Optimal non-invasive diagnosis of fetal achondroplasia combining ultrasonography with circulating cell-free fetal DNA analysis

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Running head: Non-invasive diagnosis of fetal achondroplasia

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Abstract

Objective: To assess the performance of non-invasive prenatal testing of achondroplasia using high-resolution melting (HRM) analysis. To propose an optimal diagnosis strategy combining ultrasound scan and cell-free fetal DNA (cffDNA) analysis.

Methods: Prospective multicenter study. CffDNA was extracted from maternal blood from women at risk for fetal achondroplasia (paternal achondroplasia, previous affected child or suspected rhizomelic shortening). The presence of one of the two main *FGFR3* mutations was determined by HRM combined with confirmation by SNaPshot minisequencing. Results were compared with phenotypes obtained by 3D computed tomography, post-natal examination and/or molecular diagnosis by an invasive procedure. Fetal biometry was also analyzed (head circumference and femur length) in order to offer cffDNA for achondroplasia in selected cases.

Results: Eighty-six blood samples from women at risk were collected (and sixty-five from control women). The overall sensitivity and specificity of the test were respectively 1.00 (95% CI [0.87-1.00]) and 1.00 (95% CI [0.96-1.00]). Critical reduction of femur length for affected fetuses can be observed from 26 weeks of gestation.

Conclusion: HRM combined with SNaPshot minisequencing is a reliable method for non-invasive prenatal testing of achondroplasia. Its implementation in routine clinical care combined with ultrasonography is an efficient strategy for non-invasive diagnosis of achondroplasia.

Introduction

Achondroplasia is the most common non-lethal chondrodysplasia, with an estimated rate around 1/20,000 live births,(1) and affects about 250,000 people worldwide. It is an autosomal dominant disorder with complete penetrance which is caused in more than 99% of those affected by one of two pathogenic variants in the fibroblast growth factor receptor 3 gene (*FGFR3*), namely c.1138G>A and c.1138G>C, resulting in a single amino acid change p.Gly380Arg (G380R). If prenatal diagnosis is requested (in the case of high-risk pregnancy) it is usually performed very early during pregnancy, using genetic analysis of chorionic villus samples or amniotic cells collected by invasive procedures. However, approximately 80% of cases are due to a *de novo* mutation (2) so that most cases of achondroplasia appear in what are theoretically low-risk pregnancies. Achondroplasia can be suggested prenatally by the presence of relatively non-specific sonographic features (3). Identification of short fetal limbs during the late second trimester or third trimester remains the cornerstone of the diagnosis of achondroplasia (4). Nevertheless, diagnosis of this rare disease remains uncertain in some cases, and a 3D computed tomography (CT) prenatal scan may be helpful (5,6). Molecular confirmation might also be required by invasive procedures leading to a theoretical risk of miscarriage or preterm premature rupture of membranes of 0.1 to 0.2% (7).

Since Lo and colleagues described the presence of cell-free fetal DNA (cffDNA) in the plasma of pregnant women (8), many applications have been developed, including prenatal diagnosis of achondroplasia, which served as a model for single-gene disorders suited to the development of a non-invasive detection

procedure (9). As most cases of achondroplasia result from a *de novo* mutation, the maternal DNA "background" does not interfere with the qualitative detection of the specific mutation in the maternal plasma. Different methodologies have been used for non-invasive prenatal testing (NIPT) of achondroplasia, usually in single cases or small series, all of them being retrospective (10–14).

We report here the results of a novel simple non-invasive molecular analysis of achondroplasia in a prospective multicenter cohort of pregnant women with a fetus with suspected skeletal dysplasia. A new diagnostic strategy for fetal achondroplasia combining ultrasound (US) biometric findings and cffDNA analysis is proposed.

Material and Methods

Study population

One hundred and fifty-one pregnant women were enrolled in 31 prenatal diagnosis centers in France between September 2009 and January 2016 (**Figure 1**). Eighty-six of them (patient group) were consecutively enrolled because of a high risk of fetal achondroplasia related to short long bone (below 3rd percentile) at US scan in the second or third trimester of pregnancy (n=78), a history of pregnancy with a *de novo* affected fetus (n=5) or a couple in which the father was affected by achondroplasia (n=3). All except two were singleton pregnancies. Women were offered cffDNA for achondroplasia at the time of US diagnosis. The median maternal age was 33 years (range: 18-45) and the term of pregnancy ranged from 12 to 37 weeks (median=32). Diagnosis of achondroplasia was confirmed or excluded by molecular analysis of the fetus after an invasive procedure (amniocentesis), 3D CT antenatal scan, histopathological examination of the fetus in the case of pregnancy

termination, or by clinical examination of the newborn by pediatricians at birth. If chondrodysplasia was suspected after postnatal examination, a molecular FGFR3 analysis was performed to confirm the diagnosis.

Sixty-five healthy women with a normal singleton pregnancy at the third-trimester US scan were also consecutively enrolled as controls (control group). They were of Caucasian (n=60) or African (n=5) origin. The median maternal age was 34 years (range: 22-48) and the term of pregnancy ranged from 27 to 38 weeks (median = 33).

Ultrasound examination

Prenatal US examinations were performed by referal operators in 31 French antenatal diagnosis or obstetric units, according to CNGOF (Collège National des Gynécologues et Obstétriciens Français) and CTE (Comité Technique de l'Echographie) guidelines. Conception dates used to calculate gestational age (GA) were based on a first-trimester scan (crown-rump length reported on Robinson curve)(15).

Blood sample preparation

Maternal blood samples were collected into cffDNA BCT Streck® tubes (10 mL) or EDTA tubes (5 mL) with a neutral solution of formaldehyde (1 mL/L final concentration) prepared as described previously (16). Less than 72 h after blood sampling, each tube was centrifuged at 3000g for 10 min at room temperature and the plasma was then immediately stored at −80 °C. Total plasma DNA was extracted from 1 mL of plasma using the MagNapure Compact Nucleic Isolation Kit (Roche Diagnostics, Meylan, France) according to the manufacturer's guidelines, the

adsorbed DNA being eluted with 50 µL of elution buffer. Each maternal plasma was extracted twice and all reactions were performed in duplicate. In parallel, genomic DNA (gDNA) was extracted from standard EDTA whole blood from the mother and the father using the Chemagen DNA blood kit on the JANUS Automated Workstation (Perkin-Elmer, Courtaboeuf, France).

FGFR3 mutation and paternal single nucleotide polymorphism detection in maternal plasma

High-resolution melting (HRM) PCR is an efficient and sensitive tool for mutation detection, not only in oncology but also in NIPD (17–19). We therefore decided to apply this strategy for the first time to achondroplasia. DNA extracted from maternal plasma was submitted to real-time PCR targeted to the region of the *FGFR3* gene containing the G380R variant followed by HRM and analysis of the corresponding melting curve, differences in the melting curve shapes revealing the presence of the G380R mutated allele in maternal plasma. Amplification was carried out in a similar manner to previously (20). Briefly, PCR reactions were carried out in a LightCycler LC480 Instrument (Roche Diagnostics, Meylan, France) in a final volume of 20 µL, with the High Resolution Melting Master Kit (Roche Diagnostics, Meylan, France), 3 mM $MgCl_2$, 0.5 µM of each primer (FGFR3.A: 5'-AGGAGCTGGTGGAGGCTGAC-3' and FGFR3.B: 5'-GGAGATCTTGTGCACGGTGG-3') (Sigma, Paris, France) and 5 µL of extracted DNA. The reaction mixture was initially incubated at 95°C in 10-min steps. Amplification was performed for 50 cycles of denaturation (95°C for 10 s; ramp rate, and 4.4°C/s), annealing (60°C for 10 s; ramp rate, and 2.2°C/s), and extension (72°C for 15 s; ramp rate, and 4.4°C/s) in a LightCycler 480 Instrument (Roche Diagnostics,

Meylan, France). HRM of PCR products was then performed at 95°C for 1 min, at 40°C for 1 min, and with increasing temperature from 65°C to 95°C at a rate of 1°C/s with 25 acquisitions per °C. During the same experiment, together with the corresponding genomic DNAs from the parents, a mutated DNA was used as a positive control and elution buffer as a no template control (NTC).

In the case of a negative result for the G380R variant, the ability of the method to detect a fetal variant (and as a consequence the presence of fetal DNA in maternal plasma) was evaluated through the detection of at least one other paternally inherited variant by the same PCR-HRM method according to a strategy similar to that of Chen et al (21). One set of primers was therefore designed specifically to amplify 13 single nucleotide polymorphisms (SNPs) located in different regions of the genome. This panel of SNPs was selected due to their high level of heterozygosity. After identification of SNP loci where the pregnant woman is homozygous and which differ from the father's, plasma samples were analyzed for these non-maternal alleles to confirm the presence and above all the inhibitory effect of DNA extracts. On the other hand, due to the non-specific nature of the method, all positive or ambiguous melting curve profiles were systematically confirmed by a second sequence-specific confirmation approach based on minisequencing. Briefly, following purification of the PCR products with ExoSAPIT (USB Europe, Staufen, Germany), SNaPshot analysis was carried out using the SNaPshot Multiplex kit (Applied Biosystems, Courtaboeuf, France) and the reactions were run on an ABI3130XL genetic analyzer and analyzed using the Genescan software (Applied Biosystems, Courtaboeuf, France). HRM and SNaPshot minisequencing sensitivities are reported in **Figure 2**.

Statistical analyses

Z score calculations for femur length and head circumference were done using Chitty's curves (22). Z score = (x-mean)/SD. For head circumference: Mean = -109.7+15.16*GA-0.002388*GA^3; SD = 3.913+0.2329*GA. For femur length: Mean = -32.43+3.416*w-0.0004791*w^3; SD = 1.06+0.05833*GA.

Statistical analysis was performed using the non-parametric Mann-Whitney test for quantitative variables. P values of less than 0.05 were considered statistically significant.

In order to diagnose affected fetuses, while avoiding the routine use of cffDNA for achondroplasia, as it is a highly specialized and costly assay, and limiting the use of CT scans, we provided a decisional tree. This algorithm took into account the cffDNA test results, and US biometrical and morphological findings. Femoral length z-score thresholds were retrospectively established in order to limit the number of useless non-invasive tests (true negative tests) while testing positive most of the affected fetuses.

Ethical aspects of the study

This study was approved by the institutional review board CPP ILE DE FRANCE II (2009-154). All data were de-identified to ensure patient privacy and confidentiality. In line with French regulations regarding prenatal diagnosis, written informed consent was obtained from all patients.

Results

Non-invasive prenatal testing for achondroplasia

Of 151 pregnant women in the present study (median gestational age: 32.7 ± 4.5 weeks, range: 11.6 - 38.6), 33 were positive for fetal achondroplasia (G>A variant n=31, G>C variant n=2), two of the pregnancies being twin (dichorionic, diamniotic). All these positive results were from high-risk patients (US findings n=31, father affected n=2) and were consistent with antenatal findings, a 3D CT-scan (n=15) and/or DNA analysis of the fetus after an invasive procedure (n=14) when performed, and/or clinical examination at birth (n=32). Ten terminations of pregnancy were performed in accordance with French law. All other high-risk patients were negative for the *FGFR3* variant and none of the fetuses were affected by achondroplasia. None of the 65 patients of the "control group" had a positive cffDNA assay. Three cases of Down syndrome were prenatally diagnosed in the "control group" (the pregnancies were terminated). The results of the pediatric examination at birth were normal in all infants. There were no false-positive or false-negative cffDNA results, and sensitivity and specificity were 1.00 (95% CI [0.87-1.00]) and 1.00 (95% CI [0.96-1.00]), respectively (**Figure 1**).

For three patients, analysis of cell-free DNA by HRM showed an abnormal melting curve, but were not confirmed by minisequencing. Conventional sequencing of the mother's gDNA demonstrated that these patients were carriers of the benign variant c.1150T>C (p.Phe384Leu), thus confirming the absolute necessity to confirm any abnormal HRM result. Additionally, for 23 patients negative for the *FGFR3* variant, the presence of paternally derived SNPs could not be definitively demonstrated either because we lacked the father's blood (n=12) or because none of the 13 SNP studies were informative (n=11).

Ultrasound biometric findings

Analysis of second-trimester US scans (median: 22.3 weeks) showed a significant reduction of femur length (FL) between fetuses tested for achondroplasia with a negative cffDNA result in comparison with fetuses with a positive result (**Table**). Z-scores (+/- SD) were -1.5 (+/- 1.2) vs -1.0 (+/- 1.2), respectively (p=0.03). At the last US examination (median: 32.3 weeks), fetuses with achondroplasia exhibited critical shortening of FL compared to fetuses with suspected short long bones but unaffected by achondroplasia: Z-scores (+/- SD) were -5.0 (+/- 1.5) vs -3.0 (+/- 1.2), respectively (p<0.0001) (**Figure 3**). Linear regression curves for FL of "cffDNA negative" and "cffDNA positive" subgroups crossed at 25.6 weeks of gestation (**Figure 4**). There was no difference concerning head circumference at the second-trimester US scan. However, head circumferences at the last US examination tended to be higher for fetuses affected by achondroplasia than for those not affected: Z-scores (+/- SD) were 0.4 (+/- 1.3) vs -0.7 (+/- 1.0) (p<0.001). In the patient groups, there was no difference between "negative test" and "positive test" subgroups.

Decisional tree

In order to diagnose fetuses affected with achondroplasia, we considered the following combined strategy. Any woman carrying a fetus with suspected rhizomelic shortening at US during the second trimester was first referred to an experienced sonographer in order to look for additional specific signs of achondroplasia, such as frontal bossing, depressed nasal bridge, limb bowing and/or trident hand. If there was one such additional sign, cffDNA was offered. If rhizomelic shortening was isolated (femoral length z-score ≤ -2), the US scan was repeated at 26 weeks or later and cffDNA could be offered in the case of worsening. After 26 WG, a femoral length z-score threshold of -4 was used: with this cut-off, 80% of the affected fetuses were

tested only on the basis of biometric findings. The last 20% were tested because of typical morphological signs. Indeed, this test would not have been proposed to 75% of negative patients with a femoral length z-score threshold of -4. In both situations, if diagnosis of achondroplasia was not confirmed by cffDNA, a CT-scan was considered after 28 weeks, along with an invasive procedure for aCGH analysis or any other investigations to help make a differential diagnosis (other diagnosis of skeletal dysplasia). (**Figure 5**)

Discussion

We report here a new simple approach to non-invasive diagnosis of achondroplasia based on cffDNA analysis by using HRM-PCR technology combined with minisequencing. This cffDNA approach was highly accurate in 151 pregnant women, 86 of whom were suspected to be carrying a fetus affected by achondroplasia. CffDNA detected the paternally inherited or *de novo* pathogenic variant leading to an achondroplasia phenotype in 33 fetuses. Sensitivity and specificity were 1.00 (95% CI [0.87-1.00]) and 1.00 (95% CI [0.96-1.00]), respectively. Combined with US biometric and morphological findings, cffDNA thus offers a new aid in the non-invasive diagnosis of achondroplasia.

NIPT for monogenic disorders is quite challenging due to the low level of cffDNA, but above all to extreme dilution of cffDNA in the background of maternal DNA in plasma(23) and because there is no possibility specifically to purify that fetal DNA. HRM-PCR, which can detect single base variation in a specific PCR amplicon, has been extensively used in oncology as it is able to detect minority alleles very accurately at a lower cost, without any specific expensive instrument. HRM-PCR has already been evaluated in the prenatal determination of several monogenic diseases.

Yenilmez et al. successfully detected paternally inherited fetal mutations responsible for β-thalassemia (17). All results were consistent with the genotypes obtained with conventional techniques, while HRM-PCR reduced the time needed from 3 days to 1 day. These results were recently confirmed by Zafari et al (19). Combined with COLD-PCR, HRM is more sensitive than conventional PCR for detection of very low-level mutations (24) and has been applied with very promising results to non-invasive determination of the fetal HPA-1 genotype by Ferro et al. (18) in the context of fetal-maternal platelet incompatibility. Macher et al. successfully used the same technical procedure for non-invasive diagnosis of multiple endocrine neoplasia type 2A (25).

Multiple other approaches to NIPT of achondroplasia have been evaluated, based on conventional PCR with restriction enzymes as a detection format (26), MALDI-TOF mass spectrometry (11), real-time qPCR (12), digital PCR (14) or more recently next-generation sequencing (13). None, however, has met the criteria of high sensitivity, lower possibility of DNA contamination, reduced hands-on time and technical expertise, turnaround time for reporting and cost-effectiveness, especially in terms of specific equipment requirements. Digital PCR is an attractive technology, but to date has limited clinical validation. In a single retrospective cohort of 26 fetuses at risk for achondroplasia, Orhant et al. reported 100% sensitivity and 100% specificity but with only 5 positive results (14). On the other hand, next-generation sequencing has been assessed (13) but seems ill-suited to prenatal diagnosis of rare diseases because of the number of samples vs the need for a very short turnaround time, leading to a high cost per run, which limits its use in clinical laboratories. HRM-PCR therefore appeared to be a good compromise.

Notwithstanding the test's accuracy and simplicity of use, it is important to define which patients could benefit from NIPT in order to avoid unnecessary

prescription. US biometric and morphological analysis showed that achondroplasia usually cannot be suspected until the middle of the second trimester. Critical reduction of femur length in affected fetuses can be observed after 26 weeks of gestation. The group of patients with a negative achondroplasia test was formed by a cohort of patients with heterogeneous clinical situations: most of them were affected by severe intra-uterine growth restriction, which can impact femoral length. Rhizomelic micromelia could be accompanied by other US features, such as frontal bossing, relative macrocephaly, depressed nasal bridge, limb bowing and trident hand. In our cohort, the relative macrocephaly sign does not improve achondroplasia detection. US signs of achondroplasia are of low specificity, although more specific signs, such as abnormal connection between the femoral diaphysis and metaphysis with overgrowth of the periosteum (‘collar hoop” sign), have been described (6). The advantage of 3D over 2D imaging is moderate, except that it can specify the extent of the disproportion between rhizomelic bones, the trunk and the head.(27) The accuracy of the diagnosis of achondroplasia could be dramatically improved by a late CT scan, but its use during pregnancy is controversial (28). Indeed, avoiding radiation is a public health issue, especially during pregnancy, so it is essential to promote the use of examinations that do not involve exposure to radiation.

In order to provide a cost-effective non-invasive strategy to diagnosis of fetal achondroplasia during the second and third trimesters, we designed an algorithm, which took into account US biometrical and morphological findings as well as cffDNA results (**Figure 5**). CffDNA could be proposed at any gestational age for the management of healthy pregnant women with an identified pre-existing risk factor, such as a personal history of an affected pregnancy, or for a couple where the father is affected and a carrier for the condition, even in the first trimester. Unfortunately, at

the moment, for technical reasons, it cannot be applied to pregnant women who are affected by the disease, as their plasma contains a background of mutated DNA.

Finally, HRM-PCR combined with US is a reliable and cost-effective approach to the non-invasive testing of achondroplasia. The strategy could be extended to differential diagnosis between achondroplasia and hypochondroplasia.

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Table: Femur length and head circumference of fetuses with achondroplasia

Group	Second-trimester ultrasound scan					Last ultrasound scan				
	GA (weeks)	Femur length (mm)	Femur length (Z-score)	Head circumference (mm)	Head circumference (Z-score)	GA (weeks)	Femur length (mm)	Femur length (Z-score)	Head circumference (mm)	Head circumference (Z-score)
Patients	**22.3 (1.6)**	**35 (4.2)**	**-1.2 (1.7) ***	**202 (16.7)**	**0.2 (0.7)**	**32.1 (2.3)**	**50 (4.9) ***	**-3.7 (1.6) ***	**297 (26.0)**	**0.4 (1.3) ***
NIPT positive	*22.4 (1.4)*	*36 (2.3)* †	*-1.0 (1.2)* †,§	*202 (14.1)*	*0.3 (0.8)*	*32.4 (2.3)*	*48 (3.8)* †,§	*-5.0 (1.5)* †,⟂	*305 (23.5)*	*0.4 (1.1)* †
NIPT negative	*22.3 (1.8)*	*34 (5.2)* ‡	*-1.5 (1.2)* ‡	*203 (18.9)*	*0.2 (0.7)*	*31.4 (2.4)* ¶	*50 (5.4)* ¶	*-3.0 (1.2)* ¶	*292 (27.1)*	*0.3 (1.4)* ¶
Controls	**22.3 (1.6)**	**38 (4.1)**	**0.2 (0.7)**	**198 (16.9)**	**0.3 (0.6)**	**32.4 (1.4)**	**62 (4.1)**	**-0.2 (1.1)**	**296 (14.3)**	**-0.7 (1.0)**

GA: gestational age; NIPT: non-invasive prenatal testing

*: patients group versus control group – p<0.001; †: achondroplasia-positive test subgroup versus control group – p<0.001; ‡: achondroplasia-negative test subgroup versus control group – p<0.001; §: achondroplasia-positive test subgroup versus achondroplasia-negative test subgroup – p<0.05; ¶: achondroplasia-negative test subgroup versus control group – p<0.05; ⟂: achondroplasia-positive test subgroup versus achondroplasia-negative test subgroup – p<0.0001

Figure 1: Flow chart

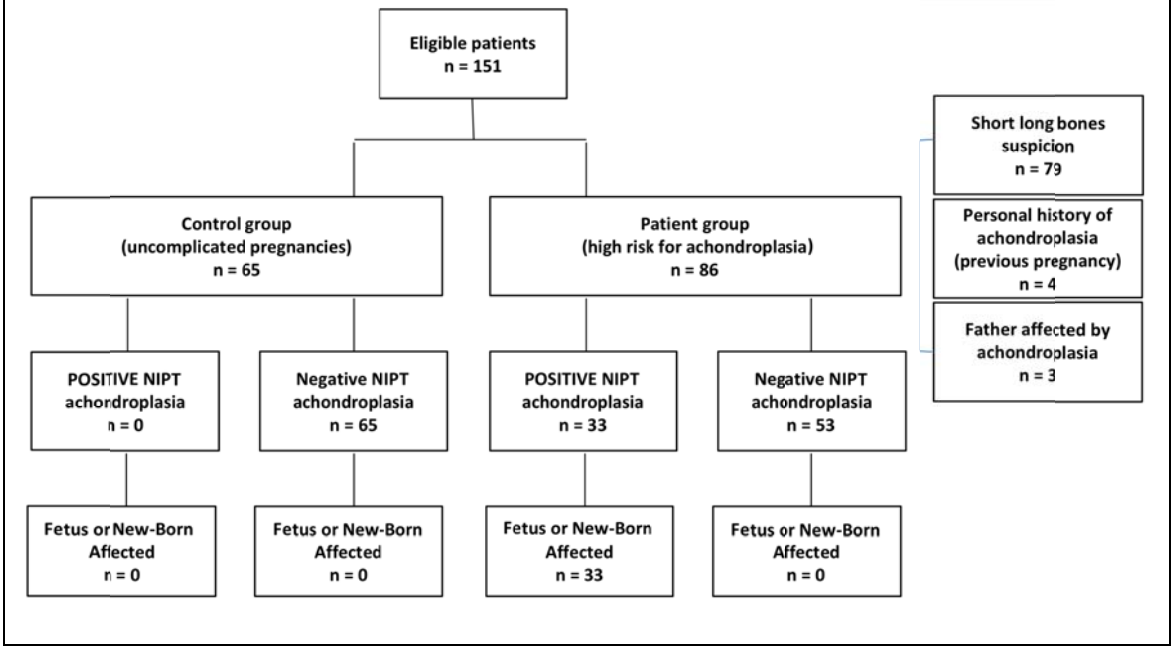
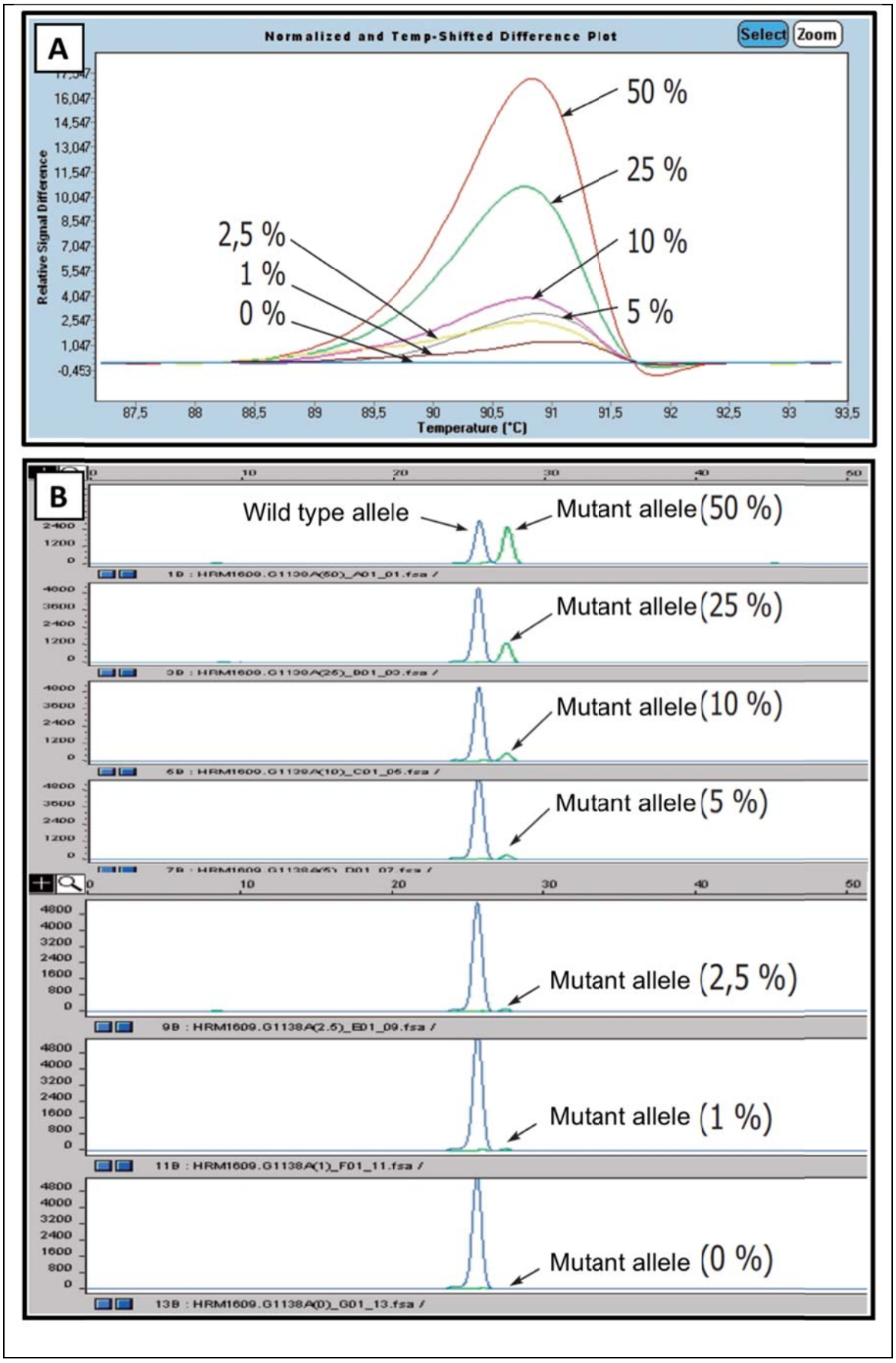
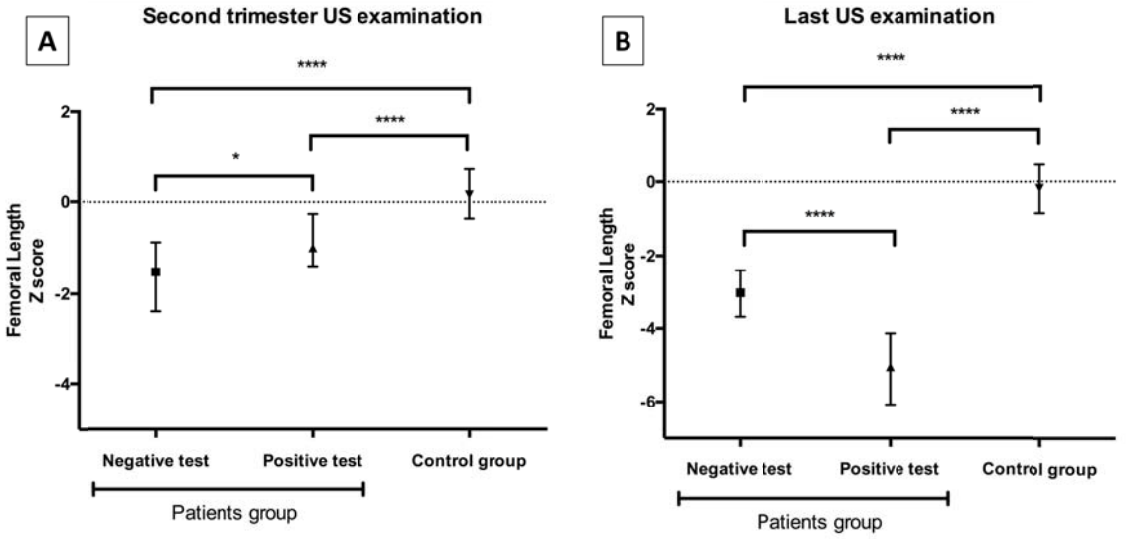


Figure 2: Sensitivity of high-resolution melting PCR and SNaPshot minisequencing for non-invasive fetal achondroplasia detection



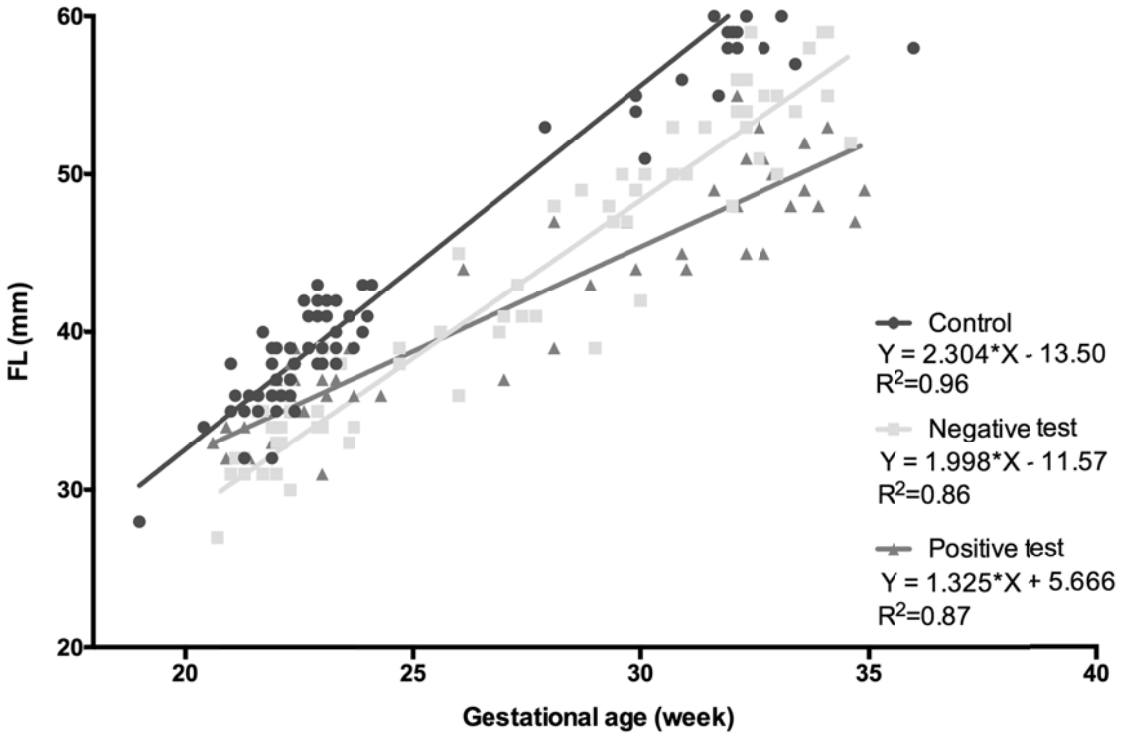
To determine the sensitivity of high-resolution melting PCR and SNaPshot minisequencing for non-invasive fetal achondroplasia detection, achondroplasia mutant DNA was serially diluted in wild-type DNA. We detected a positive signal even in a 1/100 dilution (**Figure 2A**: High-resolution melt curves; **Figure 2B**: SNaPshot electropherograms.

Figure 3: Femur length in fetuses with achondroplasia as a function of gestational age



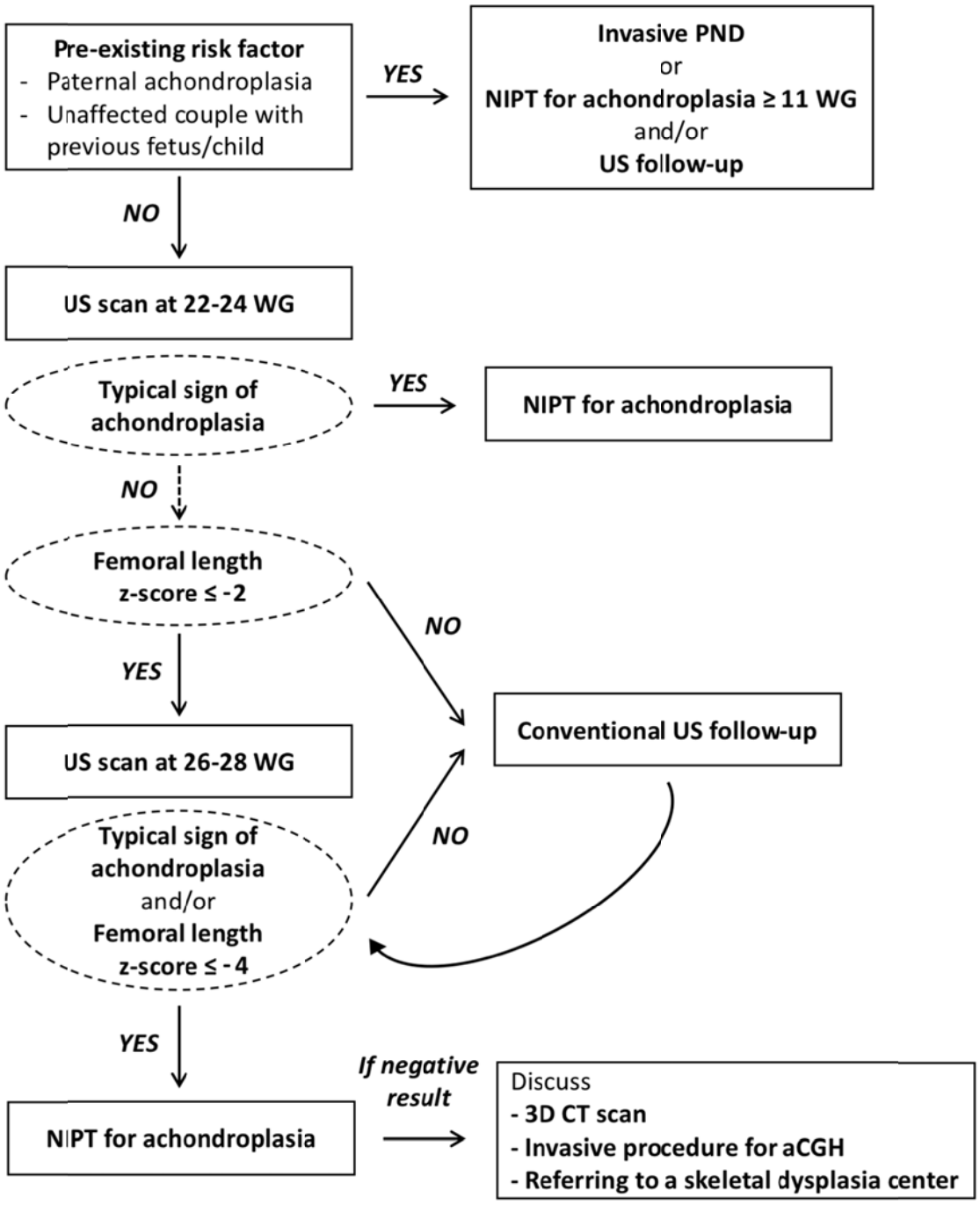
Z-scores represent deviations from the normal range and are statistically defined as Z=(x-mean)/SD. Medians for femur length Z scores were calculated for the "control group" (inverted triangle), "positive test" subgroup (triangle) and "negative test" subgroup (square) for second-trimester US examination (**Figure 3A**) and last trimester examination (**Figure 3B**). Interquartile ranges are given in brackets. * = $p<0.5$; **** = $p<0.0001$

Figure 4: Linear regression curves of femur length in fetuses with achondroplasia as a function of gestational age



"control group" (circles), "positive test" subgroup (triangles) and "negative test" subgroup (squares). Linear regression curves were calculated for each group. Linear regression curves for femur length of "negative test" and "positive test" subgroups cross at 25.6 weeks of gestation.

Figure 5: Non-invasive diagnosis of achondroplasia: decisional tree



CT: computed tomography; aCGH: array-based comparative genomic hybridization; NIPT: non-invasive prenatal testing; PND: prenatal diagnosis; WG: weeks of gestation