reported in fetuses with achondroplasia but, in these cases, this observation together with short limbs and other features, such as hydrocephalus and frontal bossing and, in one case, femoral bowing, led to a prenatal diagnosis of thanatophoric dysplasia[11].

We have demonstrated that features such as femoral bowing can occur more frequently than expected from the literature, with 29% of our cases having this feature in the third trimester. The commonality of features in achondroplasia and thanatophoric dysplasia means that misdiagnosis is understandable and it is clearly important to distinguish the two to ensure accurate parental counseling and avoidance of inappropriate intervention. Some authors have suggested the use of a femur length : abdominal circumference (FL/AC) ratio

Table 2 Aids to the differential diagnosis of achondroplasia

Investigation	*Achondroplasia*	*Fetal growth restriction*	*Down syndrome*	*Constitutional short stature*	*Other skeletal dysplasia*	*Thanatophoric dysplasia*
Detailed anomaly scan	Frontal bossing, short fingers, ± slightly small chest	No obvious anomalies	Markers of aneuploidy, AVSD, duodenal atresia	No obvious anomalies	Variable findings	Frontal bossing, short fingers, very small chest with short ribs
Growth pattern						
Femur length	Normal until > 25 weeks when growth falls from centiles, and almost ceases *ca*. 36 weeks	Variable onset of short limbs	Often < 3rd centile from early pregnancy	Often ≤ 5th centile from early pregnancy, growing at a normal velocity	Variable but often short from second trimester or earlier	Short from first trimester
AC	Within normal range	Variable onset of small AC with declining growth velocity	Normal	Normal	Usually within normal range	Normal
HC	Relative macrocephaly	Usually within normal range	Normal	Normal	Usually within normal range	Relative macrocephaly (± cloverleaf skull)
Appearance of long bones	± mild bowing of femora + rhizomelic shortening in arms	Normal	Normal	Normal	Variable pattern of shortening, ± bowing	All significantly short, bowed femora (telephone receiver handle)
AFI	Often increased	Often decreased	Normal (unless DA)	Normal	Variable	Increased
Doppler	Normal fetal and maternal Doppler	Abnormal uterine artery ± fetal Doppler	Normal	Normal fetal and maternal Doppler	Normal fetal and maternal Doppler	Not reported
Down syndrome screening	Normal	High hCG, low PAPP-A	Increased Down syndrome risk	Normal	Normal	Normal
Family/maternal history	None	Previous FGR, maternal antibodies, SLE	None	Mother and/or father short stature, familial short stature	Depends on diagnosis and inheritance pattern	None

±, with or without; AC, abdominal circumference; AFI, amniotic fluid index; AVSD, atrioventricular septal defect; DA, duodenal atresia; FGR, fetal growth restriction; HC, head circumference; hCG, human chorionic gonadotropin; PAPP-A, pregnancy-associated plasma protein-A; SLE, systemic lupus erythematosus.

of < 0.16 as a useful aid to distinguish lethal from non-lethal skeletal dysplasias[22], and this ratio might therefore be useful in distinguishing thanatophoric dysplasia, which is universally lethal, from achondroplasia. In our series data were available to evaluate the FL/AC ratio in all cases. Before 25 weeks' gestation it was < 0.16 in only one case, where the fetus was measured from 13 weeks onwards because of risk of hemolytic disease of the newborn. While the ratio was 0.13 at 13 weeks, it remained > 0.16 throughout the remainder of the pregnancy. However, in the third trimester the ratio was < 0.16 in 11 of the 26 cases (42%). This observation is in keeping with the data presented by Rahemtullah *et al.*[22], who reported a ratio of < 0.16 in two of the three fetuses with achondroplasia measured after 30 weeks in their series. These data suggest that the FL/AC ratio is not a reliable prognostic indicator in the third trimester and we would not recommend its use in the diagnosis of suspected skeletal dysplasias, such as achondroplasia, presenting late in pregnancy. We would advocate looking at the pattern of growth as a major distinguishing feature and consider that the charts presented here will be useful to the clinician in this respect. While features such as short fingers, relative macrocephaly and frontal bossing are common to both thanatophoric dysplasia and achondroplasia (Table 2), the pattern of long-bone growth is very different. The onset of long-bone shortening occurs from the first trimester in thanatophoric dysplasia but not until the late second or early third trimester for achondroplasia (Figure 1).

We recognize that the data used to construct these charts are a mix of cross-sectional and longitudinal data. This is not ideal as cross-sectional data should be used in the construction of fetal size charts, while for growth charts it should be longitudinal data[23,24]. However, the rarity of the condition means that there are limited data and with very few outliers use of all available measurements seemed justified. It was not possible to construct charts for other long bones as these are not routinely measured at the time of a second-trimester screening scan, and thus no data were available until the third trimester in all except the two cases with an affected parent.

Although the diagnosis in our series was suspected on the basis of the ultrasound findings, definitive diagnosis can only be made by detecting the mutation in the *FGFR-3* gene or by postnatal radiology. In the past, definitive prenatal diagnosis could only be made following invasive testing to obtain fetal genetic material for analysis. However, the identification of cell-free fetal DNA circulating in maternal blood[25] offers an alternative approach to definitive prenatal diagnosis and has now been reported in several cases[13,26,27]. Currently non-invasive prenatal diagnosis (NIPD) for single-gene disorders is limited to the detection or exclusion of alleles present in the fetus because they are inherited from the father or have arisen *de novo*[28]. Detection of maternally inherited alleles is not usually possible, as the majority of cell-free fetal DNA in maternal plasma is maternal in origin, only a small percentage arising from the fetus[29,30]. However, as the proportion of cell-free fetal DNA increases with gestation[29] molecular diagnosis using cell-free fetal DNA when ultrasound features are suggestive of achondroplasia seems an ideal application for NIPD in view of the third-trimester presentation and hence increased concentrations of cell-free fetal DNA.

In our laboratory we have successfully been offering fetal sex determination by analysis of cell-free fetal DNA since 2007[31] and are now developing NIPD for some single-gene disorders in families where the father carries a dominantly inherited mutation[32], or where the mutation arises *de novo*, as in the majority of cases of achondroplasia. While our experience of cell-free fetal DNA testing for achondroplasia is limited, here we report the correct diagnosis of this condition in four affected cases and exclusion in the other two cases subsequently found to have IUGR. For molecular testing to be possible there must be a high index of suspicion of the underlying cause of long-bone shortening in order to target the molecular diagnosis, and using cell-free fetal DNA this can now be done without risk.

In conclusion, the data presented here will facilitate accurate prenatal diagnosis based on the ultrasound features of achondroplasia. We have highlighted the frequency of features, some of which had not been commonly associated with the prenatal presentation of achondroplasia in the past, and produced charts of fetal size to aid the clinician. Improving the accuracy of the sonographic diagnosis of this condition will facilitate targeted molecular diagnosis, which can now be done safely using cell-free fetal DNA extracted from maternal plasma, to confirm the diagnosis and improve parental counseling.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Appendix S1 Method for extraction and analysis of cell-free fetal DNA in maternal plasma