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FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Ultrasound Assessment of Fetal Musculo-Skeletal Development

By

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ABSTRACT

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ULTRASOUND ASSESSMENT OF FETAL MUSCULO-SKELETAL DEVELOPMENT

By Pamela Ann Mahon

Epidemiological studies suggest impaired fetal musculo-skeletal development has lifelong consequences. Using three-dimensional ultrasound (3DUS) in pregnant women taking part in a population-based survey of maternal nutrition (Southampton Women's Survey), the relationship between maternal influences and fetal musculo-skeletal markers of disease was explored.

The objectives of this thesis were to devise a repeatable technique for 3DUS of fetal long bones and soft tissue volumes *in utero* and to develop protocols for measuring stored volume data accurately and consistently. Measurements were then made on a large group of fetuses and correlated with body composition and vitamin D status, to ascertain whether maternal characteristics influence fetal musculo-skeletal development.

517 fetuses were scanned at 19 and 34-weeks gestation, on a KretzGE 3DUS system. Datasets of the uppermost fetal thigh were acquired at each scan and novel techniques were used to measure fat/skin, muscle and femur volumes. Using STATA 9.2, linear regression was used to relate maternal characteristics to fetal tissue volumes and paired *t*-tests to examine gender differences.

Analysis revealed that at 19 weeks gestation within-subject coefficients of variation (CV) for linear and volume measurements were 0.6% and 3.4%, respectively. Between-subject CV for linear measurements was 7.2% and 19.9% for volumes. This strongly suggests that the measured variability largely reflects biological variability between fetuses, rather than measurement error, confirming high reproducibility for 3DUS.

Correlations showed that larger mother's muscle mass predicted greater muscle in her fetus ($p<0.001$, $r = 0.17$) and larger maternal fat/skin layers predicted greater fat/skin layers in her fetus ($p<0.001$, $r = 0.23$). Taller mothers had offspring with greater femur length ($p<0.001$, $r = 0.20$) and femur volume ($p = 0.005$, $r = 0.13$). An additional finding of importance involved 10% of the fetuses who showed splaying of the distal femoral metaphysis, a feature usually associated with vitamin D insufficiency. Relating maternal vitamin D concentrations to a measure of metaphyseal splaying of the fetal femur at 19 weeks gestation, lower maternal vitamin D status was associated with greater metaphyseal splaying ($p = 0.003$, $r = -0.15$). This demonstrates that 3DUS may help us understand fetal musculo-skeletal development and will assist in the formulation of new interventions to improve prenatal development.

Ultrasound Assessment of Fetal Musculo-Skeletal Development

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All photographs and illustrations supplied by the author unless stated otherwise.

Introduction and Aims

1. The developmental origins hypothesis of musculo-skeletal health

It has come to light in the last 20 years or so, that normal fetuses encountering adversity in the womb, experience a higher incidence of chronic diseases in later life, (Couzin, 2002). This is because it is now known that babies are 'plastic' in early life and as such can be moulded by the intra-uterine environment, (Barker, 1994; Gluckman and Hanson, 2005). In humans, birth size serves as a marker of the intra-uterine environment and individuals who were small at birth have a higher risk of adult cardio-vascular disease, (Barker, 1995 and 2004; Clark *et al.,* 1998; Barker *et al.,* 2005). This is of particular significance when thinness at birth and during infancy is followed by rapid weight gain. This type of growth pattern is most unfavourable and in previous studies has shown to lead to an increased risk of cardio-vascular diseases in adulthood, (Sydall *et al.,* 2005). Other conditions influenced by intra-uterine surroundings include type II diabetes and osteoporosis, and it is thought they originate through adaptations made by the fetus in circumstances of under-nourishment *in utero,* (Barker, 1995, 1998 and O'Brien *et al.*, 1999). These adaptations permanently change the body's structure and function culminating in later disease. Muscle and bone development, size, density and strength, or predisposition for disease, can all be influenced or limited by the nutritional state of the mother, in addition to their predetermined genetic script, (O'Brien *et al.*, 1999). Epidemiological studies suggest that weight and body composition in infancy influence the risk of osteoporosis in adult life, (Gale *et al.,* 2001; Anotoniades *et al.,* 2003). Maternal thinness, smoking and vitamin D insufficiency have been linked with impaired bone mineralisation in the offspring, (Godfrey *et al.,* 2001), but to date few investigators have assessed fetal musculo-skeletal development in the same depth.

Epidemiological studies have additionally suggested that in early to mid-pregnancy, environmental and inherited factors that influence bone growth, manifesting in variable femur length and altered birthweight, have an inverse association with systolic blood pressure at the age of six, (Blake *et al.,* 2002). This infers that the malleable environment within the pregnant uterus plays an important part in the conditioning of the individual after birth and further on into later life. Research of this nature supports the hypothesis that early uterine life can be influenced not only by genetics but by uterine environment, leading to disease consequences many years later.

Recent evidence suggests that adverse conditions *in utero* may increase the risk of osteoporotic fracture in late adulthood. Epidemiological studies have shown that weight at birth and in infancy predicts peak bone mass, which is acquired in early adulthood, and bone mass in later life. This research suggests that poor intrauterine and childhood growth are associated with an approximate

doubling of hip fracture risk six decades later, (Sydall *et al.,* 2005; Cooper *et al.,* 2000 and 2002). Other studies have shown that maternal body build, nutrition, smoking and physical activity during pregnancy are predictive of the bone mass of their offspring at birth, (Godfrey *et al.,* 2001; Javaid *et al.,* 2006).

The research shown in these studies suggest that prenatal interventions could increase peak bone mass, and thereby have biologically relevant effects on skeletal fragility in old age. It is also apparent that peak bone mass is partly inherited, but other factors might explain the variation in individual bone mass and fracture risk. It has been shown that the relations of infant weights at birth and 1 year with bone mass in later life are independent of known genetic and adult environmental determinants of bone mass. In the 2006 study by Javaid *et al.* the results provided direct evidence that the intrauterine environment, as indicated by maternal vitamin D status during pregnancy, is significantly correlated with bone-mineral accrual at age 9 years.

The mechanisms under-pinning the long-term effects of the intrauterine environment are not known, but in the case of skeletal metabolism, fetal programming of the endocrine system is one aspect. Programming is the term used to describe persisting changes in structure and function caused by environmental stimuli during critical periods of early development and is now recognised to play an important part in disease processes. Current research has suggested that some of these effects may be mediated by *epigenetic* mechanisms, such as the *methylation* status of genes that regulate fetal and placental growth, as well as specific transport systems. These factors affecting growth and development are only just being explored and the research performed here, may assist in the recognition of crucial moments during pregnancy, when the uterine environment influences skeletal muscle development, metabolism and function. This is key to prediction and avoidance of such conditions in later life.

2. The need for new techniques to investigate musculo-skeletal development

Many studies of human development have been handicapped by lack of knowledge of the prenatal phase of life, so to address this problem new imaging techniques must be formulated, so that in-depth investigation of musculo-skeletal growth and development can take place. The imaging modality most often used for exploring anomalies within the growing human fetus is medical ultrasound (US) and it is this non-invasive technology that was used in this study, to assess bone and soft tissue development *in utero*. An additional component not previously explored is the expansion of ultrasound into the world of three dimensions, which has revolutionised the field of prenatal research.

Since its inception over 30 years ago, medical ultrasound has evolved dramatically. Developments in computing and transducer materials have made real-time 2D, 3D- and 4DUS possible. A 3D volume of data can now be acquired, as opposed to a 2D picture and this volume can be manipulated and navigated through to create more familiar image planes, aiding diagnosis or enabling the measurement of previously unseen structures. Three orthogonal scan planes (A, B and C) can be displayed simultaneously so relationships of each organ, bone or anomaly can be easily identified, (Figure I.1). This can be done repeatedly on stored volumes, without the subject being present. Here, living human fetuses have been used. If this modality proves a sensitive tool, it could aid the understanding of fetal growth, development and future disease.

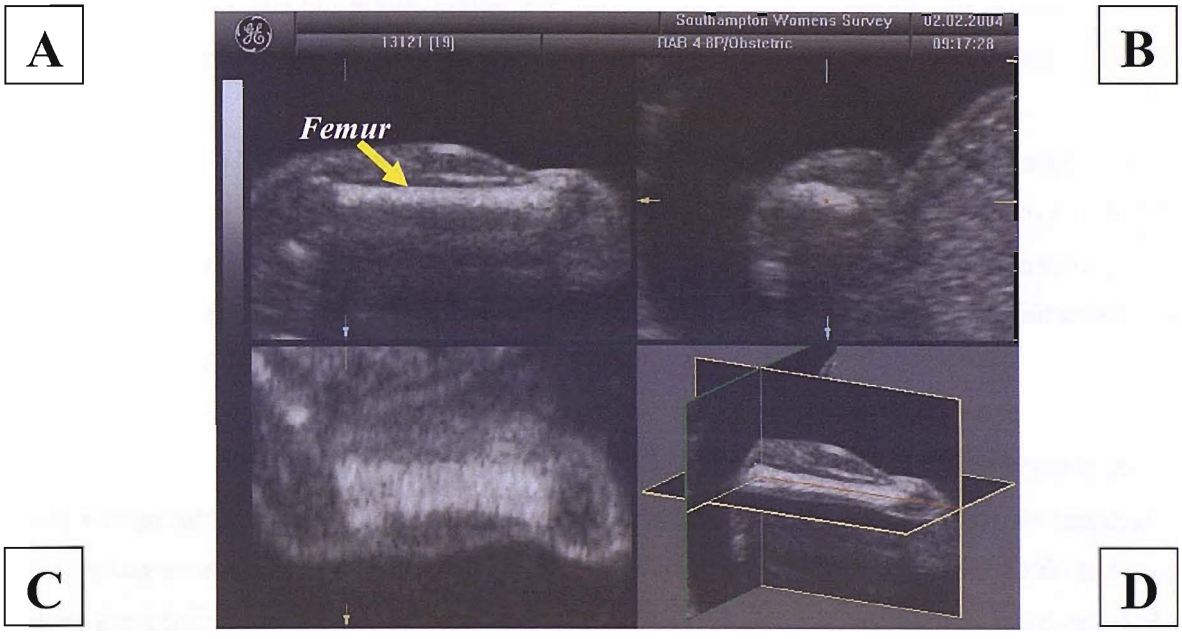


Figure I.1: Planes A, B and C showing a 19-week fetal thigh. A; conventional 2D longitudinal plane, B; transverse plane orthogonal to A and C; reconstructed coronal plane. D shows the orientation of all 3 planes. The femur is a white linear feature within the thigh tissues.

The imaging modality chosen for this study had to meet safety, ethics and practicality needs and medical ultrasound fulfilled these criteria for obstetric imaging. Measurements taken from radiographs of fetal bone are known to be more accurate than ultrasound biometry, (Piercecchi-Marti *et al.,* 2002), but in the living fetus, this is impractical as it presents safety issues and thus negates the use of computed tomography (CT) for the same reason. Published reports have described ultrasound as only moderately accurate in the detection of fetal musculo-skeletal anomalies and inaccurate in the diagnosis of specific skeletal dysplasias. Accurate prenatal imaging of skeletal structure and dimensions, remain problematic with ultrasound, whereas magnetic resonance imaging (MRI) can distinguish bone abnormalities with greater ease, (Applegate, 2004). However, MRI is cost prohibitive for routine use and the lengthy picture acquisition times needed,

does not accommodate a mobile fetus. In all respects ultrasound is considered the safest and most useful tool in imaging and it is for this reason that ultrasound has been chosen for this study.

Over 25% of all medical imaging studies worldwide are ultrasound examinations, demonstrating its diversity of use and importance, (Harvey *et al.*, 2002). For 3DUS, recent computational developments have improved image quality and data storage, with the ability to 'map' patients' anatomy so that structural relationships can be highlighted, (Nelson *et al.*, 1998). Three-dimensional *mapping* has become commonplace offering safe, early fetal imaging, generated by machines that produce high resolution, noise-free, real-time, dynamic images. Furthermore, 3DUS is affordable and produces data that can be manipulated and viewed without the subject being present. It has no known bio-effects and as it is used routinely in many centres and appears regularly in the media, recruits have prior knowledge of a technique that does not stimulate anxiety.

Used prudently, ultrasound is an invaluable research and diagnostic tool, which has proven to be less invasive and safer than other imaging modalities. It is currently considered that 3D- and 4DUS imaging poses no greater hazard than conventional 2DUS and potentially decreases ultrasound exposure to the growing fetus, (Hershkovitz *et al.,* 2002). Current research on ultrasound safety will be covered in Chapter 1 – Background.

The femur and thigh were chosen for this study for several reasons. Firstly, the fetal femur is an easily recognisable bone to image and repeatedly assess in the same plane. Secondly, its length is strongly associated with gestational age, (Chitty and Altman, 2002; Honarvar *et al.,* 2000) and thus similar associations may be anticipated with other dimensions. Thirdly, long bones and particularly the femora are the most rapidly growing bones of the fetal skeleton, sensitive to environmental change, (Eveleth and Tanner, 1990). By studying their growth and geometry a better picture of fetal skeletal development may be evaluated. The thigh tissues could also provide valuable information about nutrition and body composition, when linked with dietary and health status of the mother, (Hata *et al.*, 1998; Liang *et al.*, 1997).

3. The opportunity for this research

For the research to be successful a large cohort of pregnant women was needed and the author was fortunate to have access to the Southampton Women's Survey, (hereafter SWS). This survey was set up by the Medical Research Council Environmental Epidemiology Unit (MRC EEU), now the MRC Epidemiology Resource Centre, University of Southampton, UK. The SWS is a unique population study of young women, which encompasses information on pre-pregnant health, diet and lifestyle factors, in addition to those during pregnancy.

Between 1998 and 2002, 12,500 women aged 20 to 34, were interviewed regarding their general health, diet, activity and lifestyle. The survey participants were obtained through a selection of General Practice (GP) surgeries whose medical staff were approached and permission was obtained for their patients to be recruited. Women were asked to contact the SWS team if they subsequently became pregnant, alternatively the SWS was notified of the pregnancy by the woman's GP, as written consent for this pathway had already been obtained. In women registered with a sub-set of GPs, additional parental permission was sought for a DXA (dual-energy x-ray absorptiometry) scan to be offered to assess neonatal bone density. This sub-set formed the population from which the authors' study group was selected.

At the pre-pregnant interview trained research nurses took measurements including height, weight and skin-fold thickness, along with blood samples for measuring folate and haemoglobin levels. Any woman who subsequently became pregnant was offered three ultrasound scans at 11, 19 and 34 weeks' gestation, with an additional early dating scan if her menstrual dates were uncertain. These scans were performed at the SWS Ultrasound Unit, situated in Princess Anne Hospital, Southampton, UK. By January 2005, over 3300 pregnancies had resulted in 2500 single live babies. This reduced total was due to the exclusion of multiple pregnancies, miscarriages, terminations and recruits leaving the survey before delivery.

At each core scan, comprehensive biometric measurements of the fetus were taken by qualified sonographers, one of which was the author. The measurements comprised fetal bi-parietal diameter (BPD), head circumference (HC), thoracic circumference (TC), abdominal circumference (AC) and femur length (FL). Placental volumes at 19 weeks, amniotic fluid indices at 34 weeks and Doppler blood flow studies of the umbilical artery at 19 and 34 weeks, renal and middle cerebral arteries at 34 weeks, were also collected. At 11 and 34 weeks, maternal blood samples were taken and quantitative ultrasound of the maternal calcaneus for bone density and skin-fold measurements were performed. After the 19-week scan, maternal and paternal grip strength tests were administered by research nurses who also took paternal blood and DNA samples. Additionally at the 11 and 34-week appointments, food frequency questionnaires were completed.

Duration of gestation was derived from menstrual data, which had been confirmed by scan at the 11 week visit, or by dating scan. To recruit for the 3DUS cohort, the author selected women at their 19 week scan and they were only included onto her study if there were no medical problems which would complicate the pregnancy. The sub-set selected by the author (PM) was collected between October 2002 and June 2005, and totalled 525. At their 19 week scan, this cohort received an additional 3D scan of the uppermost fetal femora by PM who repeated this 3D volume acquisition

at their 34 week scan. Although the scans were termed the '19' and '34' week scans, a window of gestational ages was accepted at each visit as shown below:

18 weeks + 0 days to 21 weeks + 0 days (18+0 – 21+0), for the 19-week scan
33 weeks + 0 days to 36 weeks + 0 days (33+0 – 36+0), for the 34-week scan

After delivery, birthweight was measured and anthropometric measurements were taken by the SWS research nurses within 72 hours post-delivery. Samples of placental tissue and cord bloods were taken by the delivering midwives for storage and later analysis.

Neonatal DXA scans were performed within 14 days of delivery by PM and a research midwife (LG).

All women gave written informed consent before scans or blood tests and were at liberty to leave the survey at any time.

Ethics approval for this survey and the use of data collected was granted by the Local Research Ethics Committee Nos: 307/97, 089/99,153/99 (see Appendix I).

A risk assessment was carried out adhering to the guidelines of the University of Southampton and no risk to mother, baby or operators during the course of this research was identified. The pertinent paperwork for this Risk Assessment is contained in Appendix II.

4. Aims of the Study

There were 4 major aims of this piece of research which attempted to answer 4 questions.

1. Can we use medical 3DUS to visualise and quantify fetal bone and soft tissues *in utero*?

In obstetric research, ultrasound has been the technique of choice for over 30 years, not only because it is safe, but it has proven to be a reliable non-invasive method of imaging the fetus. Whilst fetal biometry is not new, the use of 3DUS to measure the growing skeleton is a recent innovation. This study aimed to devise a reproducible technique for acquiring 3D datasets and to develop protocols for measuring stored data accurately and consistently. Once PM had commenced examining the cohort using 3DUS, it was apparent that views obtained, appeared to show details not seen before, such as the medullary canal in bone and sheaths surrounding thigh muscle groups. In order to assess if the structures seen were 'real' and not artefact, a scan acquisition technique was devised and validated to corroborate the findings. Also, previous researchers overlooked potential measurement discrepancy, assuming their results to be valid. This study attempted to authenticate measurements in a scientific and logical way. With the aid of an in-house constructed test-tank, inaccuracies and limitations would be explained. Reproducibility of useful scan planes and volumes have been studied, using test phantoms in the infancy of 3DUS, (Riccabonna *et al.*, 1995), but these were limited as they used inorganic materials. It was hoped that by inserting organic material within the proposed test-tank, accuracy and technique may be improved. In turn, the machine's capabilities and limitations would be highlighted. By demonstrating reproducible and accurate techniques in both acquisition and calculation on stored data, it may be possible to make measurements of value in assessing bone and soft tissue growth *in utero*, but the machine must be able to image the required structures with sufficient detail and resolution for this to be possible. Bone is difficult to image by ultrasound in both children and adults, due to its dense nature, but the less robust skeleton of the fetus should be more accessible. With the system used here, it was hoped that visualisation of fetal bone elements would be possible.

2. Can we use 3DUS to quantify bone and soft tissue volumes *in utero* and use these measurements to examine size, differences between sexes and growth velocity of these tissues from mid to late pregnancy?

When 3DUS was first developed, it was used in fetal medicine where clearer visualisation of fetal surface features was desired. Once it was discovered that bony structures could be imaged, researchers began looking at skeletal abnormalities, particularly in the skull and spine, (Riccabona

et al., 1996; Ulm *et al.*, 1999a; Yanagihara and Hata, 2000). It took several years for manufacturers to recognise the importance of 3D mapping and measurement of anatomical structures to aid surgeons and clinicians in diagnosis and treatment planning. The facility for imaging 3 planes synoptically and taking a volume of data for analysis is now commonplace, although measurements taken within these reconstructed planes can be problematic. Volumes and variable scan planes may be useful when looking at musculo-skeletal growth in the fetus and so to explore this, measurements were taken of the fetal thigh, femur, muscle and fat / skin layers. This data was then be used to assess structure, growth patterns, relative dimensions, body composition, bone and soft tissue volumes, within the growing fetus.

3. **Can we demonstrate whether maternal influences affect the body composition of the fetus?**

By measuring maternal pre-pregnant size and body composition, a comparison database of maternal variables was prepared, against which fetal bone and soft tissue measurements, predominantly volumes, could be evaluated. Research nurses from the SWS also gathered information relating to maternal pre-pregnant diet, health and lifestyle, including smoking status, parity and vitamin D intake. This information was used to study maternal age, adiposity, leanness, BMI, smoking and vitamin D status in relation to fetal size and growth.

4. **From the associations revealed, can we speculate about the implications for the risk of adult disease in the offspring?**

To understand how maternal body composition, lifestyle and diet might influence fetal musculo-skeletal development all of the fetal scan measurements and DXA readings were correlated with maternal data to examine any growth patterns or circumstances which lead to an adaptation in fetal growth or size. Critical periods of bone or soft tissue growth were investigated, which may be influenced by maternal variables. Variations in bone shape or volume due to gender differences were explored, in addition to predicted effects of smoking and body composition. Any patterns that emerged are addressed in the Discussion section of this thesis.

Chapter 1 – Background

When research commenced, it was apparent that several fields of expertise needed to be consulted. An initial review of ultrasound revealed an abundance of technical papers explaining 3DUS production, a wealth of charts for obstetric organ volume measurements, fetal abnormality case studies and copious opinions on safety. There was an equal amount of literature and websites advertising 3DUS as the best tool for visualising the unborn child for entertainment as well as medical purposes. However, sift the literature for technical validation of this new modality and there appears to be scant evidence. Ultrasound-guided interventional procedures and vascular applications have been researched, but there is no mention of successfully imaging fetal bone or validating the use of volume measurements on the fetal skeleton, particularly in human fetuses. This background summarises pertinent literature in the following areas:

1. Developmental origins of musculo-skeletal health.
2. Maternal body composition and vitamin D status.
3. Bone development, skeletal mineralisation and ultrasound appearances.
4. Muscle development and ultrasound appearances.
5. Fat / skin layers and ultrasound appearances.
6. Dual-energy x-ray absorptiometry (DXA).
7. Current 3DUS and biometric research.
8. Choice of 3DUS systems available.
9. Ultrasound safety issues.
10. Test-tank manufacture and experimentation.

1.1 Developmental origins of musculo-skeletal health

The human fetal skeleton contains about 30g of calcium, mostly deposited in the third trimester of pregnancy, when calcium is pumped across the placenta to establish a higher concentration of ionised calcium in fetal serum than in maternal serum. This level is maintained independently of the ambient maternal concentration, which is maintained at a physiological level by increases in both intestinal absorption and release from maternal bone. Any deficit between fetal accretion and maternal intake occurs at the expense of the mother's skeleton, (Haig, 2004). Once born the on-going mineralisation of the fetal skeleton must be maintained by dietary calcium intake. It is known that low milk intake during childhood and adolescence results in decreased bone mineralisation in the adult skeleton and an increase in the risk of osteoporotic fracture, (Javaid *et al.*, 2002; Kalkwarf *et al.*, 2003), but it is not known how early in prenatal life that calcium absorption becomes critical for this programming, (Cooper *et al.*, 2000). It has been suggested that the rate of bone mineral

accrual after birth, is determined by the ease or difficulty of development *in utero*, (Rubinacci *et al.*, 2003).

A major bone disorder which is increasingly being associated with adverse fetal bone development is osteoporosis. This condition, as defined by the World Health Authority (1992), is a progressive symptomatic disease characterised by low bone mass and deterioration of bone tissue leading to fragility and fracture. It is a widespread disorder contributing to high fracture incidence and significant morbidity and mortality, and is a major public health problem in our growing populations, (Gale *et al.*, 2001). In 2003 it was estimated that £1.7 billion was spent treating wrist, vertebral and hip fractures in England and Wales alone, (Christodoulou and Cooper, 2003). Whilst current research is focussing on treatment and prevention by dietary and pharmacological means, understanding of its origins, especially in the pre-natal stage, may lead the way to a decrease in its occurrence.

It is now believed that peak bone mass achieved in childhood is one of the major determinants of the risk for future osteoporosis, (Specker, 2001), but if this is to be achieved, the uterine environment must be optimum for calcium transfer and this is only possible where enough circulating vitamin D is present. Significant calcium transfer from the mother to the fetus occurs in pregnancy as calcium is increasingly absorbed through the maternal bowel during and in the last trimester, maternal bone loses most calcium at a time when the fetal skeleton is rapidly mineralising, (Kalkwarf and Specker, 2002). What is unclear is the role of calcium absorption in the fetal skeleton and how its variability and timing of the 'critical period' affects the programming of osteoporosis.

Low protein diet in rats leads to reduced proliferation and differentiation of mesenchymal stem cells leading to subsequent 'catch up' growth. This is an important candidate mechanism for the induction of osteoporosis because peak bone mass is not attained at the correct maturity, (Specker 2001; Javaid *et al,* 2006) and a similar pattern is suspected in other mammals including humans. Insufficient protein in the maternal diet during pregnancy has been shown to cause lower fetal bone density accrual and insufficient bone mass in childhood. If catch up growth then occurs, this compounds the risk of osteoporosis in later life, (Cameron *et al.*, 2003; Oreffo *et al.*, 2003). Imprudent diet may be a candidate for low protein intake in some pregnant women, which may in turn lead to impaired bone development in the offspring.

1.2 Maternal body composition and vitamin D status

The body composition of a woman before conception has implications for the bone quality of her offspring. In previous SWS studies it has been recognised that thinner women with smaller triceps skin-fold measurements during pregnancy have offspring with lower bone mineral density (BMD) and bone mineral content (BMC) as tested by neonatal dual energy x-ray absorptiometry (DXA) scans, (Godfrey *et al.*, 2001). This effect is independent of placental size and demonstrates the link between suboptimal body composition and bone mineral accrual. Whilst thinness, a poor or unbalanced diet during pregnancy leads to decreased BMC and BMD, activity levels also play a part. Women who are more active or walk faster than others are also likely to produce neonates with lower BMC and BMD, as the 'aerobic' effect of their lifestyle helps retain vitamin D and calcium for their own active skeletons to the detriment of their fetus, (Godfrey *et al.*, 2001). Additionally these women are also more likely to be thinner as a result of their activity levels.

Over the last 100 years studies have shown that vitamin D is critically important for development, growth, and maintenance of a healthy skeleton, as its major function is to maintain calcium homeostasis (Holick, 2003; Lin and White, 2003). Vitamin D (cholecalciferol) is not in fact a vitamin but a steroid hormone and is the essential precursor of 1,25-dihydroxyvitamin D_3 (sometimes expressed as $25(OH)D_3$), the steroid hormone required for calcium absorption, bone development and growth in children. Approximately 90% of the body's vitamin D is produced by photochemistry whereby the action of sunlight on the skin (ultraviolet light) produces vitamin D, which in the pregnant mother then crosses the placenta to supply her fetus. Vitamin D is actively produced by irradiation of 7-dehydrocholesterol within the skin by ultraviolet light and there is an inverse relationship of vitamin D production in relation to pigment in the skin. Thus individuals with darker skin easily become deficient in vitamin D, especially when they migrate to more temperate climates where there is less exposure to sunlight. Religious and cultural demands which require women to cover their bodies also impacts on the amount of vitamin D that can be synthesised by some individuals, (Nozza *et al.*, 2001).

The remaining 10% of vitamin D needed for health is acquired by diet and is predominantly found in fish oils, such as those in salmon and mackerel. Additional vitamin D in the form of supplementary preparations has become popular over the last 20 years making intake easier. However, lack of dietary education or the means to buy optimum food or supplementation, has an impact on the quality of nutrition the pregnant mother receives even today.

Blood serum levels of $25(OH)D_3$ are a measure of the body's vitamin D stores and used for diagnosing vitamin D insufficiency. At present there is no international guideline on categorising

normal levels of vitamin D within blood serum, but some centres work to a value of 70 nmol/L and below as being vitamin D insufficient, whereas other groups of researchers indicate that levels below 50 nmol/L denotes insufficiency. Levels below 25 nmol/L are associated with osteomalacia or rickets and at this level the individual would be classed as vitamin D deplete.

The recommended daily intake (RDI) of vitamin D for a child is 400 IU (see Glossary of Technical Terms). In adults if there is sufficient sun the RDI is 200 IU and pregnant women are advised to have 500–700 IU daily. Studies performed in Southampton, prior to and including participants of the SWS, showed that 31% mothers were insufficient in vitamin D in their diet using the 50 nmol/L cut-off value and 18% were deficient in late pregnancy in the Southampton area, (Javaid *et al.,* 2006). This had a 'knock-on' effect to the children by the age of 9, whose bone density, examined by DXA scan, was still depleted if there had been poor circulating vitamin D in late pregnancy.

After delivery vitamin D supply to the fetus decreases, as does calcium and phosphate levels, as these are not plentiful in breast milk, but are elements needed for continuing bone formation, (Rauch and Schoenau, 2002). Thus breast fed infants are more at risk of vitamin D deficiency and some studies suggest vitamin D supplementation for these babies to maintain normal levels, (Holick, 2003; Davenport *et al.,* 2004; Haig, 2004). If levels are low then mineralisation disorders such as osteomalacia or rickets can occur in the offspring. This is a result of reduction in the structural integrity of the bone as there is a deficiency in the amount of calcium supplied to the growth plates of the bone, (Prince and Glendenning, 2004).

The vitamin D stores accrued by the fetus *in utero* are depleted within the first 8 weeks of post-natal life, (Nozza and Rodda, 2001; De Lucia and Carpenter, 2002). If the stores are low during the uterine phase, the child will be even more disadvantaged in bone health if the only vitamin D it can utilise derives from sunlight exposure and the food it receives as a baby. In children with lighter skeletons the risk of fracture and other bone conditions is higher in adulthood, (Cooper *et al.,* 2000; Dennison *et al.,* 2001; Cooper *et al.,* 2002; Javaid and Cooper, 2002; Kalkwarf *et al.,* 2002). Low weight in infancy is also associated with osteoporosis in later life, (Gale *et al.,* 2001) and so careful biometric and volumetric evaluation of body composition (bone, lean and fat mass), could help predict those at risk.

In this thesis information on maternal vitamin D status will be incorporated with scan measurements to highlight any bone changes in fetuses whose mothers were known to have low vitamin D levels. Not only will femoral geometry and volume be assessed, but information from DXA scans will be incorporated, including measurement of bone density and mineralisation.

1.3 Ultrasound assessment of bone development and skeletal mineralisation

The fetal skeleton forms as a result of maternal nutrient transfer through the placenta, with approximately 30g of calcium being laid down; 80% of this transfer occurs in the last trimester of pregnancy. The ability to provide calcium for the fetus depends on maternal calcium intake and vitamin D status, efficient intestinal calcium absorption, maternal bone turnover and renal function, acting together with the capacity for placental calcium transfer. Studies of maternal vitamin D status during pregnancy are beginning to demonstrate the mechanisms through which maternal influences affect bone mass in the child, (Javaid *et al.,* 2006).

The femur and tibia are among the most rapidly growing bones of the fetal skeleton, the lower limbs being particularly sensitive to environmental stress, (Eveleth and Tanner, 1990). As a result the femur, which has been chosen for this study, is an excellent marker of environmental change *in utero.* Vitamin D and calcium status are pertinent to fetal femoral bone mineralisation and development, (Dennison *et al.*, 2001; Cooper *et al.*, 2002). Genetics and physical activity within the uterine environment also contribute to healthy osteogenesis, as keenly as they do in childhood, (Namgung *et al.,* 2000; Specker, 2001). In addition, early muscular activity affects osteogenesis by stimulating the development of the trochanteric ossification centres, (Carter *et al.*, 1996; Panattoni *et al.*, 2000).

Even before the fetal skeleton is visible by imaging modalities, formation of bone has begun. Mammalian bone arises from the differentiation of primitive *mesenchymal tissue* and at some sites this may involve the replacement of *cartilage* by bone and in others by the conversion of a more primitive membranous template, (Scheuer and Black, 2000). By day 28 after fertilisation the lower limb buds are recognisable on the ventro-lateral body wall of the fetal pole. They initially consist of a mesenchymal core derived from the *somatic layer* of *lateral plate mesoderm* that will form the bones and connective tissues of the limb. As the limb grows, cells begin to differentiate into cartilage and muscle and between days 32 and 37 the terminal portion of the limb buds become flattened to form hand-plates and foot-plates which further separate to form digits, (Sadler, 2004).

Development of the upper and lower limbs is similar except that morphogenesis of the lower limb is approximately 1 to 2 days behind that of the upper limb. Also, during the 7th week of gestation the limbs rotate into position away from the trunk. While the external shape is being established, mesenchyme in the buds begins to condense forming the femur, tibia and fibula by day 41. At this stage the human femur would be 11-14mm in length and visible by US. The cartilaginous femur begins to form between 44 and 47 days and is complete and measuring 13-18 mm by the end of the embryonic period. At 48 days, the femoral head begins to *chondrify* and by 52 days the neck, both *trochanters* and the *condyles*, are well formed in cartilage. By the 8th week of embryonic life the

femur measures 3.5 mm in length. Joints are formed in the cartilaginous condensations when *chondrogenesis* is arrested and a joint *interzone* is induced. The surrounding cells differentiate into a joint capsule.

Ossification of the bones of the extremities begins by the end of the embryonic period and primary ossification centres are present in all long bones of the limbs by the 12th week of development. In the femur a bony collar appears in the mid-shaft at 7 to 8 weeks and about a week later, *endochondral ossification* can be identified histologically in the centre of the shaft, although not apparent radiologically until about 2 weeks later. From the primary centre in the shaft or diaphysis of the bone, endochondral ossification gradually progresses toward the ends of the cartilaginous envelope. At first, *periosteal* bone occupies more of the length of the diaphysis, but after about 32 weeks, patterns of periosteal and endochondral bone formation are seen within the femoral envelope.

By about 12-13 weeks, ossification in the shaft has reached almost to the neck region proximally and to the lower end of the diaphysis distally. From the beginning of the 2nd trimester, remodelling of the proximal end of the diaphysis at the level of the lever trochanter by *osteoclast* cells and distally, around the *medial supracondylar line* has begun. *Resorption* and *apposition* then lead to enlargement in length and width, but subsequent growth proceeds rather more slowly. At about this time, the morphology of the femoral diaphysis is sufficiently distinct to permit identification.

During the second part of gestation, the femur becomes more robust and the extremities of the shaft are modified. By the 7th prenatal month, the proximal end changes from a convex dome shape to become angulated into 2 planes which lie under the cartilaginous head and greater trochanter. In the last prenatal month, to coincide with the appearance of the secondary centre, the distal end of the ossified shaft usually develops a central depression. At term, 75-80% of the overall length of the bone is occupied by ossified shaft, the diaphysis, whose terminal ends are known as the *proximal and distal metaphyses*. At birth the diaphysis of the bone is usually completely ossified, but the two ends, the epiphyses, are still cartilaginous. Shortly thereafter, however, the ossification centres arise in the epiphyses. Temporarily a cartilage plate remains between the diaphyseal and epiphyseal ossification centres. This epiphyseal plate plays and important role in the growth in the length of the bones. Endochondral ossification proceeds on both sides of the plate and when the bone has acquired its full length, the epiphyseal plates disappear and the epiphyses unite with the shaft of the bone.

Ultrasound visualisation of the fetal musculo-skeletal system is presently not possible before the 6th to 7th week, although the bones have formed by this gestation, (Carter *et al.,* 1996). However,

recent 2D-, 3D- and 4D ultrasound developments have resulted in remarkable progress in visualisation of early embryos and fetuses from 6 week's gestation and may one day become sufficiently sophisticated for skeletal anlage to be identified, (Kurjak *et al.,* 2005).

Bone mineral content (BMC) increases rapidly from the 16th week, remaining highly correlated to gestational age during development, in both the whole shaft and all diaphyseal portions, (Panattoni *et al.*, 1999). Calcium transfer across the placenta increases dramatically during the last trimester, which is from 24 weeks to term, with peak transfer between 36 and 38 weeks. The rate of transfer is in the order of 102–151 mg of calcium per kg of fetal weight per day, (Namgung *et al.,* 2003). It is anticipated that these changes may be visible on scan images with optimised resolution and future studies may accomplish this.

At later gestations increased bone mineral changes can be seen along the length of the shaft, with the highest values of BMD contained within the mid-shaft; the lowest at each diaphyseal end, in particular the distal end, (Figures 1.1 and 1.2). This gradient heralds the appearance of trabecular bone within the diaphysis as it changes shape and micro-architecture. The distal and proximal ends remain predominantly cancellous in nature, (Chiarasini *et al.*, 1992), with greatest BMD in the shaft. Histologically, there appears to be no statistically significant difference in ossification degree and growth velocity between sexes, (Panattoni *et al.*, 1999) and it is unlikely that ultrasound would be sensitive enough to add to this. What can be hoped for presently is that at later gestations, key points in skeletal ossification may be recognised and variations identified.

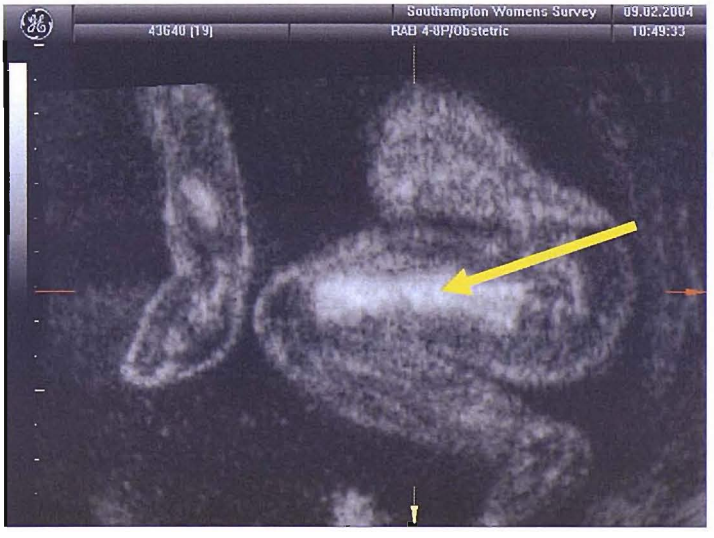


Figure 1.1: 19-week scan of the fetal femur (arrowed).

Femoral length is associated with gestational age, (Bagnall *et al.*, 1982; Exacoustos *et al.*, 1991; Hornarvar *et al.*, 2000; Gupta *et al.*, 2001; Chitty and Altman, 2002) and so it may be assumed that some other femoral dimensions may behave in the same way, such as bone volume.

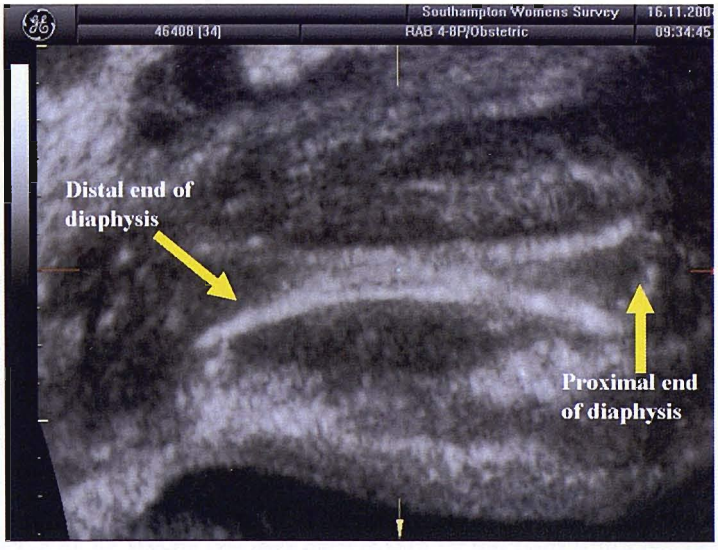


Figure 1.2: 34-week scan of the fetal femur. This demonstrates the denser diaphysis and cancellous proximal and distal ends of the femoral metaphysis.

Imaging bone by ultrasound is difficult as there is great discrepancy between the surrounding soft tissues and the mineralised bone itself. The propagation of ultrasound through bone is affected by this severe mismatch of acoustic properties and by fluid around bony structures, as in the pregnant uterus. Scattering of the ultrasound once it has arrived at the bone face is non-linear and much of the detail is lost as the echoes do not return to the transducer. Other studies using adult bone for the purpose of quantitative ultrasound in the calcaneus have highlighted the problem of discrepant tissues adjacent to each other, (Lasaygues *et al.*, 2005). This signal loss from the posterior aspect of the bone being imaged is the main reason for poor imaging of entire structures, an area which is being addressed by researchers mainly in the adult bone imaging field. This thesis attempts to evaluate the limited imaging capabilities of ultrasound on fetal bone.

1.4 Ultrasound assessment of muscle development

Genetic and intrauterine environmental factors influence fetal growth trajectory, reflected in birthweight and have long term consequences on bone and muscle mass in adulthood, (Gale *et al.*, 2002). Muscle, skin and fat layers all depend on a different set of circumstances for healthy development and as they can be distinguished apart sonographically, thickness and volume of the layers can be calculated using 3D ultrasound. Body composition studies have shown that fetal

abdominal subcutaneous fat thickness is influenced by maternal weight gain and gestational diabetes, (Rohl *et al.,* 2001). Also, with a decreased birthweight there is an associated reduction in muscle fibres, which may be detectable by ultrasound. This effect may underlie the reduced muscle strength of men and women who had a lower birthweight, (Kuh *et al.*, 2002). Mothers who smoke produce offspring with less muscle than infants of non-smoking mothers, although skeletal size and head-sparing appears to be unaffected, (Bernstein *et al.,* 2000). This affects resultant birthweight, a finding anticipated with this study. Other ultrasound studies shown an association between fetal upper arm volumes and birthweight, (Liang *et al.,* 1997; Chang *et al.*, 2002b) and increased fetal thigh volumes and increased birthweight, (Chang *et al.,* 1997b and 2003b), but the findings did not differentiate between the proportions of muscle, skin, fat and bone within the limbs.

1.5 Ultrasound assessment of fat and skin development

As shown in Figure 1.3 at 19 weeks the fat / skin layers of the fetal thigh are not distinguishable as separate layers on scan with the current technology available and therefore this structure has to be assessed as one layer at earlier gestations. For the purpose of this study this structure has been termed the 'fat / skin' layer. As fetuses develop fat begins to be accrued subcutaneously, but this does not occur until 28 weeks of gestation. After this time the layers can be distinguished separately, as seen in Figure 1.4. At 34 weeks the skin layer appears as a white structure which overlies a darker thinner line of connective tissue, arrowed in red in Figure 1.4. Beneath this layer is another white layer which represents subcutaneous fat stores. Although later gestation allows better layer differentiation, for the purpose of this study the fat / skin layer was considered together for measurement as they were at the 19 week scan.

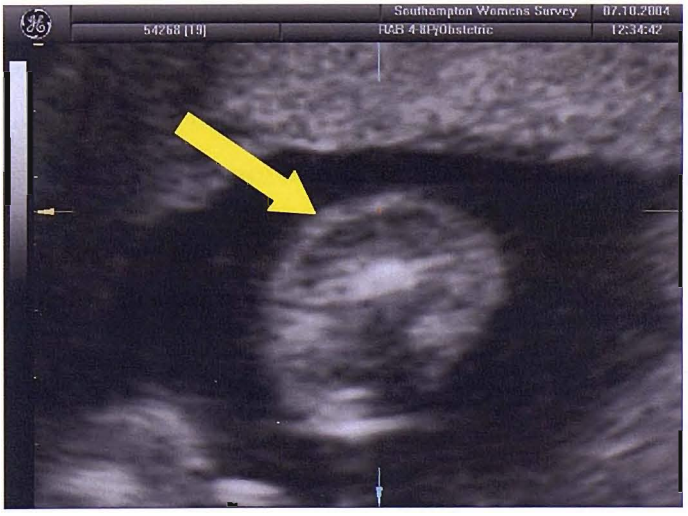


Figure 1.3: Fetal mid-thigh CSA at 19 weeks. There is no distinction between fat and skin layers at this gestation (arrowed) which show as a whiter layer.

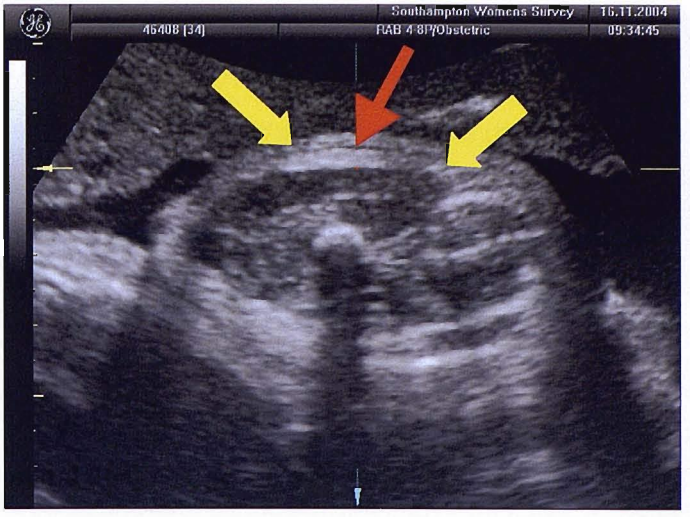


Figure 1.4: Fetal mid-thigh CSA at 34 weeks gestation. The skin layer (arrowed left) is now separated by a darker layer of connective tissue (red arrow) from the fat layer (arrowed right).

1.6 Neonatal dual-energy x-ray absorptiometry (DXA)

Studies of neonatal bone have used DXA to calculate the quantity of bone mineral content and bone density accrued by the infant during pregnancy. Bone mass and especially bone mineral density are highly associated with birthweight and birthweight is regulated partly by inherited genetics, but mainly by environmental factors. Some influences, independent of maternal factors such as gestational age, maternal smoking and nutrition are largely mediated by skeletal size and particularly adult height, (Antoniades *et al.,* 2003). The study by Antoniades suggests that maternal factors are not responsible, implying that fetal nutrition and placental factors play a key role in the development of the skeleton. Lower bone mineral density has also been shown to be associated with lean body mass in adults. In addition muscle strength is associated with bone mineral density, which is known to be under stringent genetic control and twin studies have been performed to assess the degree of genetic control that may be exerted, (Arden and Spector, 1997). Bone mineral density, total bone area and bone mineral content can all be derived using DXA and it was decided in this to utilise this modality to test relationships between bone geometry, growth and bone mineral accrual. Bone mineral density is not a measure of mass and does not equate with bone strength, but it is a useful indicator of bone mineral accrual. Bone mineral content and total bone area are useful variables to quantify bone development and size, as seen in other studies, (Heaney, 2005). There are now dedicated DXA machines capable of imaging the neonate with low radiation exposure. The equipment is able to assess bone mineral content, bone mineral density and total bone area and in addition can give calculations of lean and fat body mass, displayed on screen, (Figure 1.5).

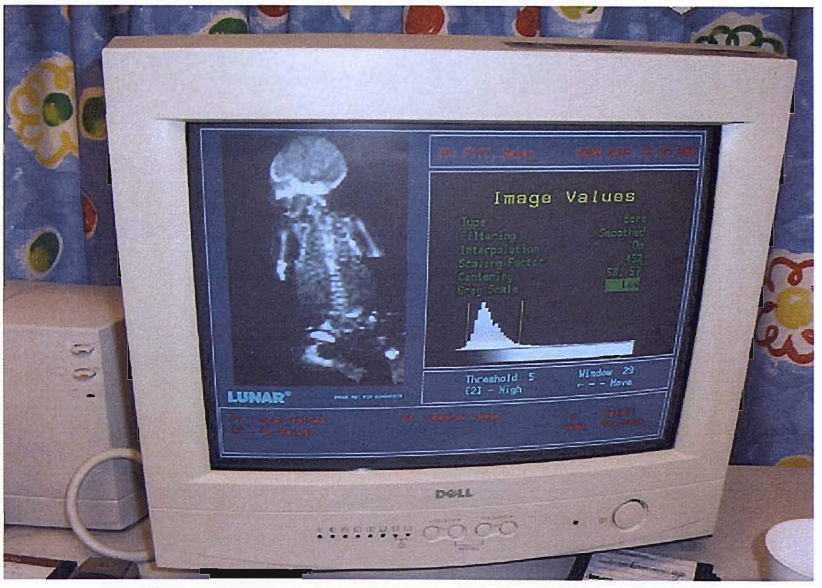


Figure 1.5: Typical image acquired during a neonatal DXA scan. Excessive leg movement can be seen as truncated bone in the lower third of the image.

1.7 Current 3DUS and biometric research

Experimental 3DUS appeared over 10 years ago, but it had to wait for computational advances to make it feasible in the daily working environment, allowing complicated imaging and calculation. Three-dimensional ultrasound was first used to quantify fetal organ volumes, (Kuo *et al.*, 1992), and later fetal abnormalities, (Lees *et al.*, 1994; Merz *et al.*, 1995); the fluid-filled environment of the uterus lending itself to this new innovation. Later, intricate studies involving the moving fetal heart, (Chang *et al.*, 1997) and additional studies of fetal anatomy and disease, (Merz *et al.*, 1995) pushed forward computational and data acquisition technology. With the backing of Professor Merz in Austria, who assisted in the development of 3D for fetal imaging, 3DUS gained popularity, albeit in the research setting. Once the value of this modality had been realised, evaluation of its usefulness in the obstetric sphere became necessary. In 1998 a study of normal and abnormal fetuses in each trimester, was performed to highlight any advantages of 3DUS over and above 2DUS, (Hata *et al.*, 1998). It was claimed that clearer visualisation of abnormalities was possible with 3D, but in reality, all diagnoses were made using 2D, although in specific circumstances 3D confirmed and further demonstrated the diagnosed anomaly. The systems in the early 1990s, produced images, which by today's standards are crude and hard to interpret, (Figure 1.6). As processing became faster and resolution better, not only could surface-rendered images be produced (Figure 1.7), but measurements could be taken from orthogonal planes and volumes calculated and navigated through to obtain planes not visualised before.

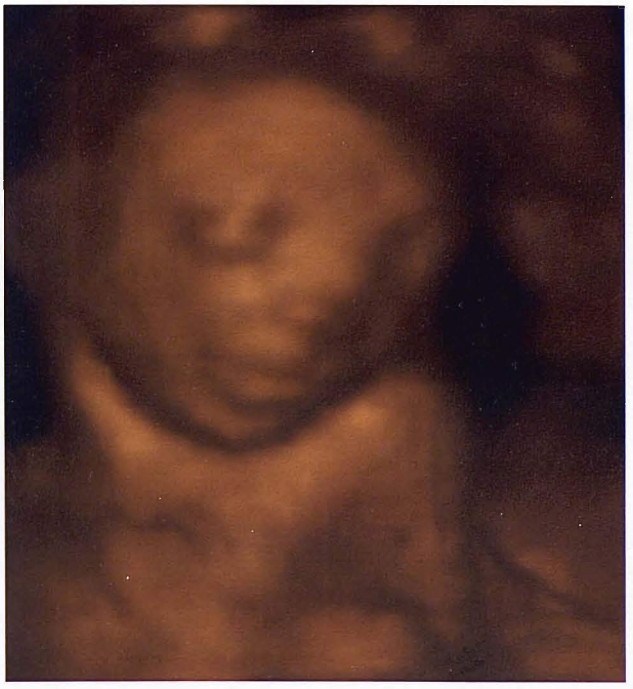


Figure 1.6: An early generation surface-rendered 3DUS image of a 25-week fetus. (Reproduced by courtesy of Dr.Karim Kalache).

The development of measurement packages revolutionised 3DUS as a research tool and many groups began constructing centile charts for specific organs and populations, to aid in the diagnosis of anomalies and disease. In Taipei, Taiwan, F. M. Chang and colleagues began exploring 3DUS for volume assessment of the fetal liver, (Chang *et al.,* 1997) and heart volume (1997a). His colleagues, lead by C. H. Chang, then began producing centile charts for fetal organ volumes; cerebellar volume (2002, 2002a), adrenal glands (2002b), brain volume (2003) and liver volume (2003a). Other researchers also recognised the potential of 3DUS in obstetrics and published findings on brain development, (Pooh *et al.,* 2000; Cohen *et al.*, 2001; Endres and Cohen, 2001), liver growth, renal volume, (Yu *et al.,* 2000) and fetal gender, (Michailidis *et al.,* 2003). By the beginning of this century there had been an explosion in technological development of 3DUS systems and researchers from around the world began to apply the technique to other areas of imaging and interventional studies. Multi-plane ultrasound guidance for biopsies, cyst drainages and surgical procedures followed, but the field was still dominated by obstetric and particularly fetal medicine practitioners.

Three-dimensional ultrasound assisted in the evaluation of small for gestational age fetuses and fetal extremities were examined for volume quantification. Volume studies of the thigh, (Chang *et al.*, 1997b; Song *et al.,* 2000) and upper arm, (Liang *et al.*, 1997; Chang *et al.,* 2000) were used for predicting the infant's weight at birth and more recently for detecting signs of intra-uterine growth

retardation (IUGR), (Chang *et al.*, 2005). Recently, the use of liver volume to predict fetal growth restriction has also been published by the same team, (Chang *et al.*, 2006).

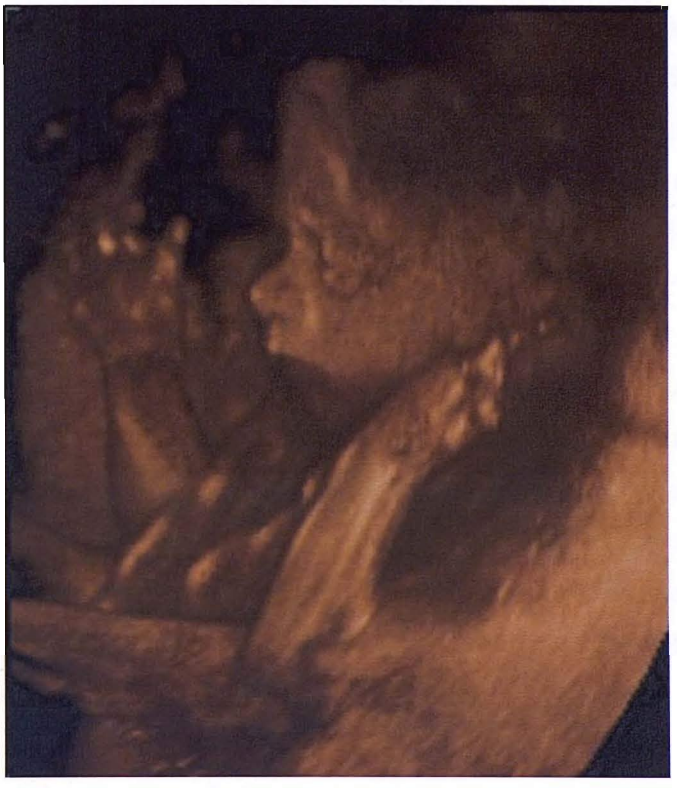


Figure 1.7: Surface-rendered image of a 25-week SWS fetus acquired by the KretzGE Voluson® 730, taken in 2004.

By the early 2000s the images produced by the new wave of systems far out-shone their predecessors as seen in Figure 1.7. Most of the publications referring to 3DUS explore soft-tissue anomalies where the *surface-rendered* image of the fetus comes into its own. In some studies 3DUS has been hailed as an important tool for aiding the fetal–maternal bonding process, because of the ease with which the untrained eye can distinguish the fetal face, (Ji *et al.*, 2005). However, a recent study using 2D-, 3D- and 4DUS conversely concluded that 4DUS did not significantly change the mother's perception of her child or changed the antenatal attachment. The importance of the Sonographers' expertise and explanation of the scan as it proceeded has been realised as the most important aspect in the acceptance of 3D imaging, not the imaging *per se,* (Rustico *et al.*, 2005).

Occasionally, publications address skeletal questions, predominantly for spine anomalies (Riccabona *et al.*, 1996; Ulm *et al.*, 1999; Yanagihara and Hata, 2000; Benoit, 2003), others for characterising skeletal dysplasias, (Garjian *et al.*, 2000). The infrequency of these skeletal studies indicates the difficulty of imaging fetal bone, but some attempts such as Schild's application of

3DUS to lumbar spine volumes, (Schild *et al.*, 1999 and 2000) for anomaly detection, have tried to break new ground. Fetal tooth buds, (Ulm *et al.,* 1999a) and palate deformities, (Campbell *et al.*, 2005) have also been assessed using 3DUS. Planar images are usually more useful than rendered images for interpretation of normality, so the surface-rendered images are more often used for skin-covering anomalies rather than deep structures. Recently, multi-slice view (MSV) imaging, or 3D extended imaging, has been developed to aid fetal diagnosis, although this modality is in its infancy, (Leung *et al.,* 2005). Adult bone and its imaging difficulties has been recently studied, but this utilised bone samples and in no way compares with the moving, fluid environment of the pregnant uterus, (Lasaygues *et al.,* 2005).

Of greatest interest in recent years has been the publication of a paper by the prolific team led by C. H. Chang, who used fetal humeral volume to assess gestational age and predict birthweight, (Chang *et al.*, 2003b). The technique involves tracing around the shaft of the bone at intervals and allowing the software within the machine to create a volume. What was not made clear in this study, and indeed in any other 3DUS study of bone, were any difficulties encountered and, to date, no validation of the technique has been formally addressed. Thus the need for 3DUS bone imaging evaluation, as proposed in this thesis.

1.8 Choice of 3D US systems available

Three-dimensional ultrasound is useful for prenatal recognition of malformations and anomalies within the fetus and placenta, as it produces a volume of data, which can be displayed in a variety of ways. An organ or bone can be 'repositioned' for easier evaluation and the three orthogonal planes generated can be displayed simultaneously. How this is achieved depends on the type of system being used. Some systems employ a *free-hand* technique where the operator glides the transducer across the skin in a linear fashion, to acquire the image, such as the TomTec system, the Stradx system developed in Cambridge University, (Prager *et al.,* 1998) or the Acuson 'Freestyle', from Siemens. Recently, steps have been made to lessen the calculation time needed for free-hand systems by a team from Taiwan, who have developed a system for creating volume datasets of adult muscle and skin layers, but it may be a long time before this technology will be ready for the unpredictable fetus, (Huang *et al.*, 2005).

Recently, a new 3D modality has been developed called *fractional thigh volume technique*, which takes a sub-volume that includes 50% of the femoral diaphyseal length. The volume is centred on the mid-femoral shaft and is split into 5 sections by the software. These sections are manually traced around by hand from the axial views and thigh volume is calculated from the sum of these slices, (Lee *et al.,* 2004). This is still in its early stages and it is unknown whether it is more accurate than the techniques used in this study. Large areas of the body can be investigated with

this free-hand method, but it is time and operator dependent. Other systems operate a mechanical scan head, where moving parts are built into the transducer, which is held in the correct place by the operator. A motor sweeps the transducer through an arc, acquiring volume data as it passes. This has the advantage that the acquisition time is reduced, operator dependency is minimised and there is less time for the fetus to move during acquisition. Movement artefact is the most usual loss of scan information in 3D imaging. Systems using mechanical sweep technology are now produced by Siemens, Phillips, Sony, Toshiba, ATL, GE and Medison. The system chosen for this study was a KretzGE Voluson® 730 which utilises a mechanical sweep. This renders it more suitable for reproducibility testing as it can recreate the same sweep technique each time a volume is acquired and is not influenced by variation in operator method.

1.9 Ultrasound safety issues

Diagnostic ultrasound has an enviable safety record in comparison with radiographic examinations, which have known genetic and somatic risks associated with ionising radiation, or thermal damage from magnetic resonance imaging, (Duck, 2002). This makes ultrasound the method of choice as high frequency sound waves within the diagnostic range do not cause deleterious effects in the fetus, (Kieler *et al.*, 1998; Barnett *et al.*, 1997 and 2000; Abramowicz *et al.*, 2000; Duck, 2002). The British Medical Ultrasound Safety Group published guidelines in 2000, observing that thermal hazards exist with some diagnostic equipment, if used imprudently. However, if temperature changes within the area being examined are kept below 1.5°C, even indefinitely, no damage to mammalian tissue, including human embryos or fetuses, was observed, (BMUS, 2000).

It should always be borne in mind that exposing the fetus to ultrasound with no anticipation of medical benefit is not justified and must be used prudently. In 2DUS the subject is scanned until the operator is convinced that all structures and pathology have been seen or recorded on film, which may take many minutes. The brief acquisition time needed for a 3DUS volume data-set and the fraction of a second dwell-time for each line of data to be pulsed and return to source, illustrates that 3DUS does not increase the cumulative power output of the equipment, but reduces it. The safety of 3DUS has yet to be formally studied, but due to the speed of volume acquisition and the flexibility of image processing, it is expected that it poses no greater hazard than 2DUS to mother, fetus or operator, (Duck *et al.*, 2002; Hershkovitz *et al.*, 2002). Current ultrasound safety guidelines are included in Appendix III for reference.

1.10 Test-tank manufacture and experimentation

The ability of an ultrasound system to measure small on-screen features with precision is particularly important in fetal imaging, (Lessoway *et al.,* 1990; Dudley and Chapman, 2002) and may only be possible if the system is regularly maintained and calibrated, (Berg *et al.*, 2002). Test devices known as *phantoms* are used to calibrate new ultrasound systems. They can be bought commercially for periodical quality assurance tests and are scanned using the hand-held transducer. The resultant images are then measured for comparison with the test object, which is constructed from materials of known size and density, (Goldstein, 2000). Phantoms are usually constructed to mimic anatomical features and are very expensive to produce, due to the materials from which they are constructed and the low volume of manufacture. They commonly consist of metal pins and rods or urethane shapes embedded in a firm gel that has low scattering properties to avoid noise in the resultant image, (Madsen *et al.*, 1998). The gel is matched to the acoustic properties of mammalian tissue and can be made to a variety of recipes, (Duck, 1990; Fish, 1990; Madsen *et al.,* 1998; Goldstein, 2000; Wu, 2001 and Dudley *et al.,* 2002).

With the invention of 3DUS, new phantoms were created to help students and practitioners navigate their way around previously unseen planes. For obstetrics applications, test phantoms similar to those seen in Figure 1.8, are designed to assist in anatomical recognition of the fetus and orientate the scan planes during clinical training.

For the purpose of this study, a commercially available phantom would not be adequate, as they do not contain materials that accurately mimic bone and they are prohibitively expensive. To assist in identifying difficulties with bone imaging, for this study, a basic test-tank was constructed, into which organic and non-organic materials were placed, submerged in water. The manufacture of this test-tank is described in Chapter 3 – *In vitro* Evaluation of Techniques for Fetal Musculo-Skeletal Measurements.

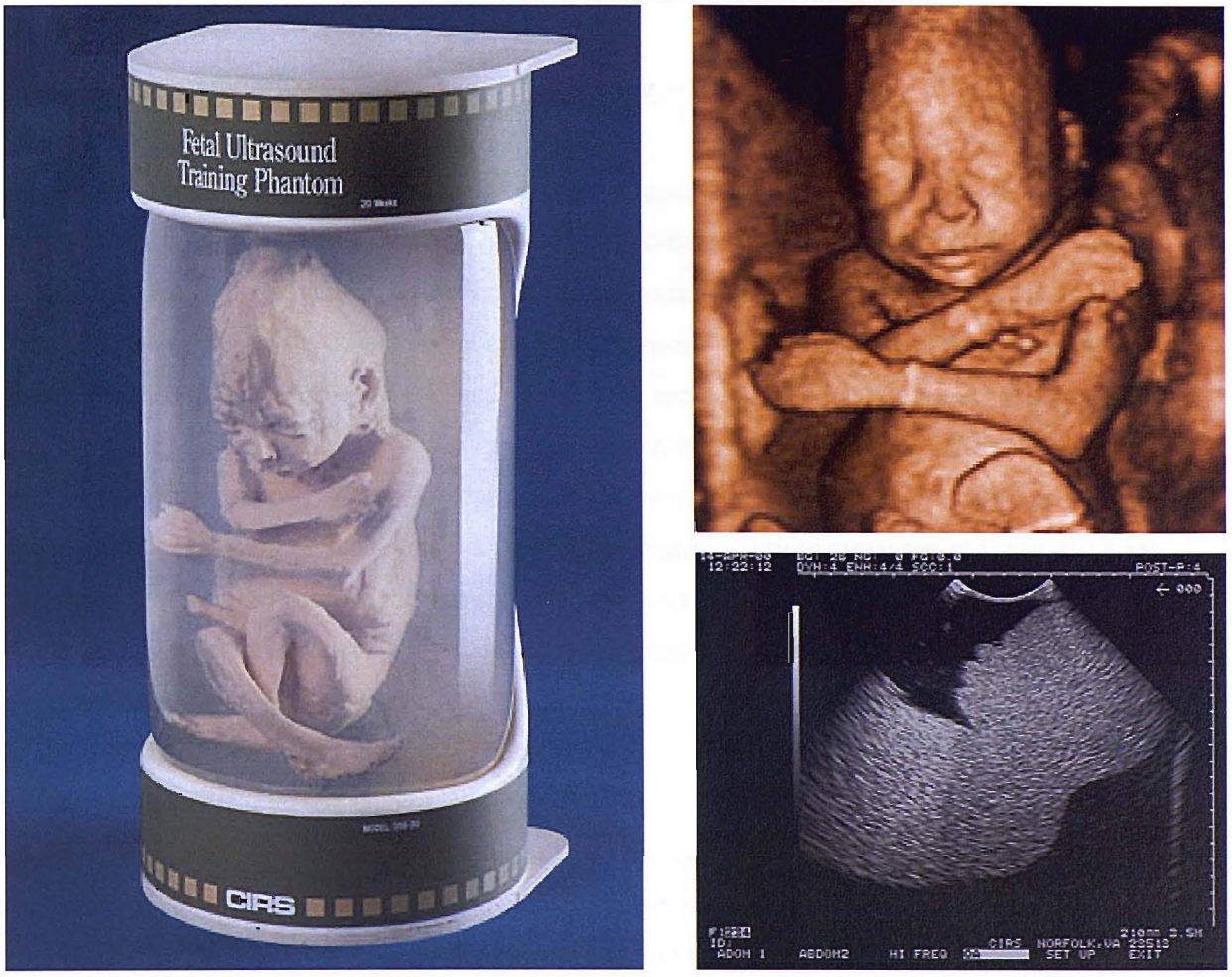


Figure 1.8: The CIRS 3D Fetal Ultrasound Training Phantom (left panel). Bottom right panel shows the resultant 2D image obtained by scanning through the plastic outer jacket and top right panel shows the resultant 3D volume acquisition image in surface-rendered mode. Images reproduced by courtesy of CIRS Tissue Simulation Technology, Norfolk, Virginia, USA.

For ease of understanding of the difficulties encountered with imaging the fetus and in particular, the fetal skeleton, the next chapter deals with the understanding of how an ultrasound image is produced, improved and interpreted, and how initial trials assisted the evolution of reproducible techniques for the purpose of this study.

Chapter 2 – Theoretical Aspects of Methods Development

Introduction

Long bones, particularly the femora, change and grow rapidly within the developing fetal skeleton. In this study the femur has been used, as it is easily identified, shows linear growth and can indicate changes in skeletal development, metabolic and chromosomal disorders, (Gupta *et al.*, 2001; Javaid *et al.*, 2002; Parilla *et al.*, 2003). Conventional measurements of the femur are reproducible, but innovative cross-sectional areas (CSA) and volumes are more challenging. However, these parameters may be more sensitive at showing variation in different diseases or conditions than linear or traced dimensions. For example, a femur altered by rickets could theoretically have an increased CSA at the diaphyseal ends or a large bone volume. A linear measurement such as femur length would be of little value in this case. Attention must be paid to thigh tissues, where CSA and volumes could be used to reflect muscle and fat development. To understand the difficulties that may occur in performing these measurements knowledge of ultrasound production is essential. Until this study, the linear measurement of femur length has been the only measurement routinely used in obstetric scanning. By using the orthogonal planes displayed by the 3D system, other measurements have been derived and it is these new measurements that will be tested for reproducibility and usefulness in the obstetric research setting. Each of these measurements has its own difficulty in execution. Linear measurements are easier and more reproducible than trace measurements that depend on the skill of the operator to manually trace around the object on-screen. Volume measurements depend on trace measurements being taken in systematic order so their values can be added together and calculation of volumes between each slice can be created. This adds inaccuracy into the method and so by assessing each type in turn, usefulness of each measurement can be evaluated.

Core measurements and acronyms explained

The author (PM) is an accredited radiographer, specialising in obstetric ultrasound imaging. Part of her role is to scan the fetus during pregnancy, take images of normal and abnormal fetal anomalies and make measurements on the static images recorded of fetal structures. This usually entails making linear and trace measurements on-screen during the examination. Although 3DUS is a specialist technique, with practice the acquisition of three-dimensional volumes and measurement on those volumes was possible and new measurements were then devised, which supplied useful data for the purpose of this research. In published studies the most utilised fetal bone measurement has been femur length (FL) and for the purpose of this study, it was necessary to devise other

linear, trace and volume measurements representative of each of the fetal thigh tissues, in addition to bone length. This comprised 15 core measurements as shown below:

The proposed measurements for bone were:

Femur length	(FL)
Femur mid-shaft transverse section diameter	(FMSTS)
Femur mid-shaft antero-posterior section diameter	(FMSAP)
Femur proximal cross-sectional area	(Proximal CSA)
Femur mid-shaft cross-sectional area	(Mid-shaft CSA)
Femur distal cross-sectional area	(Distal CSA)
Femur proximal antero-posterior diameter	(FPAP)
Femur distal antero-posterior diameter	(FDAP)

For soft tissue measurements:

Mid-shaft skin-thickness	(MSST)
Mid-thigh cross-sectional area	(MTCSA)

For muscle measurements:

Mid-thigh muscle cross-sectional area	(MTMCSA)
(derived by subtracting mid-shaft CSA from MTCSA)	

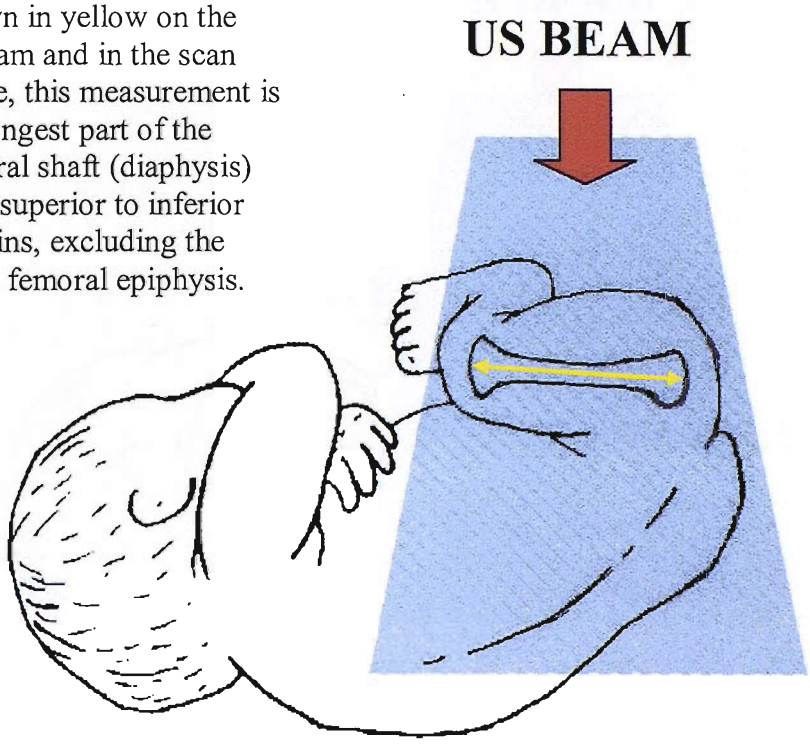
For volume measurements of all tissues:

Thigh volume	(TV)
Fat / skin volume	(SV)
(derived by subtracting MV and FV from TV)	
Muscle volume	(MV)
(derived by subtracting FV from the volume)	
Femur volume	(FV)

A diagrammatic representation of each measurement and its appearance on scan images are seen in the next section. This is to assist the reader with understanding the biometric parameters to be measured and the acronyms by which they are known. Other technical terms are included in the 'Glossary of Technical Terms' at the end of the thesis. In order to understand the ease or complexity of each measurement, a small knowledge of ultrasound and how it creates the views on which the measurements are taken, is necessary and so the diagrams will be followed by an explanation of the Technology that provides the ultrasound data.

FL
(Femur length)

Shown in yellow on the diagram and in the scan image, this measurement is the longest part of the femoral shaft (diaphysis) from superior to inferior margins, excluding the distal femoral epiphysis.



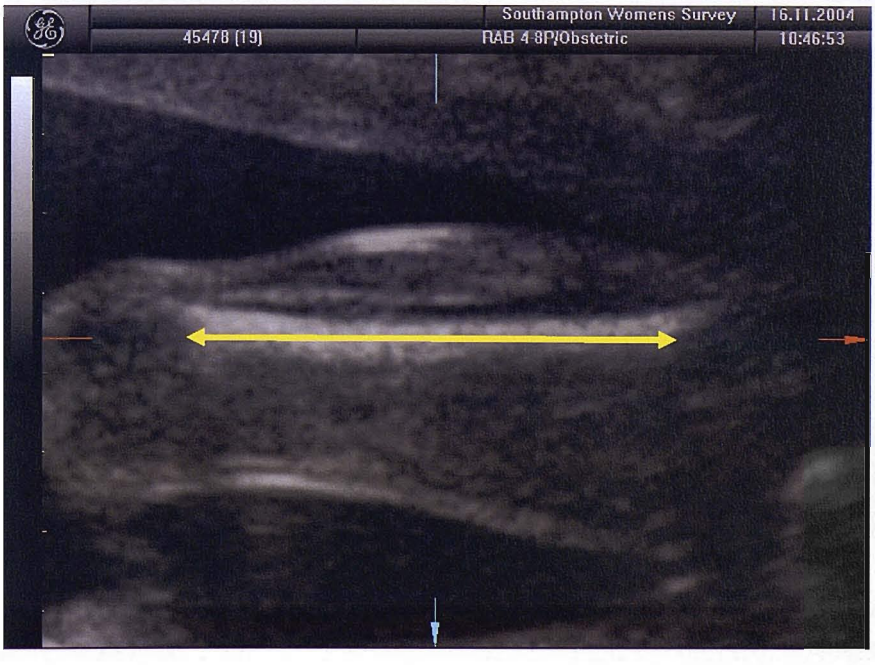
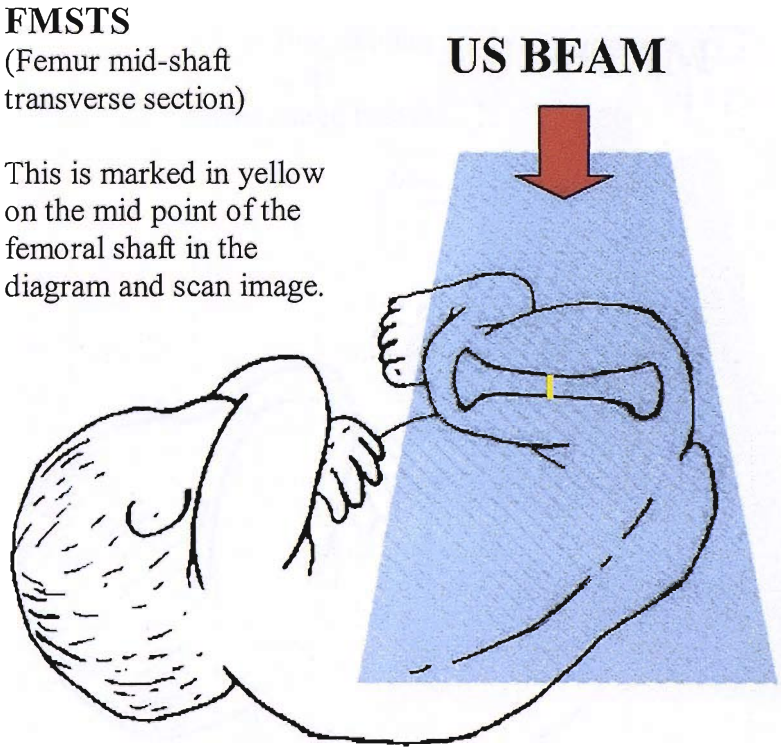


Figure 2.1: Femur length (FL) measurement; shown in yellow superimposed over a 34-week scan of the femur.



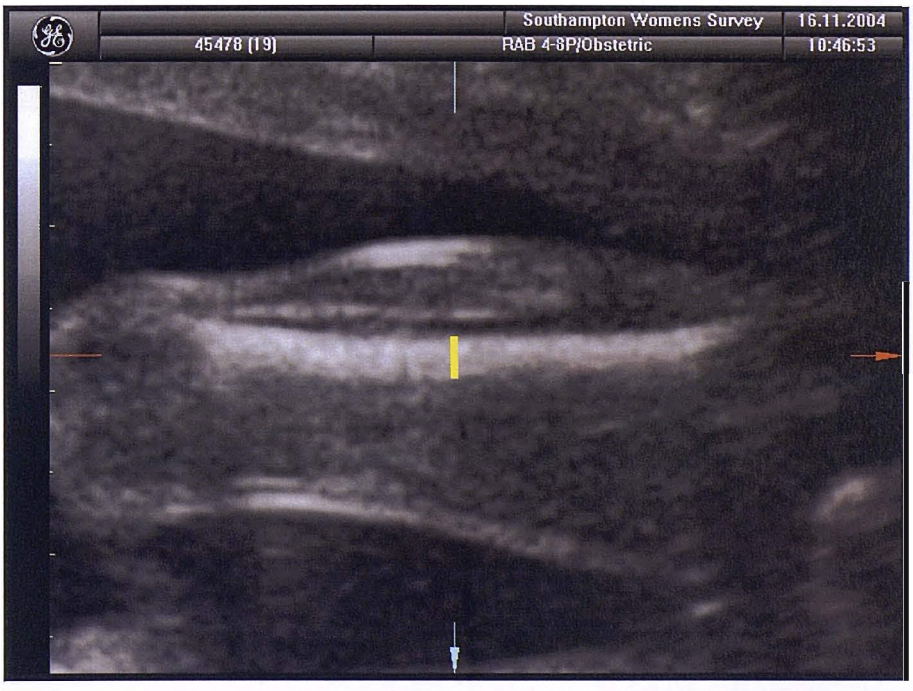
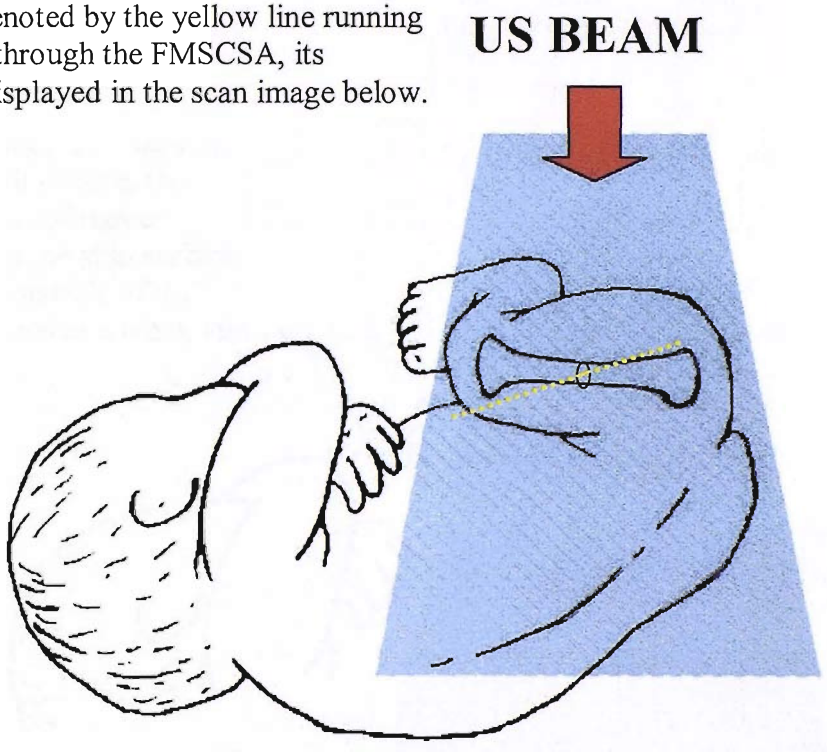


Figure 2.2: Femur mid-shaft transverse measurement (FMSTS); shown in yellow superimposed over the mid-shaft of a 19-week femur scan.

FMSAP

(Femur mid-shaft antero-posterior diameter)

This is the denoted by the yellow line running horizontally through the FMSCSA, its orientation displayed in the scan image below.



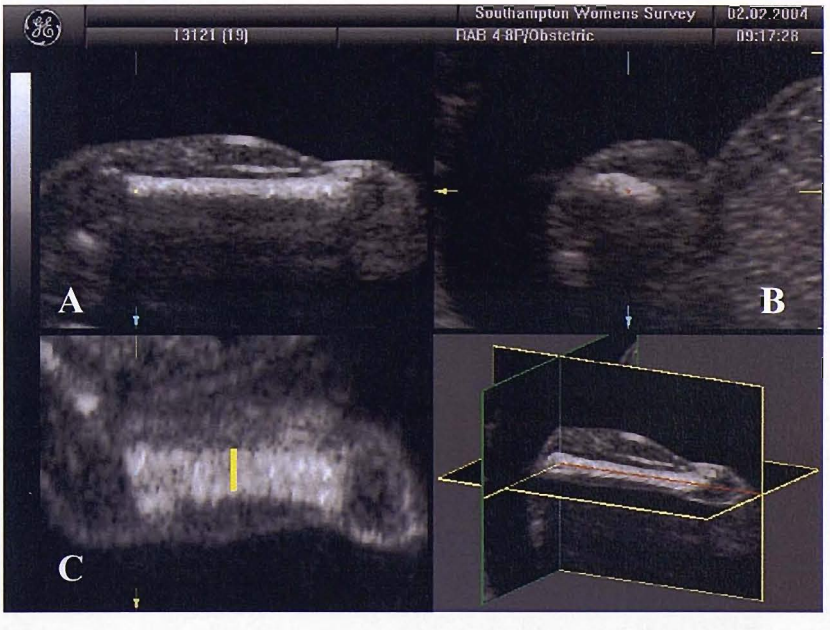
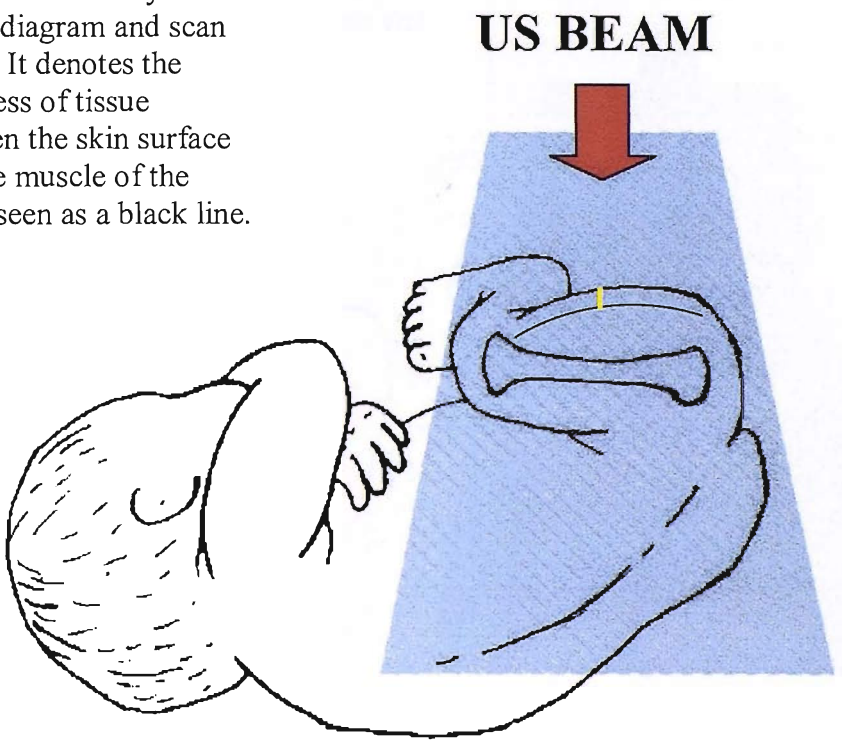


Figure 2.3: Femur mid-shaft antero-posterior measurement (FMSAP); shown in yellow superimposed on the reconstructed C plane of a 19-week femur scan. This is 90 degrees (orthogonal) to the FMSTS shown on the previous Figure 2.2.

MSST
(Mid-shaft skin thickness)

This is marked in yellow on the diagram and scan image. It denotes the thickness of tissue between the skin surface and the muscle of the thigh, seen as a black line.



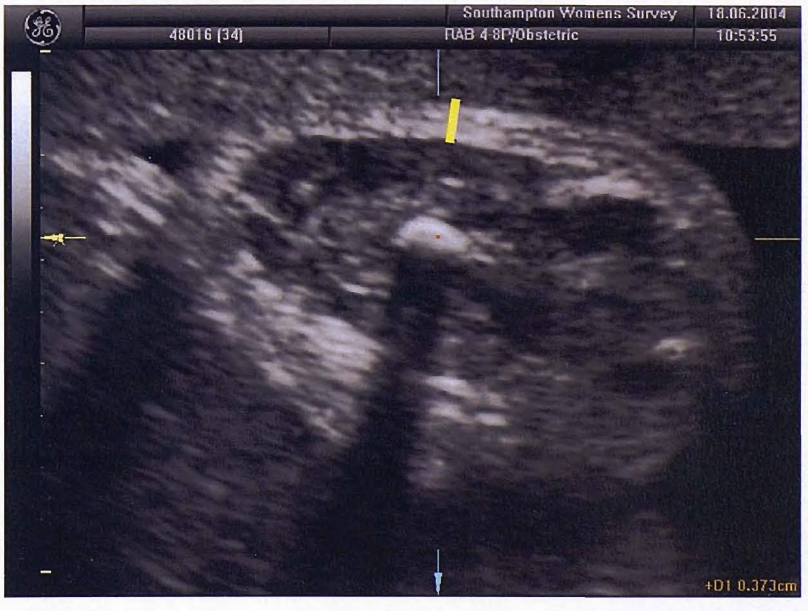
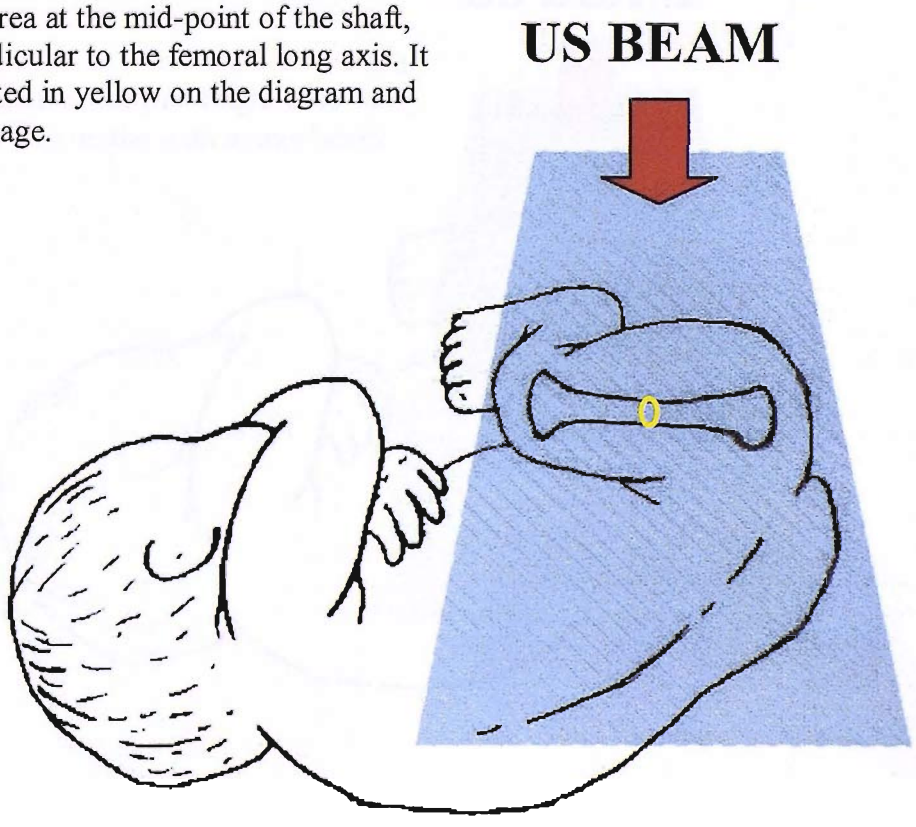


Figure 2.4: Mid-shaft skin thickness measurement (MSST); shown in yellow superimposed over the fat / skin layer of the thigh at the mid-shaft of the femur, perpendicular to the axes of the layers. It includes both fat and skin layers, but not muscle.

Mid-shaft CSA

Femur mid-shaft cross-sectional area

Trace area at the mid-point of the shaft, perpendicular to the femoral long axis. It is denoted in yellow on the diagram and scan image.



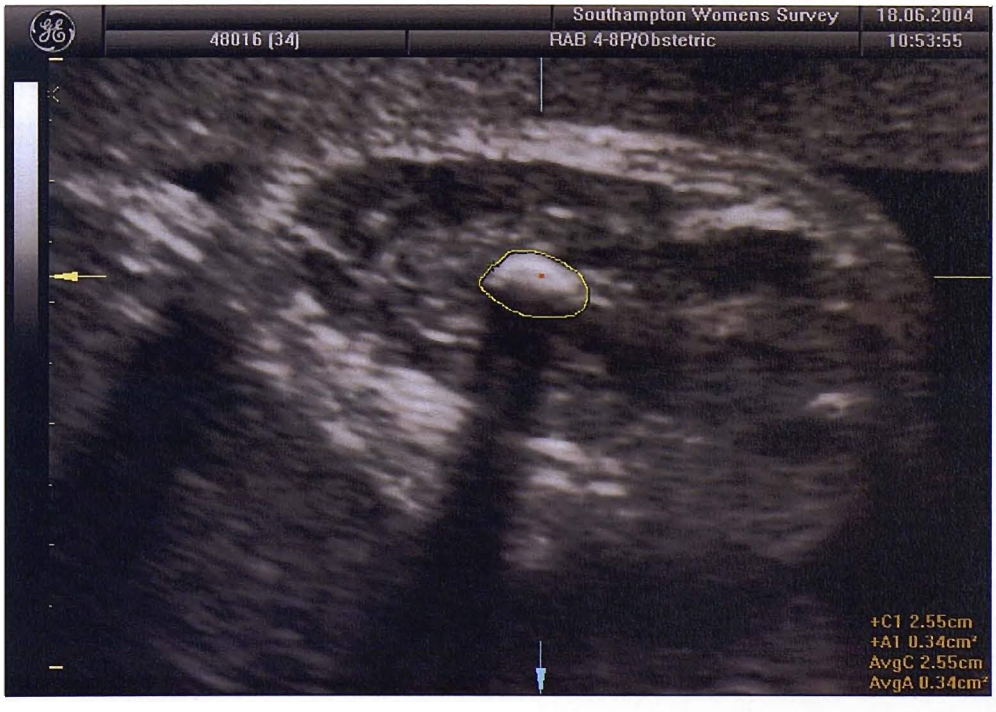
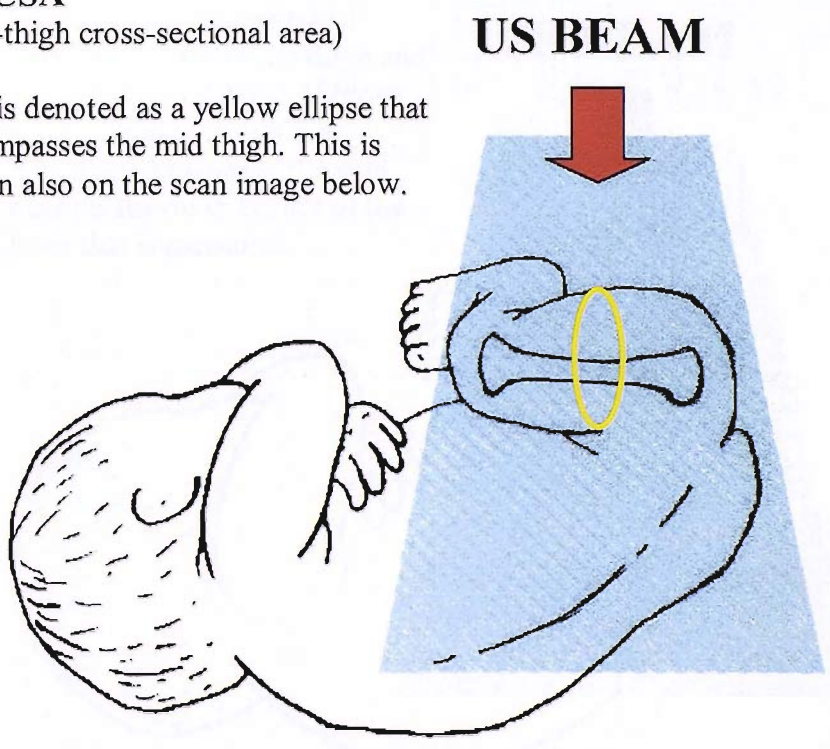


Figure 2.5: Mid-shaft CSA measurement (FMSCSA); shown in yellow superimposed over the mid-shaft of a 34-week femur, shown in cross-section on the scan image.

MTCSA
(Mid-thigh cross-sectional area)

This is denoted as a yellow ellipse that encompasses the mid thigh. This is shown also on the scan image below.



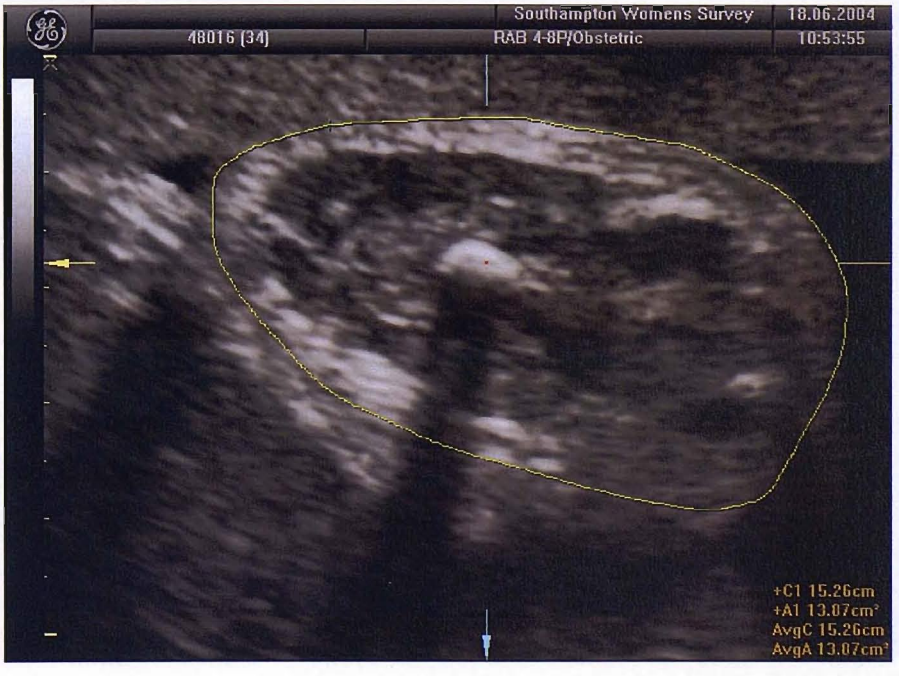
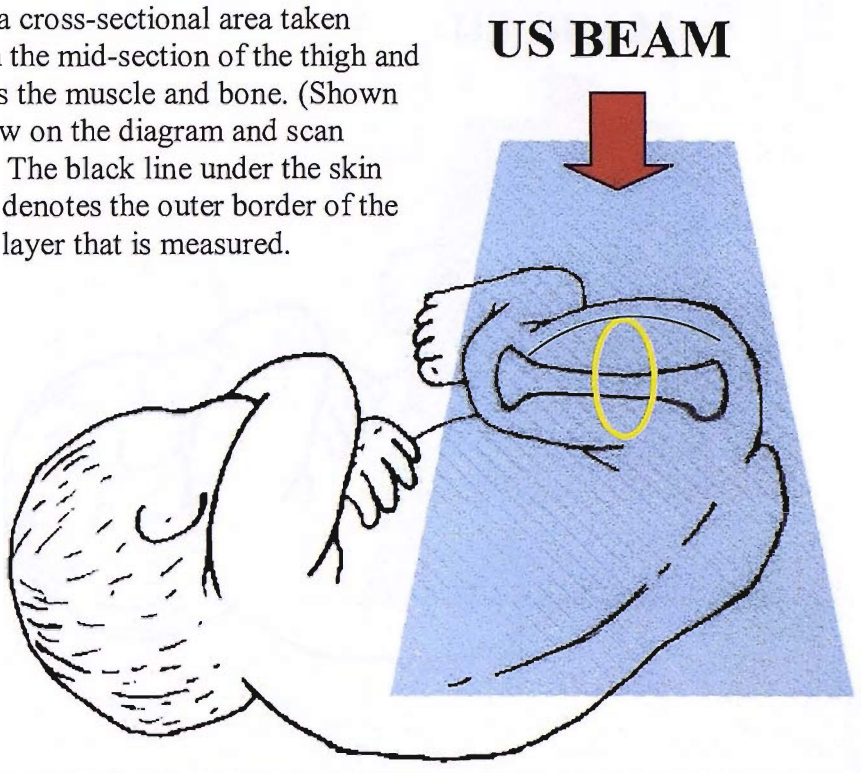


Figure 2.6: Mid-thigh cross-sectional area measurement (MTCSA); shown in yellow superimposed over the skin surface of a 34-week mid-thigh section.

MTMCSA

(Mid-thigh muscle cross-sectional area)

This is a cross-sectional area taken through the mid-section of the thigh and includes the muscle and bone. (Shown in yellow on the diagram and scan image). The black line under the skin surface denotes the outer border of the muscle layer that is measured.



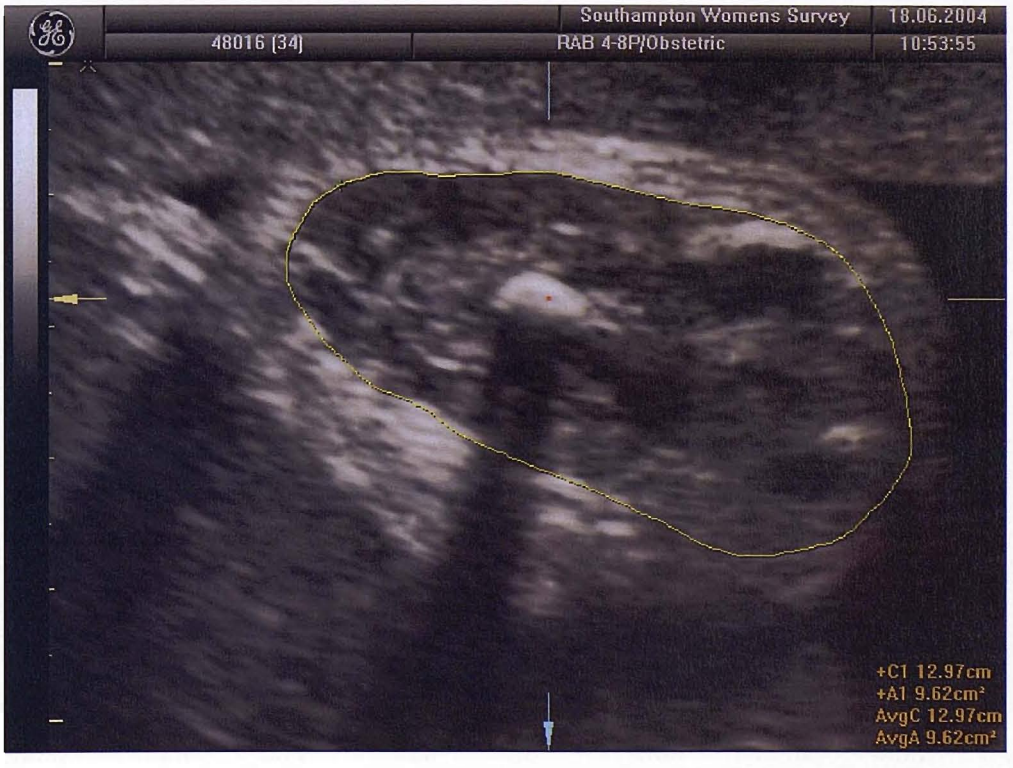
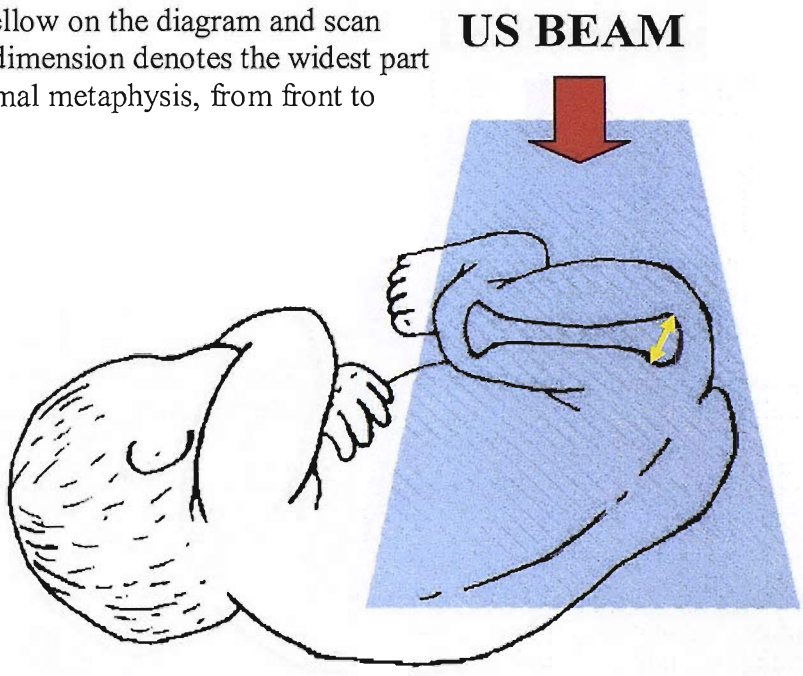


Figure 2.7: Mid-thigh muscle cross-sectional area measurement (MTMCSA); shown in yellow superimposed over the outer edge of the muscle layer at the mid-shaft point of the femur at 34 weeks, in cross-section.

FPAP

(Femur proximal antero-posterior diameter)

Shown in yellow on the diagram and scan image, this dimension denotes the widest part of the proximal metaphysis, from front to back.



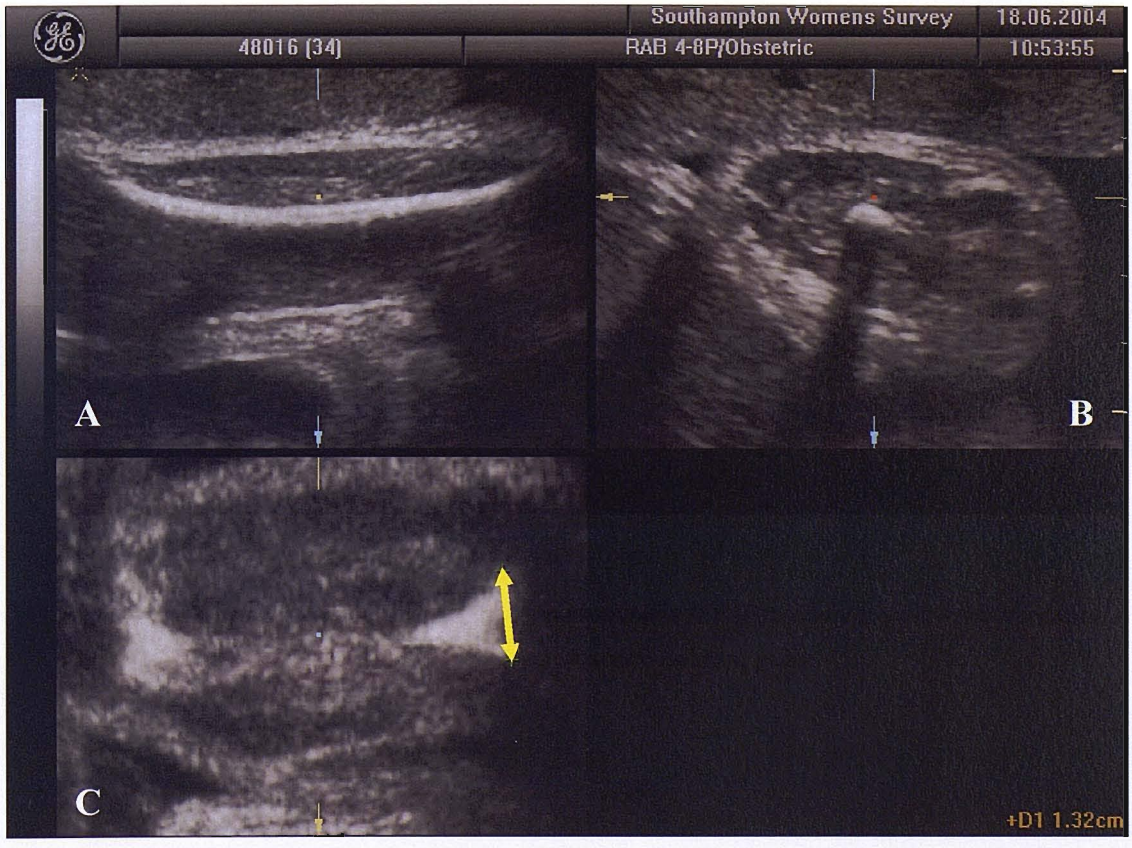
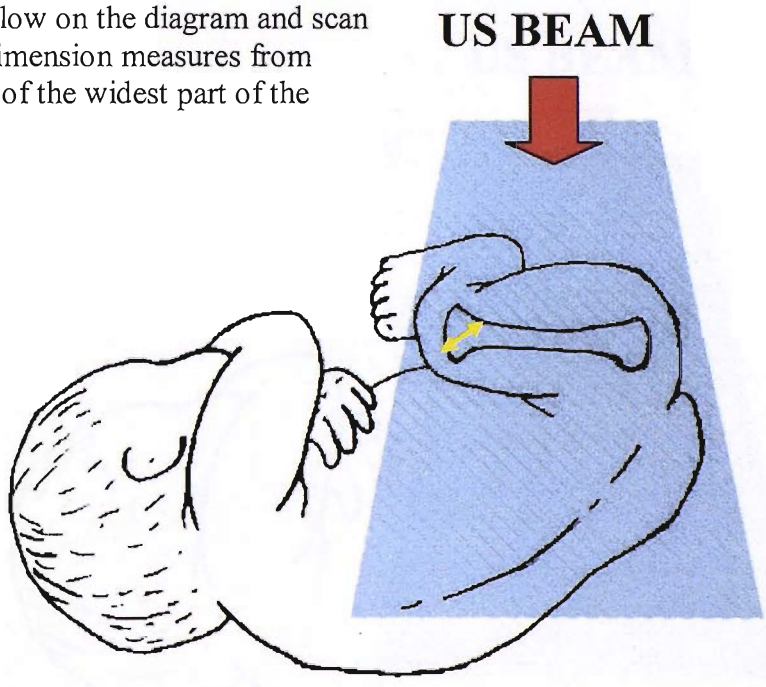


Figure 2.8: Femur proximal antero-posterior measurement (FPAP); shown in yellow superimposed over the widest part of the proximal end of the femur at 19 weeks, on the C plane.

FDAP

(Femur distal antero-posterior diameter)

Shown in yellow on the diagram and scan image, this dimension measures from front to back of the widest part of the distal shaft.



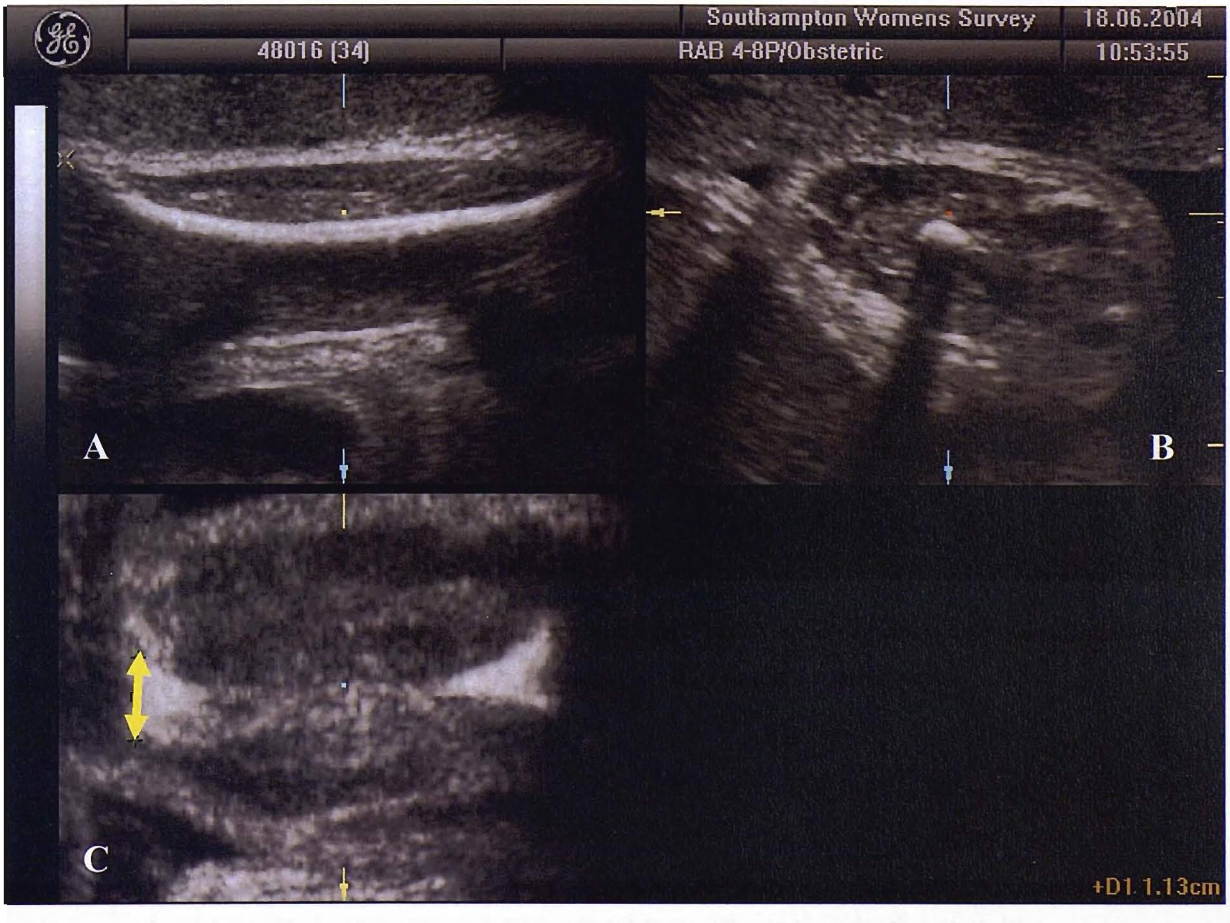
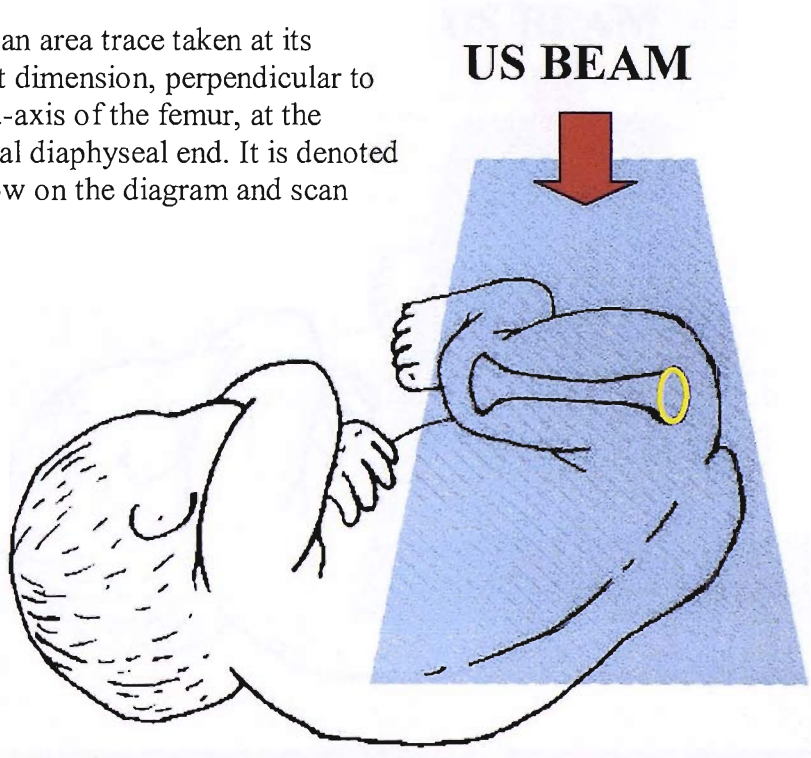


Figure 2.9: Femur distal antero-posterior measurement (FDAP); shown in yellow superimposed over the widest part of the distal end of the femoral metaphysis at 19 weeks, on the C plane.

Proximal CSA

(Femur proximal cross-sectional area)

This is an area trace taken at its greatest dimension, perpendicular to the mid-axis of the femur, at the proximal diaphyseal end. It is denoted in yellow on the diagram and scan image.



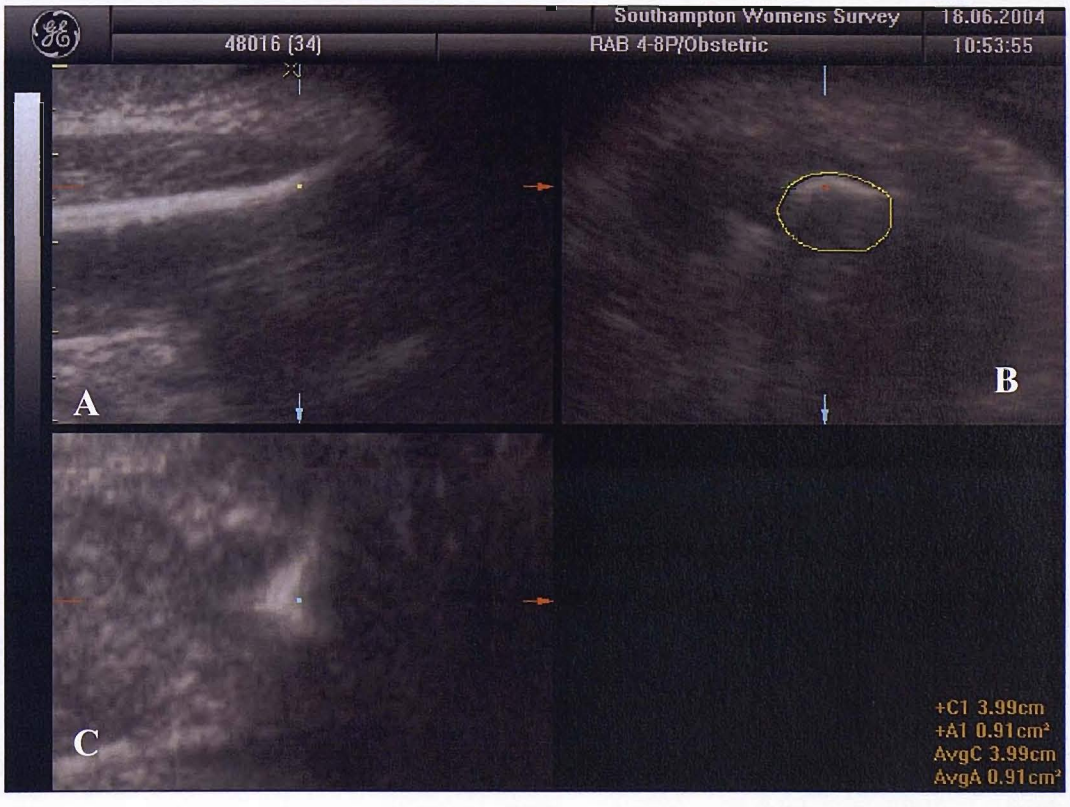
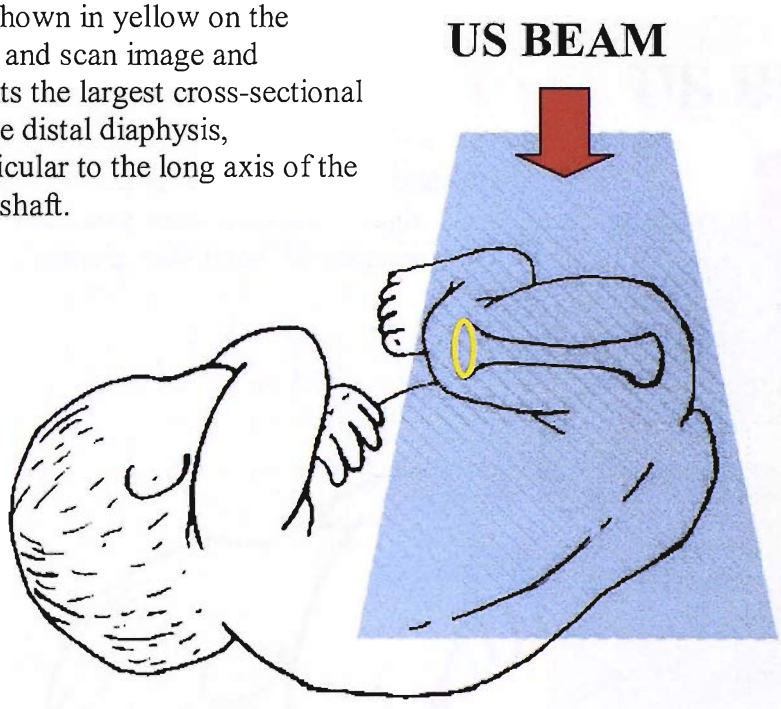


Figure 2.10: Femur proximal cross-sectional area measurement (proximal CSA); shown in yellow superimposed over the proximal end of the femoral metaphysis, perpendicular to the long axis of the shaft at 34 weeks.

Distal CSA

(Femur distal cross-sectional area)

This is shown in yellow on the diagram and scan image and represents the largest cross-sectional are of the distal diaphysis, perpendicular to the long axis of the femoral shaft.



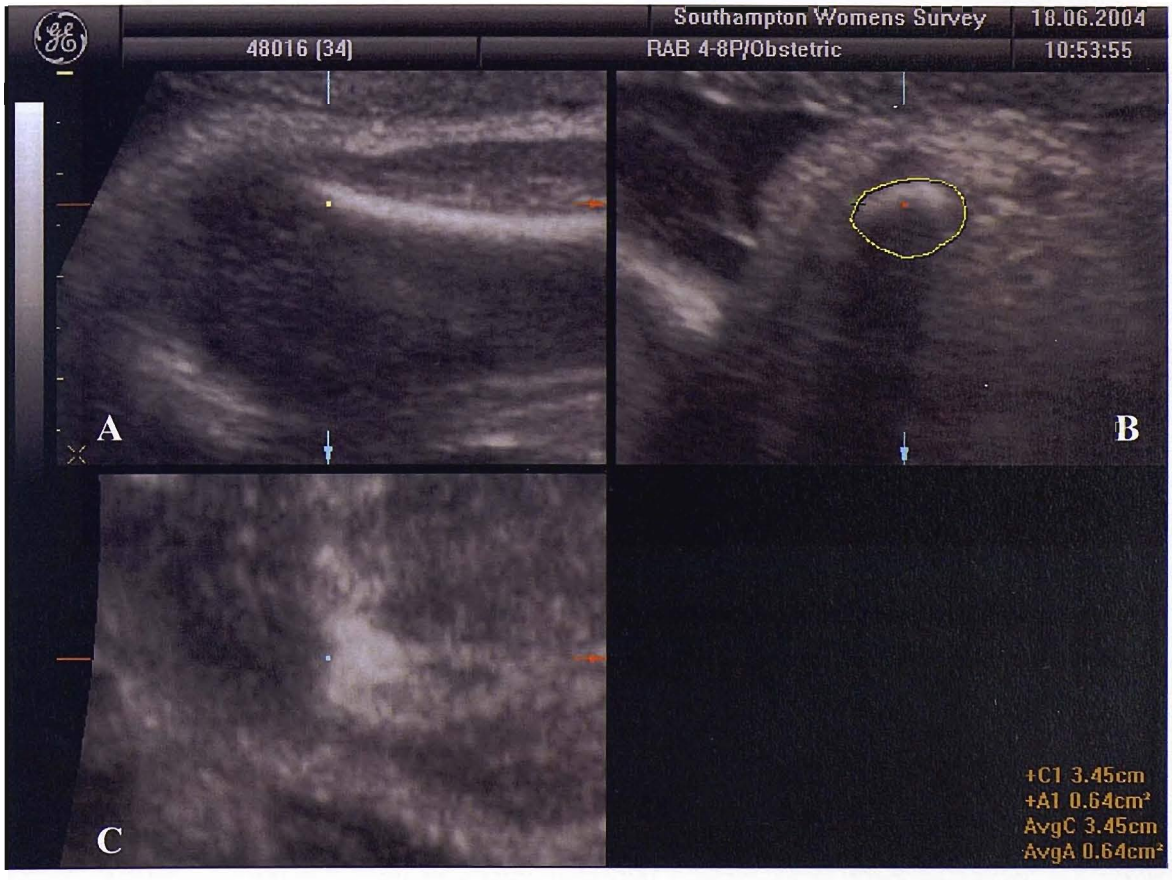


Figure 2.11: Femur distal cross-sectional area measurement (distal CSA) shown in yellow superimposed over the distal end of the femoral metaphysis, perpendicular to the long axis of the shaft at 34 weeks on the B plane.

VOLUME SLICES

For **TV** (thigh volume), **MV** (muscle volume and **FV** (femur volume).

Cross-sectional areas can be taken parallel to each other along the long axis of the limb.

The cross-sectional areas are traced around as for all other area measurements. Thigh slices are shown in yellow on the diagram.

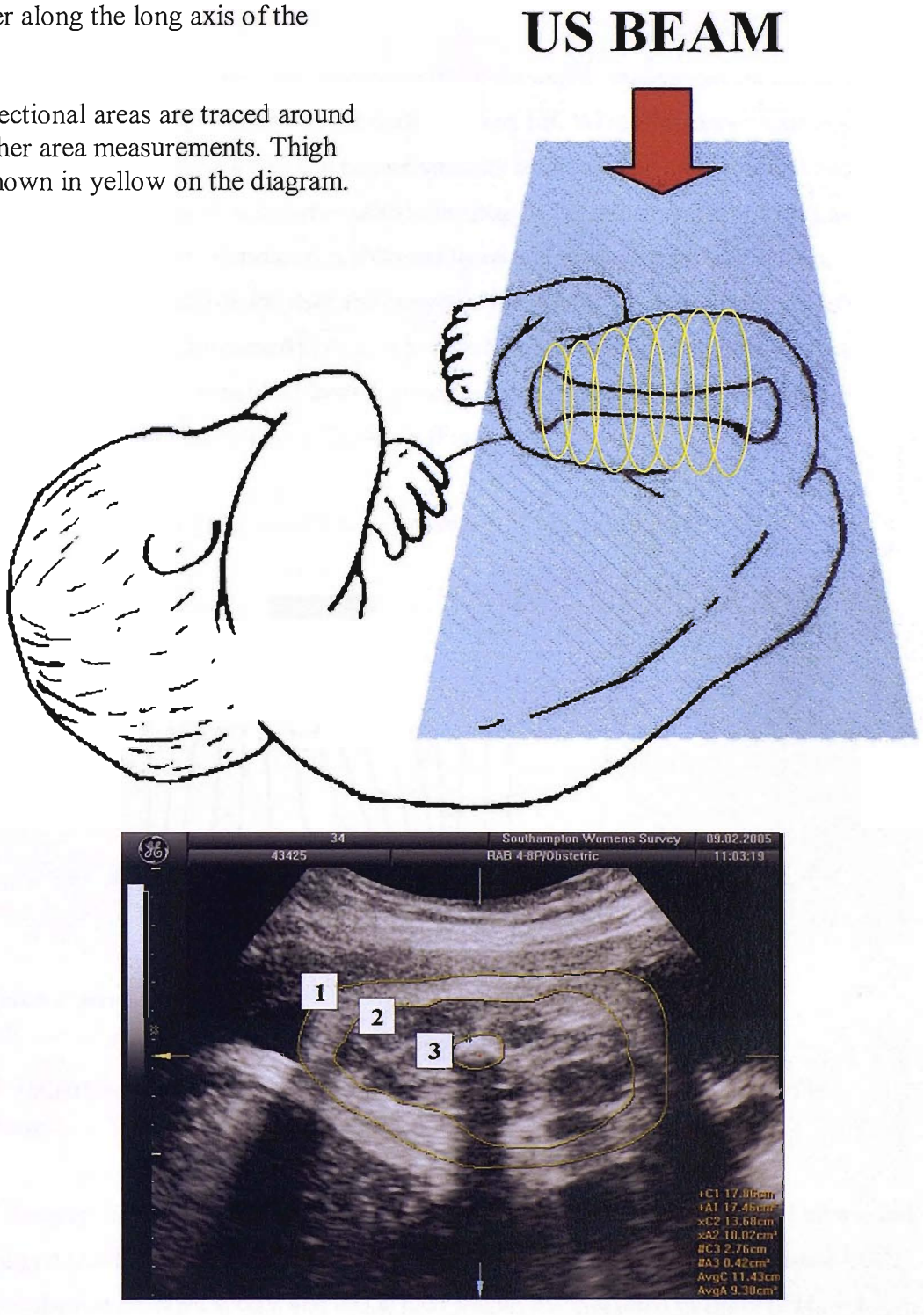


Figure 2.12: Volume slice measurements: 1 shows the skin edge around which thigh volume (TV) traces are taken, 2 shows the fat/muscle boundary where muscle volume (MV) traces are taken and 3 shows the bone edge around which femur volume (FV) traces are taken. The distances between the slices can be selected by the operator as little as 1mm apart. The system's software calculates the volume between each slice, adds the slices together and displays the total volume on screen (bottom right-hand corner).

Technology

2.1 Explaining ultrasound

Ultra, meaning *above* or *over,* when used in conjunction with sound, implies that the sound being produced is above the range of hearing for the normal human ear. Whilst we cannot hear or see the waves produced, the waves themselves can be mechanically manufactured and projected into materials. In medical ultrasound, sometimes called *sonography*, the transmission of high frequency sound into the body through a *transducer,* is followed by reception of the returning echoes, processing of those echoes into digital data and display of the data as an image. The high frequency sound wave produced by an ultrasound system, is a disturbance caused by local pressure change or movement of particles within a material from their normal resting-place. This disturbance is passed on as vibrations, away from the source of the wave, (Figure 2.13).

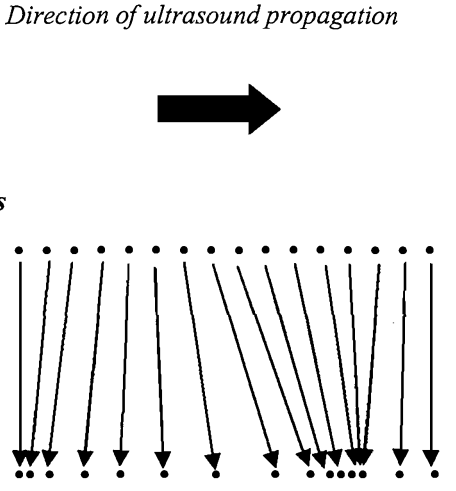


Figure 2.13: The positions of particles of a medium at rest and when disturbed by the passage of an ultrasound wave.

In medical imaging, the transducer is held onto the skin of the body and emits pulses of ultrasound, which propagate into the tissue, causing disturbance away from the source. Different tissue molecules oscillate at different speeds and this is their *frequency*, measured in Hertz (1 Hz = 1 cycle per second). For a wave of ultrasound the relationship between the tissue (f) and the speed through which the wave passes (v) varies with differing tissues (λ). This can be expressed as:

$$v = f\lambda$$

At around 20 kHz the sound is no longer audible and so the disturbance is known as *ultrasound.* When the ultrasound wave encounters a change in the material through which it passing, the boundary between these non-similar materials reflect, refract or allows transmission of the beam. The portion of the beam returning to ultrasonic transducer is sensed, amplified and converted into an electrical signal to form image data, (Figure 2.14).

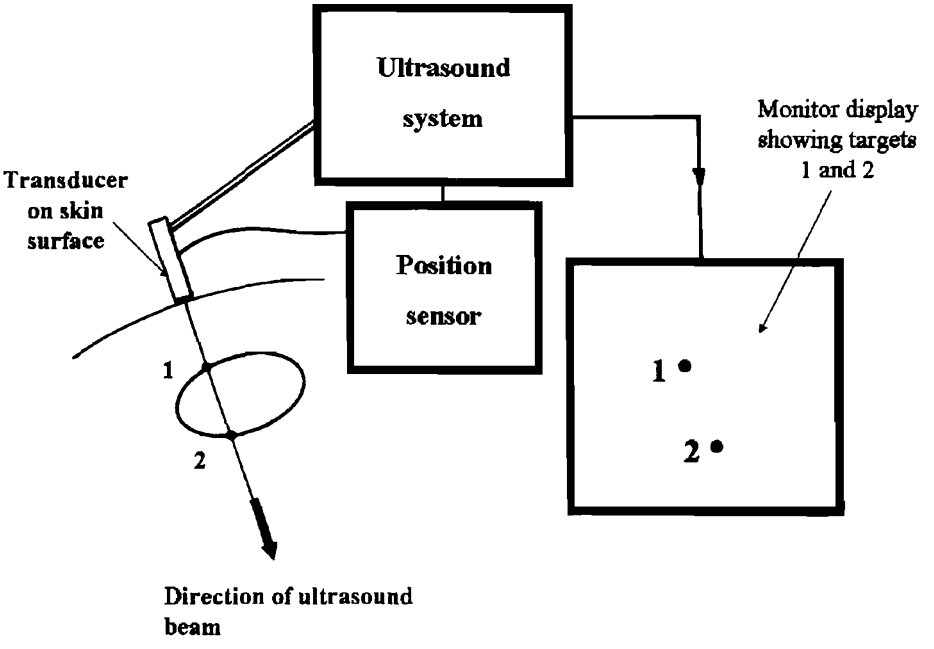


Figure 2.14: Diagram of an ultrasound system's operation. Targets 1 and 2 are scanned by the transducer, which uses the returning signal and position sensor to display the targets on the monitor in the same position as in the tissue.

If the speed of the beam is assumed (v), the time (t) it takes for the beam to propagate through the material on which it is placed, strike a boundary further away and return to source, the distance (d) from the transducer and hence the position of the boundary is located. Two of these parameters need to be known in order to calculate the third as seen in the relationships below:

$$v = \frac{d}{t} \qquad d = vt \qquad t = \frac{d}{v}$$

It is by these simple means that a 'target' or area of interest can be located by the ultrasound transducer and can be re-created for display on-screen as a pixel of data, converted to a shade of grey corresponding to the intensity of the echo; the greater the echo intensity, the brighter the shade of grey. As each echo is received, it is arranged along a line within the ultrasound image display.

Within the transducer a crystal array emits ultrasound by means of the *piezo-electric effect*. This is where a material with inherent contractile properties, such as a quartz crystal, can be flexed and contracted by the application of an electric current. If the current is switched on and off repeatedly (pulsed) then a by-product of this phenomenon is the production of a high frequency ultrasound wave. The wave produced is directed into the subject by holding it against the skin with coupling gel (silicon and water) sandwiched between the transducer outer casing (usually latex) and the subject's skin (Figure 2.15). As ultrasound does not pass through air, the gel acts as a 'bridge' between the convex surface of the transducer and the soft tissue of the subject, ensuring a good contact through which ultrasound can pass.

The transducer is both transmitter and receiver, collecting echoes from one scan line before the next pulse is emitted. As the wave progresses through the tissue, it strikes anatomical structures and the resultant image is generated from density and location information. The image is constantly updated by pulsing (pulse repetition frequency or *PRF*) and if repeated at 32 frames per second, creates images that can be resolved by the human eye on-screen as 'real-time' moving images. As ultrasound travels well through fluid-filled structures it is ideal for imaging the fetus within the amniotic fluid.

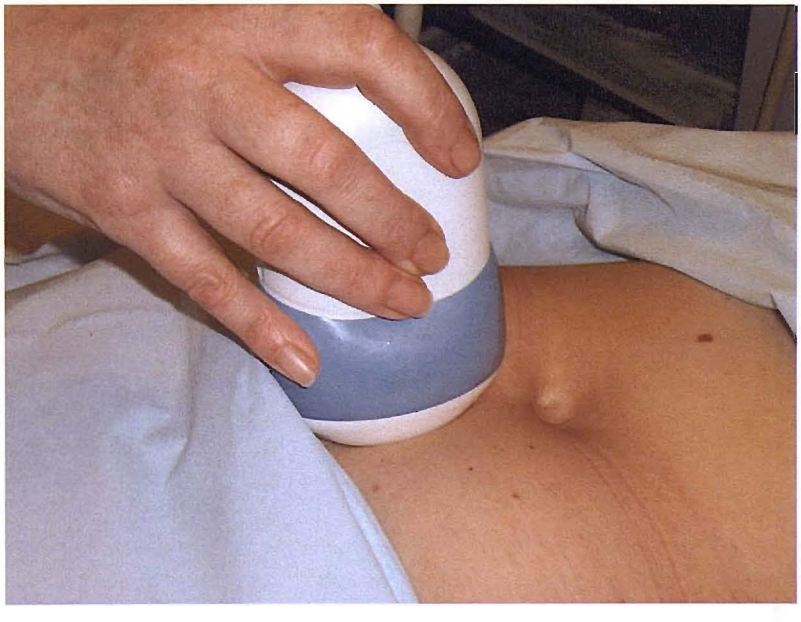


Figure 2.15: Scanning position for the 3D transducer on the subject's abdomen.

2.2 Choice of technique and system

In order to create a 3D volume using 2D ultrasound, several methods of adaptation of the standard technology have been devised. The machine acquired for this study uses a mechanical 'sweep' system that is simple to use, making volume data acquisition reproducible. The transducer is held still while an internal mechanism allows the crystal array to rotate through an arc, transmitting and receiving ultrasound as it proceeds, (Figure 2.16). The time taken for each sweep can be altered by the operator to suit the resting state of the fetus. This time can be varied between 1 to 9 seconds.

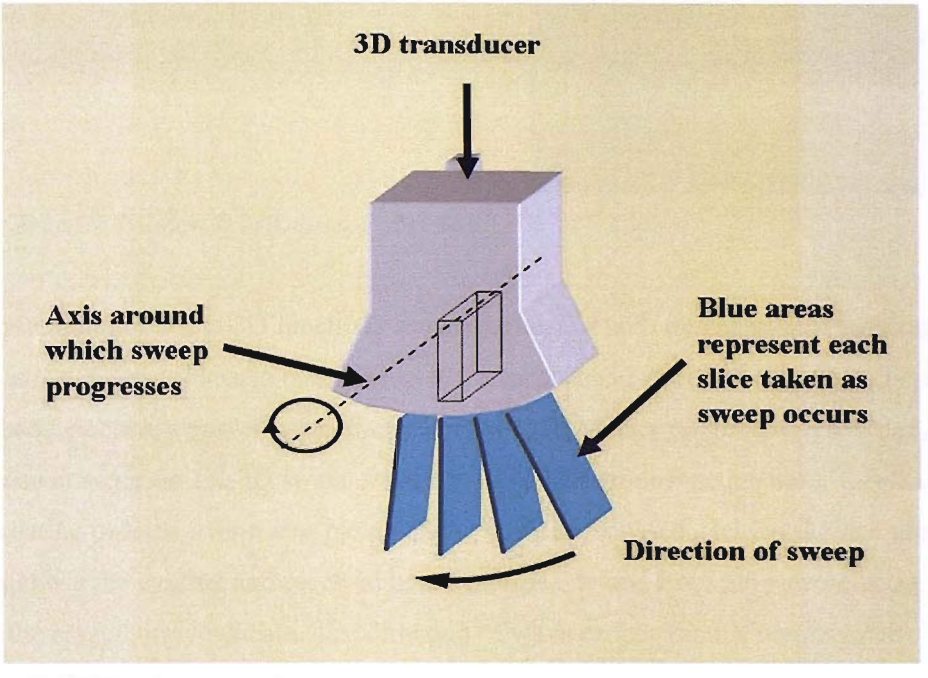


Figure 2.16: The mechanical sweep method of 3DUS acquisition. The blue planes represent images taken as the mechanical sweep passes through an arc. (Diagram courtesy of Dr. Dean Barratt, University College London).

The system chosen was the KretzGE Voluson 730® system (Kretztechnik AG, Zipf, Austria), (Figure 2.17) which has optimum quality resolution, necessary for imaging small structures and uses the mechanical sweep method of volume acquisition.

Figure 2.17: KretzGE Voluson ® 730 ultrasound system.

With this system both 2D and 3D functions can be performed with one hand-held transducer, which is larger than the 2D transducer in order to house the mechanical sweep mechanism, (Figure 2.18). This broadband electronic curved-array transducer has a frequency range of 4–8 MHz and a controllable scan angle for the 3D sweep from 15° to 75° for volume acquisition. It is faced with a protective coating of latex over a *lens* by which the beam is focussed. Behind the lens are *matching layers*, to equalise the emitted and received beam energies, to and from the piezo-electric quartz material of the crystal array beneath. The direction of wave propagation is perpendicular to the long axis of the transducer.

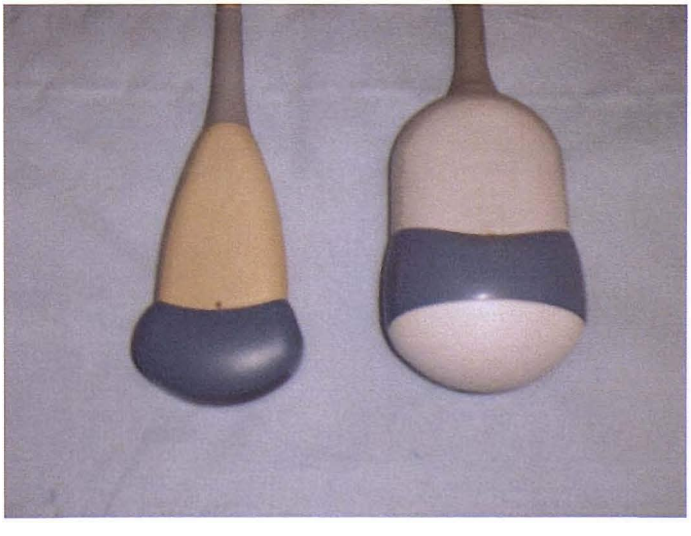


Figure 2.18: KretzGE transducers. Left: standard 2D transducer. Right: 3D transducer, which is larger to accommodate the mechanical sector sweep array.

The 3D multi-element transducer allows several focal zones to be activated, for optimal resolution at variable depths within the image. At the press of a key, the 3D function is switched on and the mechanical sweep motor drives the scan-head within the housing through an arc, pulsing ultrasound waves into the subject and receiving the returning echoes between the pulses , as it passes, allowing a 3D volume data set to be acquired. After a few seconds, the A, B and C scan planes are displayed synoptically on the screen, reconstructed from the acquired volume, (Figures 2.19, 2.20 and 2.21).

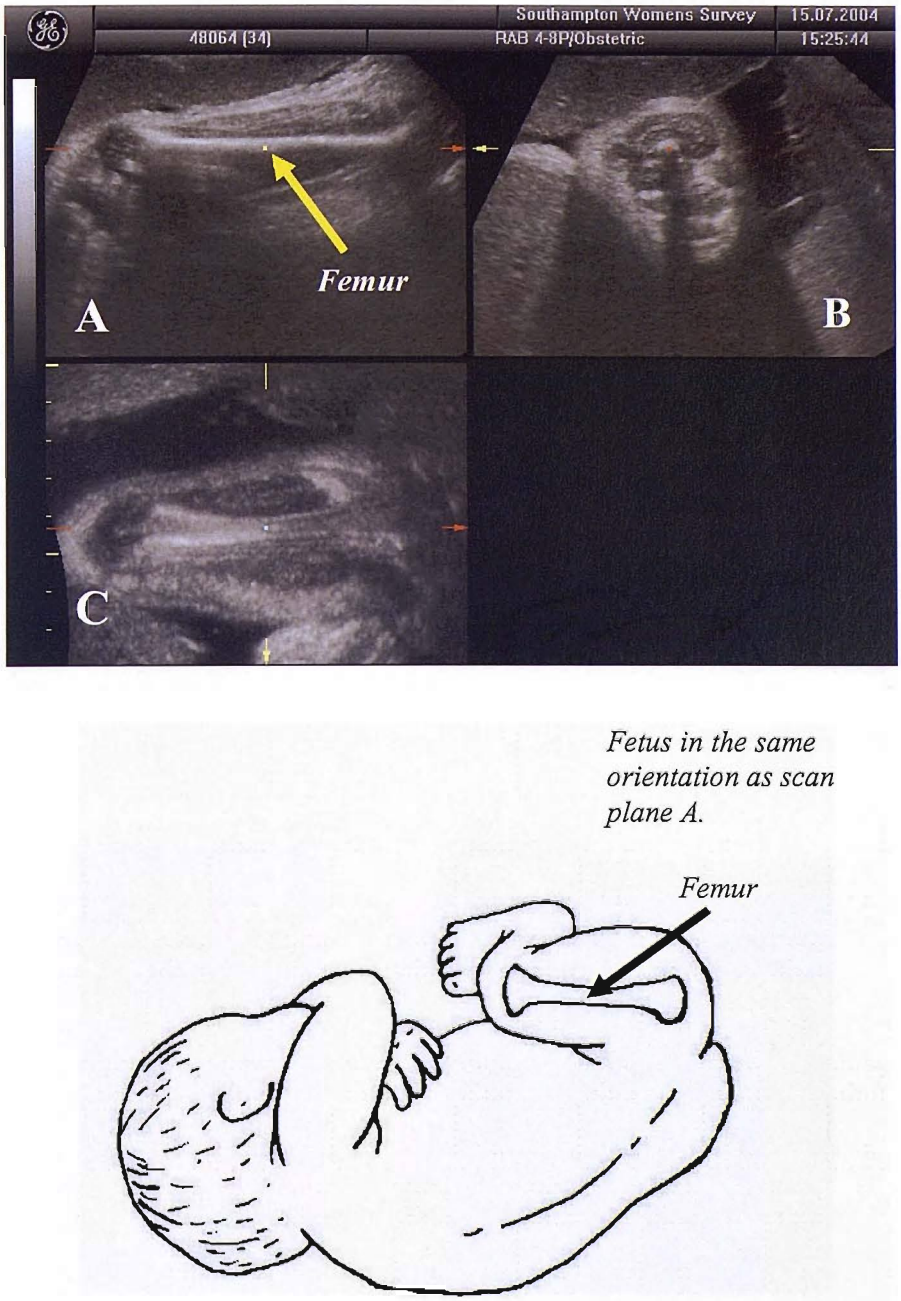


Figure 2.19: 3D planes as displayed on the system's monitor. A: longitudinal, B: transverse and C; coronal or horizontal. The lower diagram shows the fetus in the same orientation as the scan image in plane A.

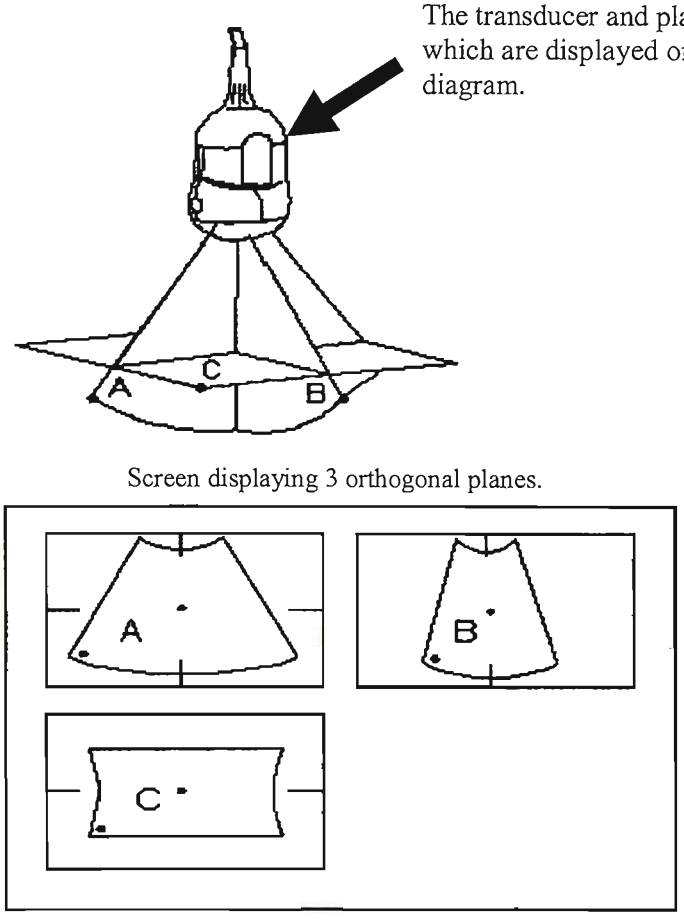


Figure 2.20: Diagrammatic reconstruction of how each plane represents the data acquired.

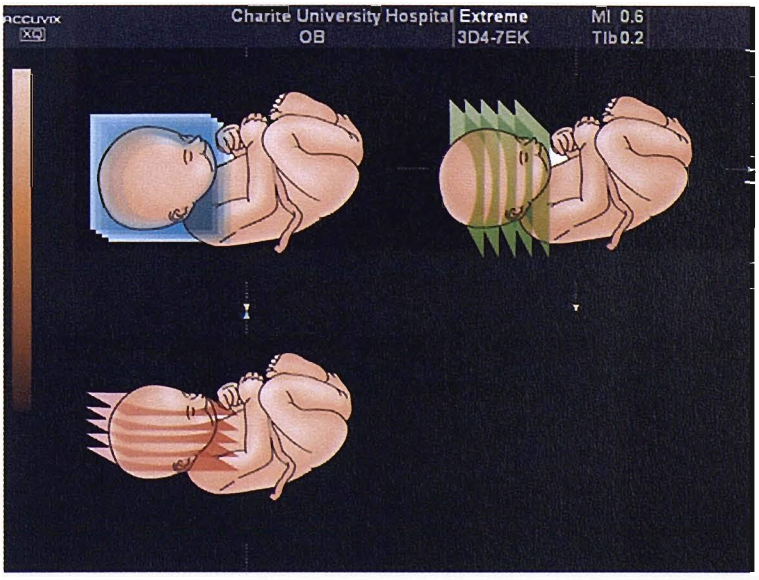


Figure 2.21: Diagrammatic representation of how the 3D planes relate to each other. Blue: A plane, green: B plane and red: C plane. (Picture courtesy of Dr. Karim Kalache).

The system's ability to display a crisp image on screen depends on the density of lines of information that can be stored; 350 lines for 2D data and 250 on 3D volume data. On-screen these lines of information are displayed at 16 frames per second, decreasing with larger volume size, higher quality image setting and chosen frequency. The viewing monitor has a resolution of 1280 x 1024 pixels.

The transducer has a software safety device (©k-Temp) that will not allow the transducer face to exceed 41°C. Optimal temperature for clinical operation in both transducer and system, is between 10°C and 40°C, recommended by the American Institute of Ultrasound in Medicine Bioeffects Committee (1989), further supported by the European Committee for Medical Ultrasound Safety, (2000).

Methods Development

2.3 Real-time scanning for optimal image quality

To ensure that optimal volume data is acquired by ultrasound, first the fetus has to be scanned using 2DUS, with the mother lying supine on the examination couch next to the ultrasound system. Warm coupling gel (silicon–water mix) is applied to the maternal abdomen to prevent an air-gap between the transducer and skin. In real-time, the operator can assess fetal position, fetal activity levels and adjust the controls for the optimum region of interest (ROI) as seen in Figure 2.22.

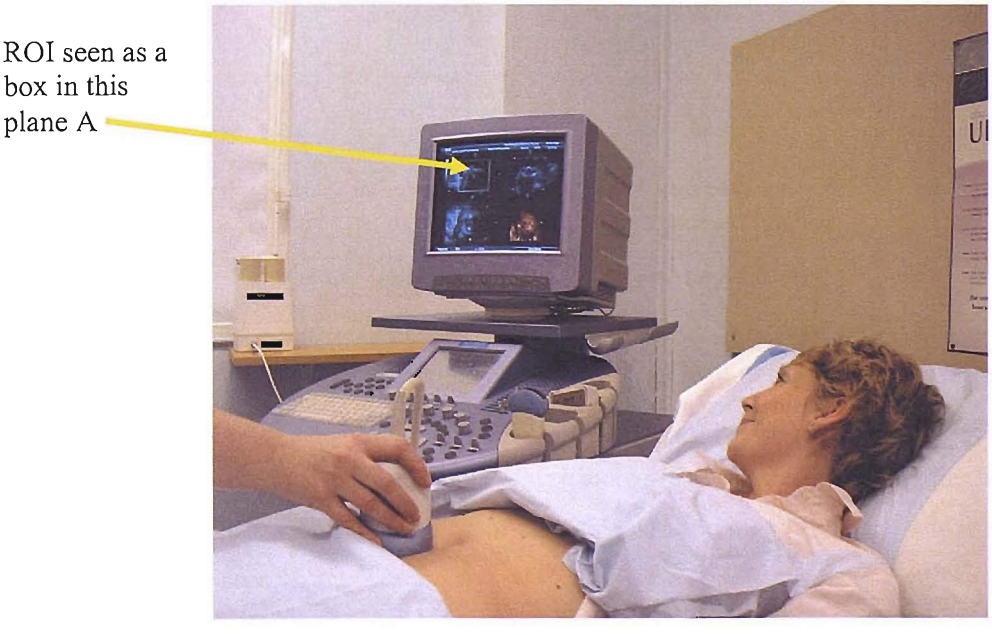


Figure 2.22: Clinical scanning and positioning of ROI; the ROI appears as a box in the A plane.

The standard technique for obtaining an optimum view of the fetal femur, involves rotation and angling of the transducer over the uppermost fetal thigh and femur, with the lateral border of the femur being perpendicular to the long axis of the beam direction. The leading focal zone should be positioned just above the structure of interest, such as the fetal thigh. The ROI is denoted on-screen by a highlighted square, (Figure 2.22). This is an indicator of the extent of data that can be collected with one sweep. This should be positioned and widened to include all of the soft tissue of the fetal thighs if possible. The image should be magnified so that all of the soft tissue, the hip and knee joint and lower limb directly beneath the upper can be seen. Ideally the fetus should have one thigh 'stacked' on top of the other, without being pressed together, so that amniotic fluid outlines the skin surface. In this position the shadowing from the dense bone of the upper femur usually lies directly and uniformly, over the lower leg, (Figure 2.23). This ensures good positioning but renders acquisition and measurement of the lower femur unfeasible.

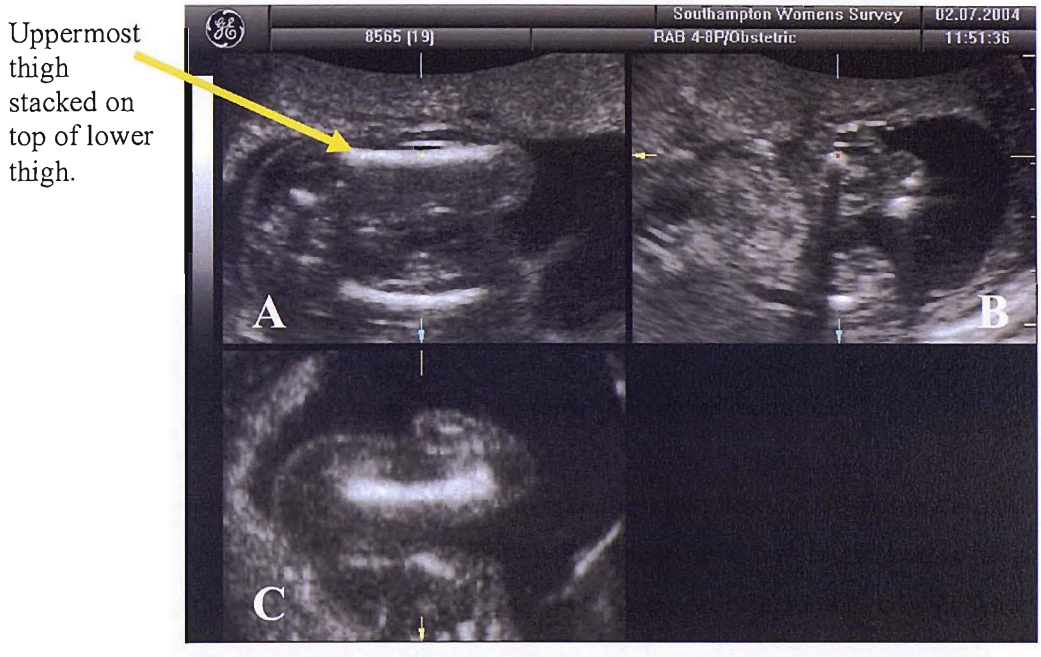


Figure 2.23: Fetal femora in the 'stacked' position for volume acquisition.

Poor positioning or lack of fluid (oligohydramnios) may lead to soft-tissue distortion and if the fetus is too flexed or has its lower limbs pressed together it may be difficult to judge which surfaces are that of the thigh or another structure. Where there are structures overlying the fetal thigh, such as part of the lower limb, an upper limb or the spine, shadowing will affect how much of the thigh and femur can be seen.

Obese women have excess adipose tissue that makes imaging difficult. Also fetuses lying breech or straddling the maternal spine, or very active fetuses whose movements were faster than the time needed to acquire the image, limit the examination. Analyses of all acquisitions in this study were

attempted, reviewed and some discarded for poor quality. If the fetus has hydramnios (excess liquor in the amniotic cavity), it may often mean that the baby will have room to move vigorously and keeping acquisition times low can decrease the chance that the fetus may move. Movement can have disastrous effects on the image if fetal movement is faster than the scan acquisition, (Figure 2.24).

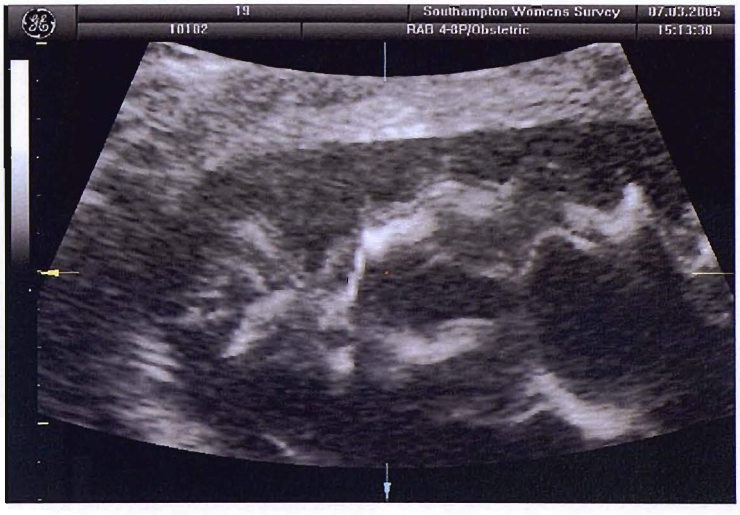


Figure 2.24: Movement artefact on-screen produced when the fetus moves during scan acquisition.

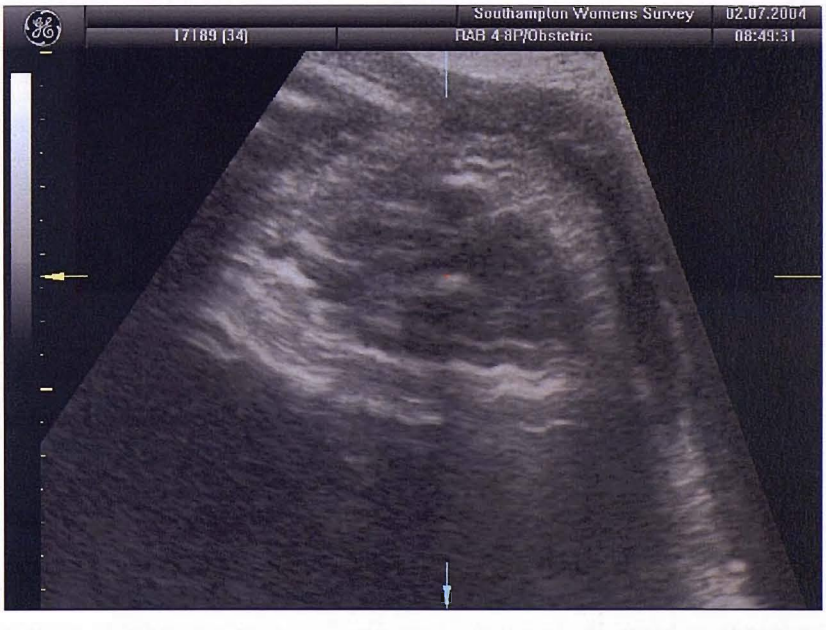


Figure 2.25: Subtle movement artefact from fetal 'breathing practice'. This is seen on a cross-sectional plane through the fetal thigh and appears as a 'ripple' effect.

Normal fetal movements such as 'breathing practice' can also cause artefact on scan, (Figure 2.25), which appears as ripples in a structure which would normally show smooth linear layers. Whilst ultrasound can visualise soft-tissue structures with ease, some factors reduce resolution. The on-

screen image can be optimised by use of manual controls, while in real-time 2DUS mode and a few moments spent adjusting these controls can improve quality. The picture display can be adjusted for brightness and contrast. High contrast is usually set for bone imaging, so that edges can be clearly defined. For soft tissue, less contrast is desirable with more shades of grey (grey-scale). The same anatomical structures can appear completely different with sub-optimal settings, (Figures 2.26 and 2.27).

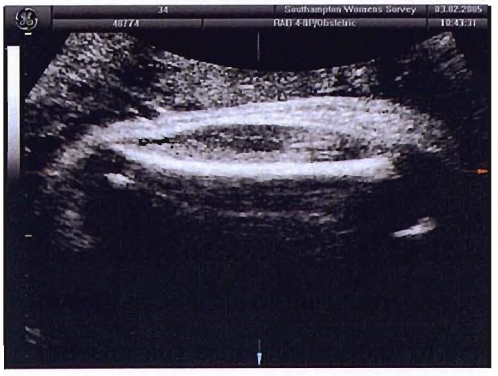


Figure 2.26: High contrast settings on a scan image.

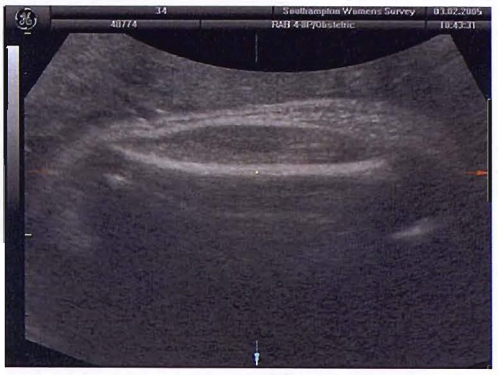


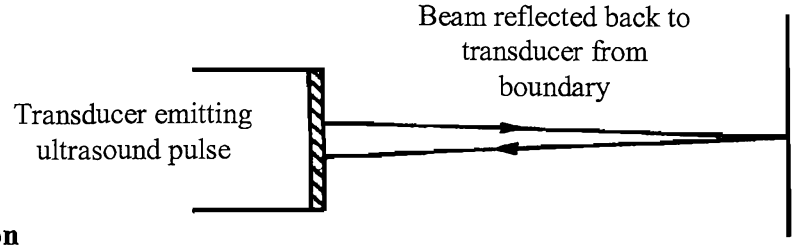
Figure 2.27: Low contrast settings on a scan image.

To amplify the echoes sensed by the transducer, overall *gain* can be adjusted. This affects the overall grey-scale of the image on-screen, so that even the weakest signals can be seen. However, if the returning echoes in one part of the image need to be boosted, such as the echoes from beyond a denser structure, the time gain compensation (TGC) facility can be used to enhance deeper echoes. This aids visualisation of the posterior border of the femoral shaft or the furthermost border of the fetal thigh. Care must be taken not to set the TGC too high or low, as it may create or hide real structures within the image. Within the fetal thigh, there are dense sheaths enclosing muscle groups and if the gain is set too high, these structures take on a bright echo, similar to that produced by bone. These can be mistaken for parts of the femur.

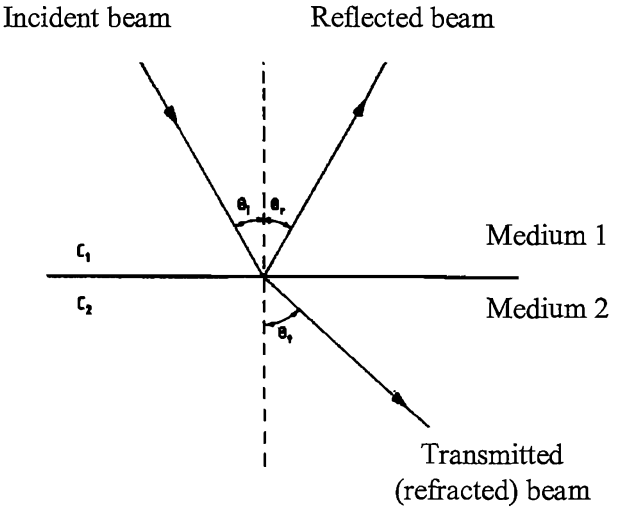
Fluid-filled structures can cause *posterior-enhancement* where the ultrasound passes easily though less dense material such as the amniotic fluid. Intense echoes may be created at the furthermost side of the cavity, causing 'pseudo structures' but this can be minimised by adjusting the TGC. When an ultrasound pulse is produced, it will either be propagated by the closest tissue next to it allowing transmission of the wave or it will reflected, scattered, diverted or absorbed. All of these effects or *artefacts* conspire to improve or degrade the 2DUS image displayed on-screen and behave in the same way for 3DUS, (Nelson *et al.*, 2000).

Speckle occurs when scattering structures are so finely spaced that they cannot be resolved by the system, (Figure 2.28). The resultant interference of waveforms creates a 'busy' image where structures cannot be accurately assigned an address. This is apparent when scanning over the dense

bone of fetuses at later gestations, as there is more mineralisation within the femoral shaft than previously. A bright speckled echo results and some of the beam will reflected away as *specular reflection.* This also occurs when the wave front strikes a surface that may or may not be perpendicular to the direction of the incident beam, (Figure 2.28). The amount of beam *reflected* to the transducer will vary depending on the angle at which the beam strikes the boundary. If the dense bone of the femur or an overlying limb causes this to happen, then data can be lost.



Reflection



Refraction

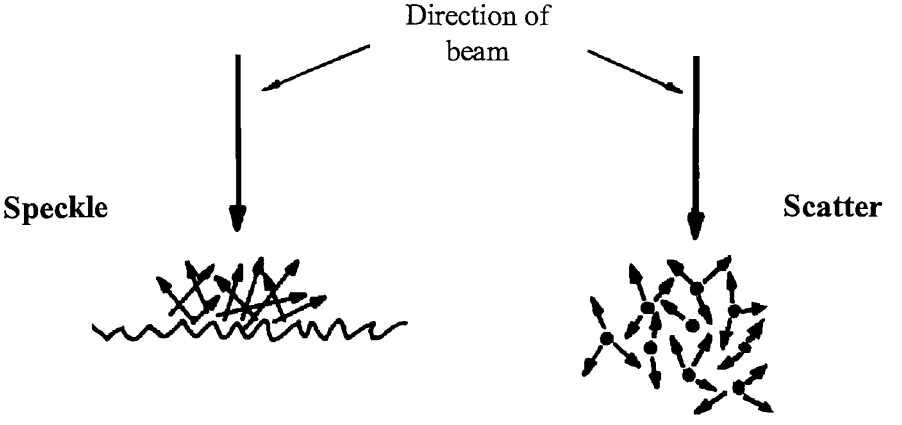


Figure 2.28: The nature of ultrasound at boundaries.

If the beam strikes a rough boundary surface, much energy is lost in many directions by *scattering*, this effect being more apparent with higher beam speed, (Figure 2.28). This may not be a problem for obstetric scanning, unless the tissue of the placenta or another part of the fetus comes directly between the beam and the target of interest. The speed at which the beam passes through a material can also dictate how much of the beam is directed away from its original course when it strikes a boundary. This *refraction* allows sound to be lost, as the denser the material at the boundary, the more likely sound will be refracted away from the direction in which it came and therefore will not contribute information to the resultant image.

The speed of sound within materials also varies depending on which medium it is passing through. In mammalian soft tissues the average speed that propagates the beam has been calculated and is universally recognised as being 1540 m/s, (Duck, 1990; Fish, 1990). In bone this speed varies between adults, children and fetuses, cortical and trabecular bone and even wet or dry bone. Speeds range from 3250 m/s in the living adult femur to 2109 m/s for a child's, (Duck, 1990). This presents a problem with exact calibration within the ultrasound system as the quantities of bone within a 3DUS volume dataset will be variable and sometimes very small. The behaviour of ultrasound within bone is less predictable than when imaging large expanses of soft tissue such as the adult abdomen.

The intensity of the beam can be reduced or *attenuated*, when it passes through a medium, as it is converted to heat and so does not form part of the returning echoes. A long path for the beam to and from the ROI results in more attenuation. The depth at which a structure appears in the image is consistent with its actual distance from the transducer face, provided the speed in the tissue is constant, such as 1540 m/s in mammalian tissue. In practice if the fetal thigh is 4 cm from the transducer face at the maternal abdominal surface, then it will appear 4 cm from the upper border of the on-screen image. At deeper levels the amplitude of the waveform decreases as it traverses distant tissues and the loss of this amplitude is brought about by absorption or scattering of the beam. Not only will data be lost by this phenomenon, but artefacts will also degrade the image. While artefacts can occur in the simplest path from the transducer through the medium and back to the transducer, some image loss is compounded by the reconstruction of the data in the 'horizontal plane' (C plane) in 3D imaging.

There is the facility to select up to 3 *focal zones* whilst live scanning. A focal zone is where the beam is electronically controlled to sharpen the image at a focal point. However, if the number of zones is increased, then pulse repetition frequency (PRF) decreases and in 3D imaging, the length of the acquisition time increases. Whilst images with more information will be produced, movement artefact may escalate.

If the mother has increased adipose tissue or muscle within the anterior abdominal wall, there will be a subsequent decrease in the velocity of ultrasound within that tissue and greater distance between transducer and target. This causes image degradation in 3D images. Adipose tissue is constructed from many small fat components creating tiny boundaries that scatter, giving a noisy bright echoic image. This can be partly compensated for by use of the TGC.

Operator skill is needed to select the optimal 2D settings and obtain the exact orientation of transducer on the fetus and once the settings and position have been selected, 3D volume data can be acquired. It is essential that the best quality 2D image is obtained during the real-time scanning period to obtain the best 3D volume.

2.4 Volume data acquisition technique

Once the optimal view of the fetal thigh is seen in real-time 2D, 3D mode is selected and a 'volume data box' is superimposed on-screen. The operator positions this box over the most appropriate part of the image before the start key is pressed. The 2D image represents the central 2D position of the volume. The internal mechanism sweeps the crystal array from one margin to the other, the angle selected by the operator. This entire ROI will be stored during the sweep. The time this takes depends on the depth of ROI, angle of sweep, power settings and number of focal zones. For images containing greatest information, but at a speed least likely to acquire movement artefact, a sweep between 3 and 6 seconds is usual. Figure 2.29 shows the resultant 3 planes and surface-rendered plane (optional) that are acquired during volume acquisition. The ROI is seen in the top left A plane as a rectangle in the central part of the image.

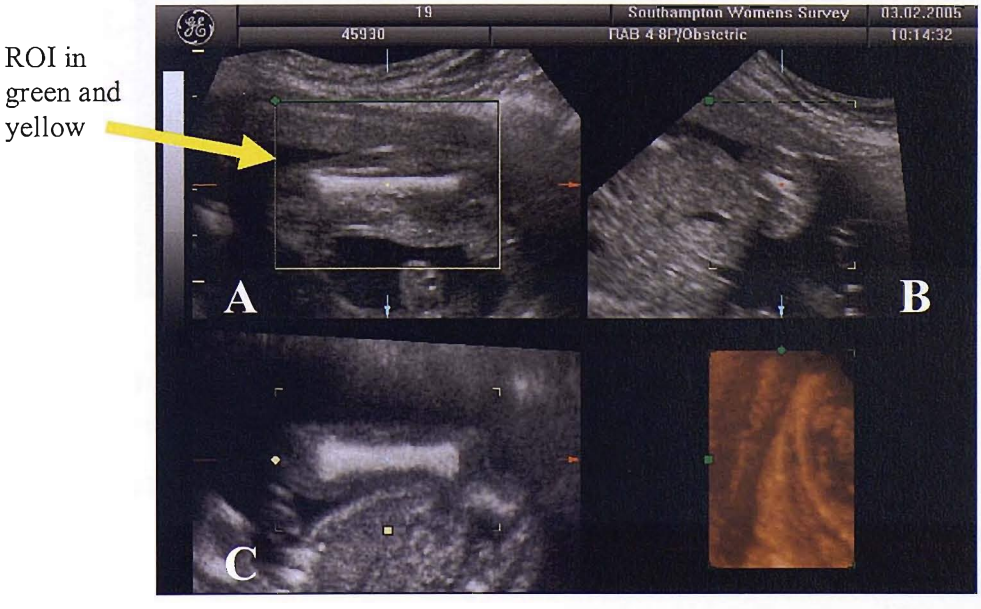


Figure 2.29: The ROI as displayed on-screen; a green and yellow box in plane A.

The transducer must be held steady on the maternal abdominal wall, and as the sweep progresses and real-time images are displayed on screen so that any fetal or maternal movement, respiration or blood vessel pulsation can be assessed as to its effect on the volume being acquired. It is advisable to wait for the fetus to settle before volume acquisition is attempted. Additionally, suspension of maternal respiration for the few seconds needed is preferable. In practice, it may not be possible at later gestations to suspended respiration, as maternal lung capacity decreases when lying down. Movement artefact can be seen if the operator's hand is not steady or the fetus moves during acquisition. It can also occur if the area of interest includes the maternal aorta, or if the fetus is lying over the pulsating aorta. Turning the mother onto her side or waiting for the fetus to change position are the practical solutions to these problems.

The uppermost fetal thigh must be within the volume data box when scanning in 2D real-time mode and the transducer must always be held in the correct orientation to avoid confusion in data interpretation. After the completion of the sweep and a few seconds of calculation, three images are now displayed simultaneously representing longitudinal (A), transverse (B) and horizontal (C) sections within the scanned volume, (Figure 2.30).

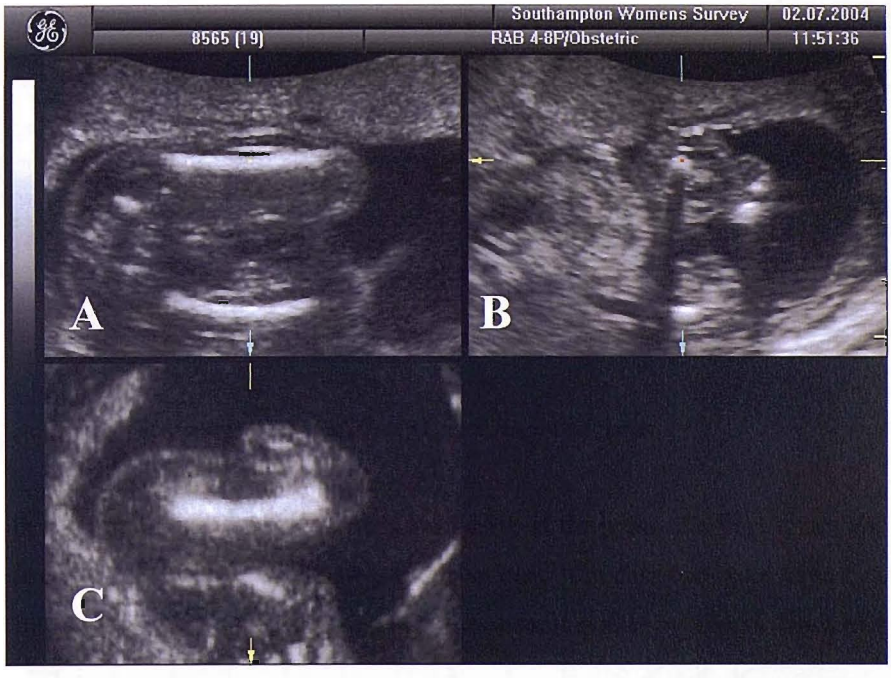


Figure 2.30: The displayed planes of a 19-week fetal thigh and femur.

The data can be stored onto the system's hard drive at the press of a key. It can also be exported to a remote computer or transferred onto a compact disk. The data can be reviewed at any time and can be interrogated from any angle, using planes unachievable by 2DUS. Each of the three images can be navigated through to create any desired section within the volume by means of position

controls. When scrolling through an image plane the central position within the volume is indicated by coloured reference points on the other two images, enabling the object of interest to be interrogated simultaneously in three axes perpendicular to each other. The central position is shown on plane B as a red dot in Figure 2.30.

2.5 Measuring on stored data

Retrieved data can be manipulated by rotation around the X-, Y- or Z-axis on the stored volume, which are 2D rotations on-screen, controlled by dials on the control panel of the system. The images can also be magnified to aid interpretation, although a highly magnified image will suffer from loss of resolution as the pixel borders become apparent. Contrast and brightness can also be enhanced at this stage. The digital memory can be understood by imagining a matrix of cubes or *voxels*, which have been assigned a grey-scale to each particular address, (Figure 2.31). If the voxel size is too great (i.e., bigger than the target it is imaging) the information displayed will be formed using the dominant amplitude echo at that site and this may lead to edge blurring within the resultant image. For imaging small structures it is necessary for a high voxel density for storage and a high pixel density for the image display.

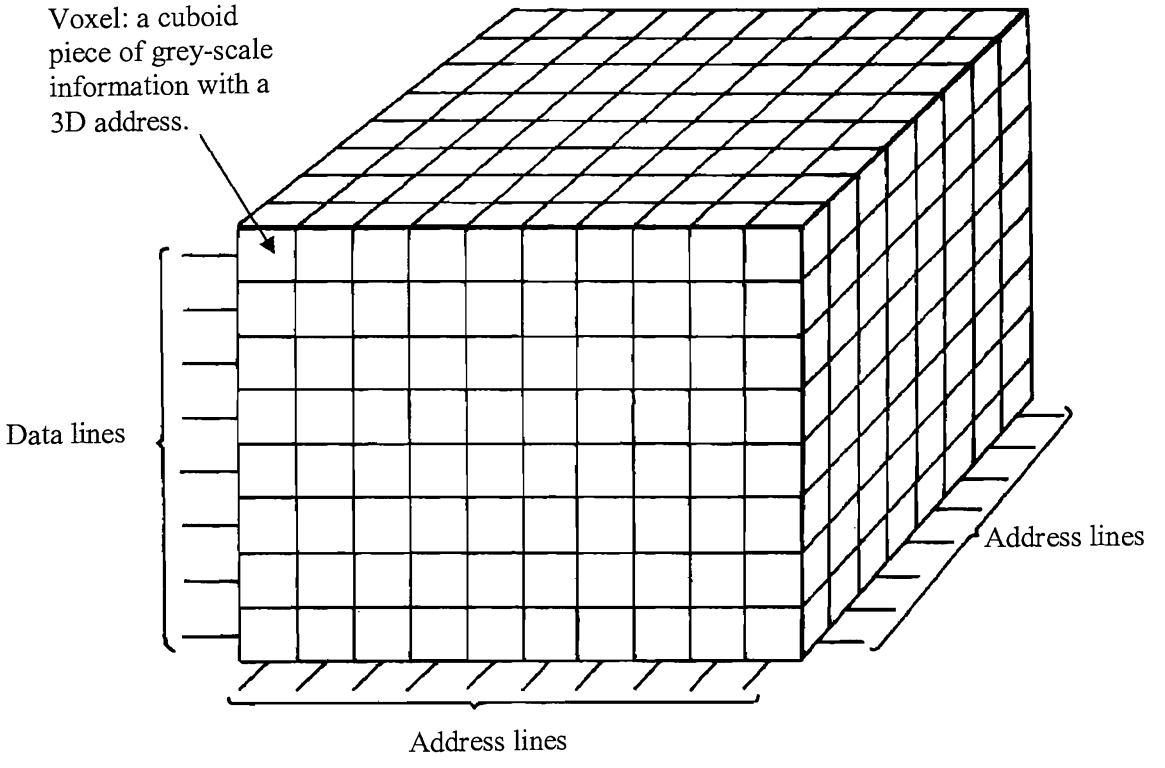


Figure 2.31: Diagrammatic representation of digital data storage of 3D information in voxel matrix. Each 'voxel' of information is assigned a grey-scale level before display.

The retrieved image can have measurements made upon it on-screen in three ways:

1 Linear

2 Traced

3 Volume calculation or *3D multi-plane mode*

Below is a tabulation of the core 15 measurements previously described and on which plane they are measured, followed by a brief description of how they are achieved on stored data.

Measurements to be taken on each stored volume acquisition				
Measurement	**Acronym**	**Type**	**Unit**	**Plane**
Femur length	FL	Linear on 2D	cm	A
Femur mid-shaft transverse section	FMSTS	Linear on 2D	cm	A
Femur mid-shaft antero-posterior view	FMSAP	Linear on recon*.	cm	C
Femur proximal cross-sectional area	Proximal CSA	Trace on 2D	cm^2	B
Femur mid-shaft cross-sectional area	Mid-shaft CSA	Trace on 2D	cm^2	B
Femur distal cross-sectional area	Distal CSA	Trace on 2D	cm^2	B
Femur proximal antero-posterior view	FPAP	Linear on recon*.	cm	C
Femur distal antero-posterior view	FDAP	Linear on recon*.	cm	C
Mid-shaft skin thickness	MSST	Linear on 2D	cm	B
Mid-thigh cross-sectional area	MTCSA	Trace on 2D	cm^2	B
Mid-thigh muscle cross-sectional area (derived)	MTMCSA	Trace on 2D	cm^2	B
Thigh volume	TV	Multi-plane mode	cm^3	B
Fat / skin volume (derived)	SV	Multi-plane mode	cm^3	B
Muscle volume	MV	Multi-plane mode	cm^3	B
Femur volume	FV	Multi-plane mode	cm^3	B

*Table 2.1: Tabulation of the 15 core measurements. * Recon – on reconstructed data C plane image. N.B. MTMCSA and SV are derived values.*

2.5.1 Linear measurements

FL, FMSTS, FMSAP, MSST, FPAP and FDAP are all linear measurements. A linear measurement is made by placing electronic callipers at each end of the structure to be measured on any of the images displayed, (Figure 2.32). The measurement is then displayed on-screen in centimetres. Each measurement was taken 3 times, noted down and averaged before use in the statistical analysis.

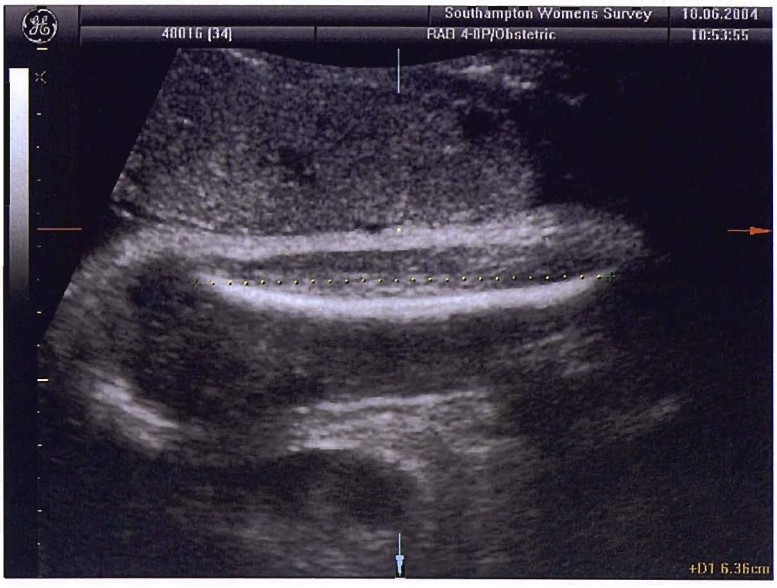


Figure 2.32: Linear measurement of 34 week femur length (FL).

2.5.2 Trace measurements

Mid-shaft CSA, MTCSA, MTMCSA, proximal CSA and distal CSA are all trace measurements. Trace method is used for area calculation and can be performed on any plane. A movable cursor activated by the track-ball facility on the control panel allows the operator to trace around the border of structure, such as the MTCSA. The circumference and cross-sectional area are displayed on-screen in cm and cm^2 respectively (Figure 2.33) once the trace is enclosed.

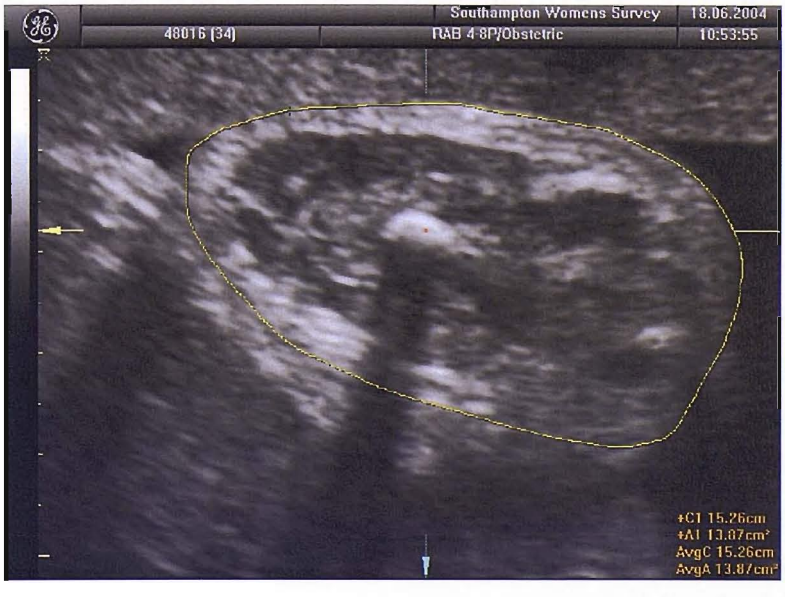


Figure 2.33: Trace measurement of MTCSA at 34 weeks; on-screen callipers are used to trace around the skin surface at the mid-thigh point.

2.5.3 Volume measurements

TV, SV, MV and FV are all volume measurements. The volume calculation facility (3D multi-plane mode) is a method by which area traces parallel to each other can be made through an object, such as the fetal thigh or femur, on any plane, (Figure 2.34). As each trace or 'slice' is made, the system calculates the volume as it progresses and the size of the increment can be selected by the operator as measuring progresses. In this evaluation, intervals of 3 mm were used along the femur, thigh and muscle to calculate the volume at 19 weeks and at 5 mm for the 34-week scans. Increments between slices as small as 1 mm can be made, which is useful where the bone or organ shape is irregular. The resulting measurements are displayed on-screen in cubic centimetres (cm^3). The larger the number of slices, the more precise the volume will be, (Riccabona *et al.*, 1995).

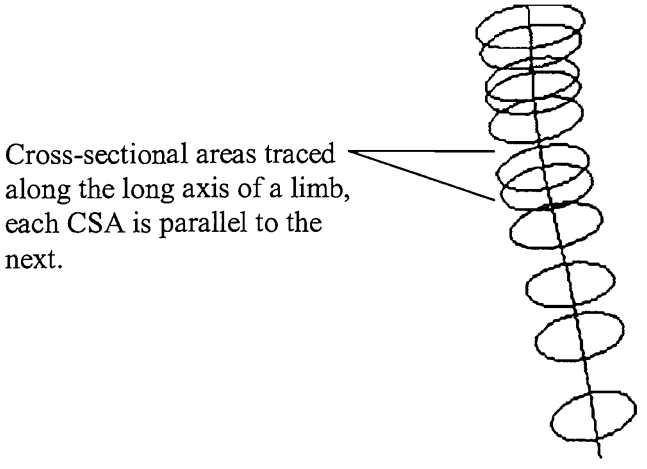


Figure 2.34: Volume production by cumulative CSA measurements; these are added together along the length of the limb or bone to create a volume (cm^3). Software estimates the tissue volume between slices.

2.5.4 Measurements taken on reconstructed oblique planes

FMSAP, FPAP and FDAP are all linear measurements taken on reconstructed oblique planes. Sonoview® software allows linear, trace or volume measurements to be taken on any of the displayed planes. Any of the planes can be rotated away from the initial orientation in which they were acquired. This means that some adjusted images will be oblique and therefore reconstructed in nature. The FDAP is a linear measurement and whilst the measurement technique is standard, it is being performed on the reconstructed C plane that is affected by distortion inherent in the reconstruction process, (Figure 2.35). This affects resolution and makes calliper placement difficult.

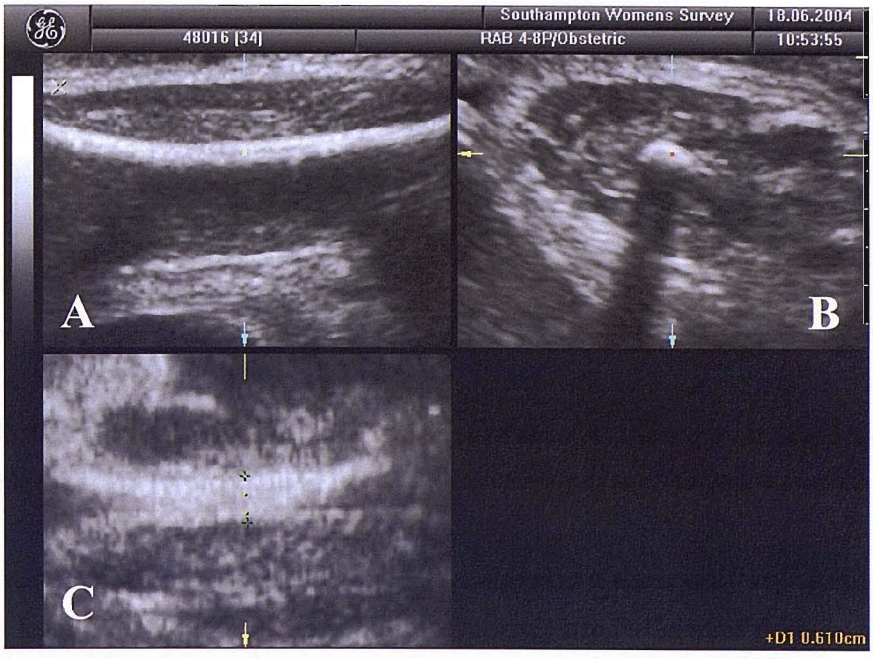


Figure 2.35: Measurement on an oblique reconstructed plane (C). Note how the bone edges are unclear where the cursors are being placed. This loss of resolution leads to measurement inaccuracy.

The distance between the transducer and femur also affects the resolution in this plane. At depth, there is loss of information due to an increase in voxel size furthermost from the transducer. This makes the image appear blurred which worsens the further away from the transducer the femur is placed. Boundaries may be difficult to assess subjectively by the operator and consequently difficult to measure. The manufacturers have also reported a scaling problem within reconstructed planes, which also causes poor resolution, (Pers. comm., Hans Gassert, KretzGE).

The discrepancies outlined here affect the accuracy of the acquisition technique and precision of on-screen measurement. To assist in the identification of which measurements are most prone to discrepancy, a test-tank was constructed in which to test objects of known dimension. The results of these in vitro tests are described in Chapter 3 – *In vitro* Evaluation of Techniques for Fetal Musculo-Skeletal Development.

2.6 Neonatal DXA scans

In anticipation of combining data from fetal ultrasound scans with additional neonatal and maternal data, results from the neonatal DXA scans performed on the SWS babies was also collected. As part of the SWS pregnancy protocol a DXA scan for bone quality was offered for each neonate immediately after delivery. From this scan information regarding bone mineral content, density of the bone and size of skeletal envelope could be collected. Ideally the DXA scan was arranged

within 14 days after birth, so that information could be gathered before weaning had an effect on bone quality of the infant. At the scan visit, a trained researcher undressed the baby and swaddled it in a cotton towel so that clothes fastenings would not cause defects on the scan image and recorded results. The child was given a pacifier or fed with milk if required to calm them. Ideally, the baby was allowed to fall asleep in the warm and darkened scan room, before placing them onto the scan bed in the supine position on a radiolucent mattress on the Lunar DPX system, (Figure 2.36). A sandbag was placed over the loose end of the towel at the baby's feet to prevent movement. If the baby moved during the scan (which took about 10 minutes for a total body scan) then a single repeat was attempted after the baby had been pacified. The mother or father attending the scan gave written consent before the scan was performed and could be present throughout the scan.

Figure 2.36: The Lunar DPX unit used for neonatal DXA scans.

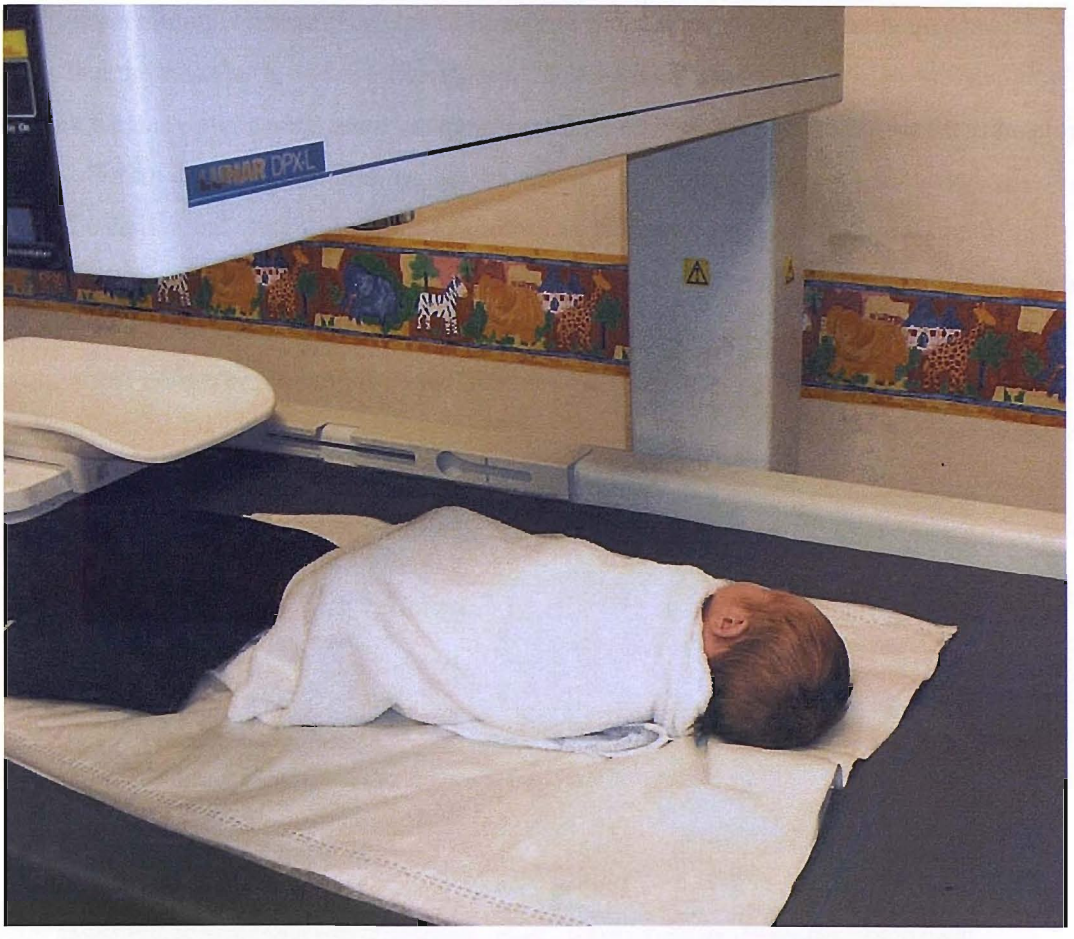


Figure 2.37: A sleeping infant undergoing a DXA examination. The sleeping infant is placed on the black radiopaque mattress. The gantry above the baby moves slowly down the length of the baby emitting x-rays as it proceeds. The x-radiation is detected by sensors beneath the table.

As the scan takes place the moving machine arm positioned above the baby, (Figure 2.37) motors gently along the table with the x-ray tube sweeping from side to side as the movement progresses, in a *raster* pattern. As it passes it emits x-radiation in a slim pencil beam that passes briefly through the sleeping infant beneath, through the radio-lucent mattress and table top, before striking the DXA detector under the table. In this way a radiographic image is made up line by line by a single vertical beam of radiation. This is time-consuming, taking up to 10 minutes for the whole body of the infant to be covered, but it gives accurate readings of size and density. If a conventional radiographic of the total neonatal body was taken, it would be much faster (i.e., hundredths of a second), but the image would be subjected to magnification produced by the much larger field of radiation needed to form the image and the radiation dose could be up to 40 times higher. The dose of radiation produced by the DXA unit is 1.0 mSV of radiation for the total body scan, which is much less than the 7.0 mSV everyone in Britain receives as background radiation in 1 day. Thus the dose administered is not considered a danger to the infant.

The DXA readings obtained from the total body scans of each newborn give indications of bone area (BA), bone mineral content (BMC) and bone mineral density (BMD). The bone area represents the entire skeleton and is an indication of skeletal size and is quantified as a two-dimensional measure and is expressed in square centimetres (cm^2). The BMC gives an indication of how much calcium has been laid into the skeletal envelope during pregnancy and the system calculates the equivalent weight in grams (g), although it is not a true 3D representation of the skeletal mass. The BMD is a derived value which tries to address the 3D nature of the skeleton and is expressed as grams per square centimetre of body mass (g/cm^2). The resultant image from the DXA equipment before processing is shown below.

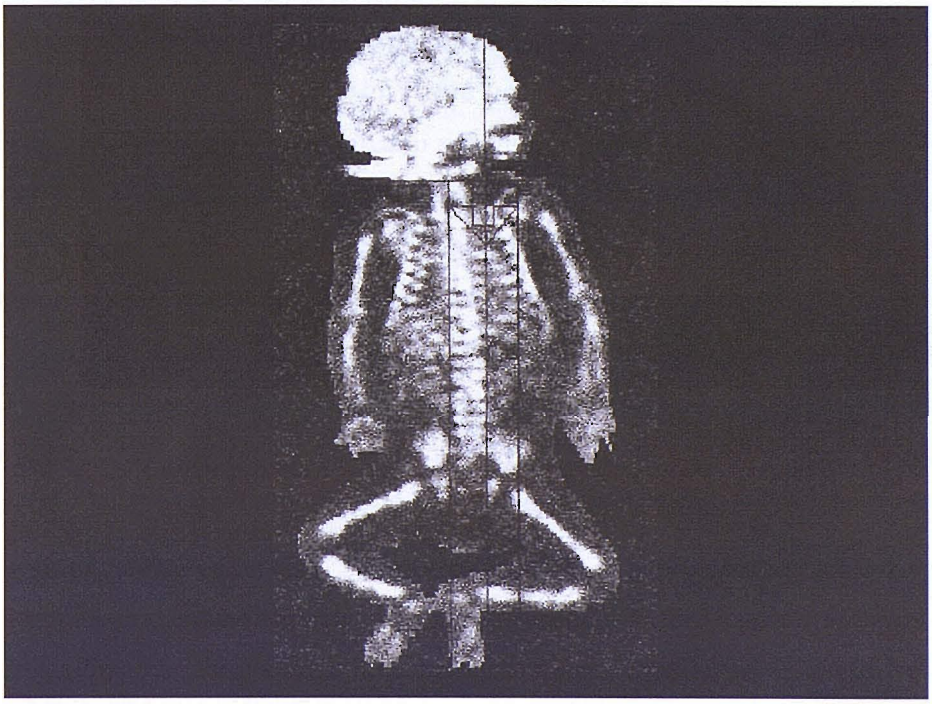


Figure 2.38: DXA image produced of a total body neonatal scan before analysis.

Analysis of the DXA scan takes place after the infant has gone home and takes the form of placing an electronic grid around separate parts of the neonatal skeleton (Figure 2.38) manipulated by the operator (PM). Each area such as head, spine, ribs, arms, legs and pelvis can be isolated and the readings automatically calculated by the equipment for bone area, BMD and BMC. In this study the total body bone area, total BMC and total BMD were used for analysis. An initial trial of the first 55 DXA subjects was assessed to see if there were any relationships between neonatal DXA variables and 19-week scan measurements. It was anticipated that there would be some relationship between the amount of bone already accrued in a 19-week skeleton and the bone mineral accrual by the time the baby was delivered and so this trial was to see if this line of enquiry was worth pursuing. The results are tabulated in Table 2.2.

DXA and scan variables at 19 weeks – First trial of 55 subjects			
	19 weeks		
	β coefficient	p	n
Femur volume and bone area	56.8	**0.001**	*47*
Femur length and bone area	24.2	**0.023**	*53*
Femur volume and BMC	33.4	**0.002**	*47*
Femur volume and BMD	0.02	0.32	*47*

Table 2.2: Associations of neonatal DXA results and selected fetal bone variables at 19 weeks.

Femur length and volume at 19 weeks were correlated with bone area, BMC and BMD by DXA at birth and it could be seen from the table that there were some significant results. Femur volume was significantly associated with bone area ($p = 0.001$), BMC ($p = 0.002$), but not BMD ($p = 0.32$). Also, femur length was associated with bone area ($p = 0.023$). The β coefficient value is an estimate of an analysis performed on variables that have been standardised, so that it can be calculated for every cm^3 increase in femur volume there is a corresponding increase of 56.8 cm^2 in bone area. When the volumes were split into quarters this relationship becomes more apparent, (Figure 2.39). With increasing bone volume at 19 weeks, there is an increase in neonatal bone area and bone mineral content at birth, suggesting that 19-week femur volume is predictive of bone size and mineral content at birth. The relationship was not true for bone mineral density.

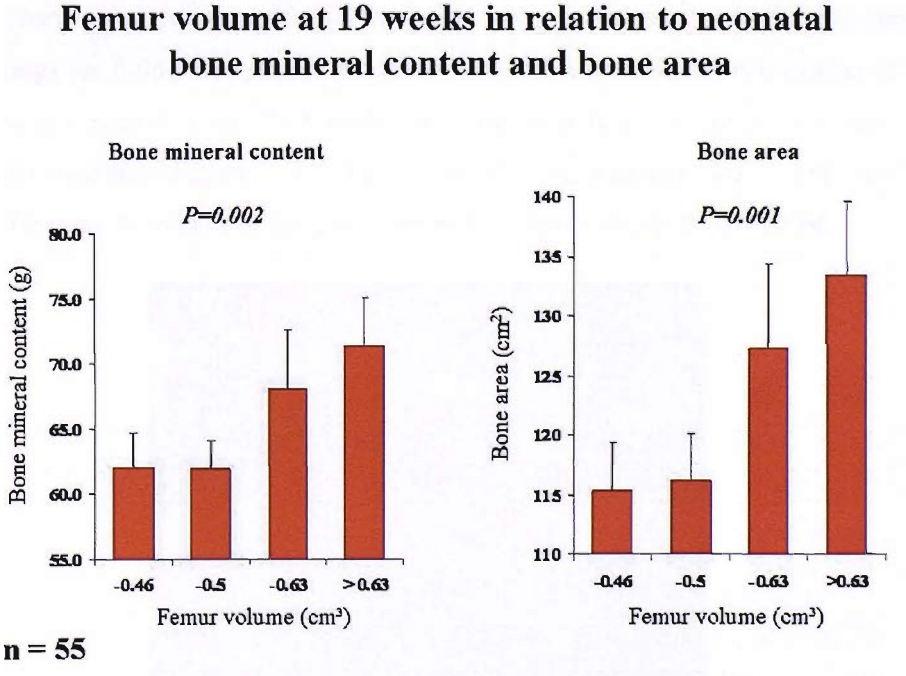


Figure 2.39: Histogram showing the relationship between BMC, BA and skeletal size. As each cm^3 of bone is accrued in skeletal size (FV), BMC and bone area increases. Values are means and standard error.

The promising results shown by these correlations indicated that it would be of value to encourage the parents of the cohort babies to return for a DXA post-delivery and so recruitment of babies for DXA scans was continued. This was done by information leaflets given at the time of the 34 week scan and was reinforced by the research nurses who measured the babies post-delivery. Written consent from the parents was obtained before DXA scanning commenced.

Results of Methods Development

2.7 Ultrasound imaging difficulties

The refinement process for 3D volume data sets involved the refining of the scan image quality and standardisation of measurement technique on the stored volumes. This took several months to develop as both processes encountered difficulties which had to be overcome, as outlined here.

2.7.1 Movement

When visualising a moving object such as an active fetus, it was necessary to obtain the correct orientation of the fetus before setting up the acquisition process on the KretzGE system. Once the transducer position was orientated, the acquisition time had to be kept to a minimum, without reducing the quality of the image on-screen. The screen image quality directly reflects the image quality and resolution of the stored volume images. By trial and error the machine settings were perfected by adjusting number and position of focal zones activated, size of ROI, the angle of sweep, TGC, harmonic imaging settings, contrast, brightness and adjusting machine presets to optimum settings for fluid-filled structures. If the fetus did move then the acquisition time could be shortened or a wait ensued whilst the fetus became comfortable in another position before acquisition was repeated, (Figure 2.40). All volume attempts were stored on the system's hard-drive and at the time of processing for measurement the best volume was selected.

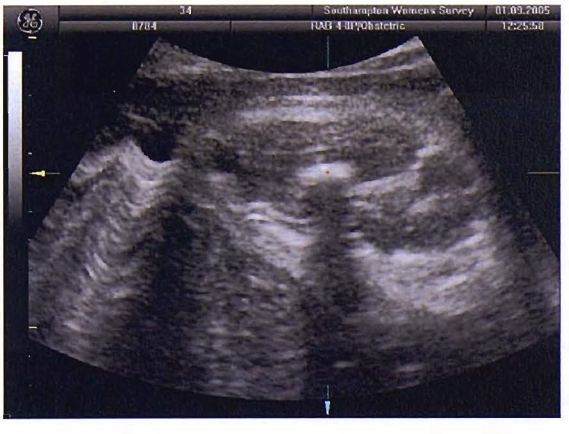


Figure 2.40: Movement artefact on a scan image; artefact is seen as wavy lines through the soft tissue layers.

2.7.2 Reconstruction

It was evident from the 3D volume data sets that some images were sub-standard. These were usually the images recorded on the C plane (Figure 2.41) and this made measurement problematic. The core measurements of FMSAP, FPAP and FDAP were taken on this plane and were the most difficult to perform as the edges of the bone appeared blurred. It was therefore difficult to judge consistently where the callipers had to be placed. What made these measurements more difficult was the machine's reliance on 'smart processing' whereby the placement of a cursor by the operator was not always accepted by the equipment, as it straddled a boundary between two pixels on screen. The system automatically selected which pixel was 'best fit' and allocated that pixel for the cursor placement. This meant that the boundary of the structure being measured was subtly shifted by the system. This discrepancy was most apparent to the operator when the image being processed was magnified on screen for ease of use and it was noticed that the system would realign the calliper by approximately 1 pixel. Thus measurements may not always entirely have been the choice of the operator, although the discrepancy was small.

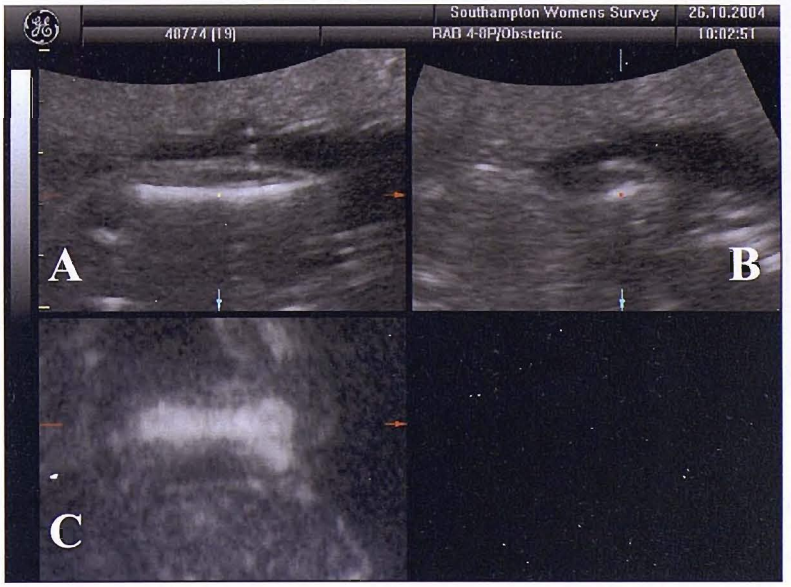


Figure 2.41: Blurred edges of structures as seen in the C plane.

2.7.3 Shadowing

Since medical ultrasound has been in existence bone imaging by sound waves has been problematic. Ultrasound is an invaluable imaging tool for soft tissues and fluid-filled structures such as muscle / skin boundaries, organ margins, cystic structures and vessel borders. Bone presents a challenge to an insonating power such as ultrasound, due to its dense nature and

reflective properties. It also presents a distinct boundary between the hard mineralised bone and the soft tissue immediately surrounding it. As explained previously the ultrasound beam striking a dense medium will be refracted and reflected, with a greatly diminished transmitted beam travelling on into the bone itself. Returning signals from within bone also suffer from the same refraction, reflection and absorption that the outward bound wave endured. Thus much of the information that could be contained within the bone and soft tissues beyond is lost. The resultant image shows a bright white leading edge echo and a black shadow where the information is missing, (Figure 2.42). This is typical of the interface between soft tissue and higher density structures. This discrepancy in tissue types also causes problems for other applications of ultrasound such as *quantitative ultrasound*, which is used to investigate bone density, (Lasaygues *et al.*, 2005).

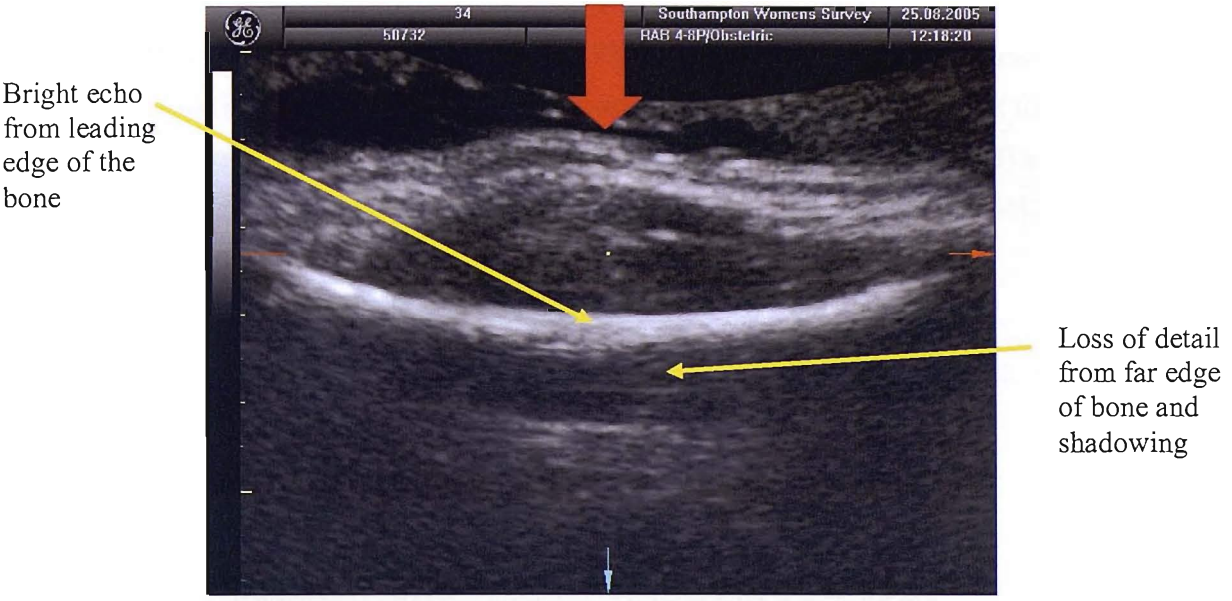


Figure 2.42: The effect of shadowing on scan images. The intense echo produced from the leading edge of the femoral shaft insonated by the beam from above (beam direction in red). Note the loss of detail in the furthest border of the bone and the tissues beyond, in the shadow of the bone.

Fetal limbs often interfere with the image (Figure 2.43) and the practical solution to this is to wait for the fetus to move. At 19 weeks external pressure from the operator manipulating the maternal abdomen has no effect on fetal behaviour, but at 34 weeks, the fetus can respond to external pressure and may move more dramatically than necessary to achieve clear access to the thigh. Thus patience is needed until the fetus removes the overlying limb from the ROI.

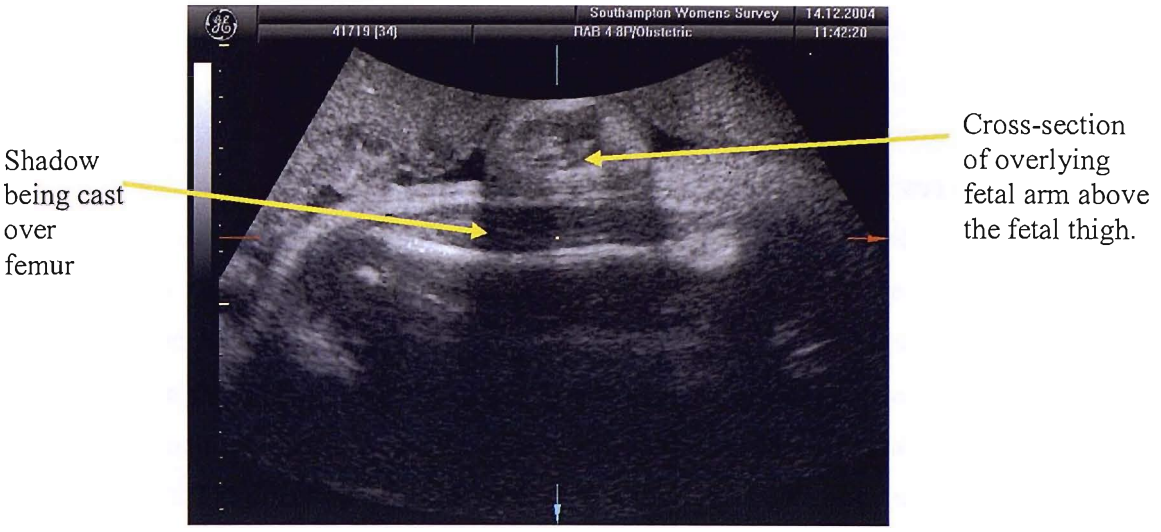


Figure 2.43: The effect of overlying limbs on a scan image.

During the first trials of scanning fetuses *in vivo*, shadowing was most apparent on the stored data sets of the 34 week scans where the bone density had increased sufficiently to block the passage of ultrasound. The effect was not so evident at 19 weeks where both leading (proximal) and most distant (distal) edges of the bone shaft could be distinguished on screen, (Figure 2.44).

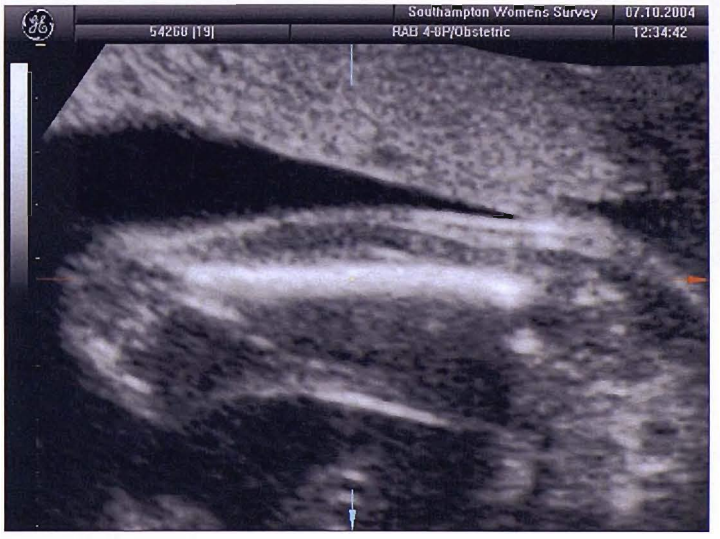


Figure 2.44: The effect of bone density on scan images. At 19 weeks there are homogeneous echoes within the bone, minimal shadowing beneath it and clearer detail in the far field.

By utilising the Sonoview® software on a remote computer with a high resolution screen, low returning echoes from shadowed areas and high density white 'flares' could be manipulated digitally to enhance echoes contained within the volume and thus increase the information that could be resolved by the human eye. Previously unseen boundaries could then be measured with greater ease by the operator.

2.7.4 Estimation of fat / skin layers

In order to measure the fat and skin elements of the fetal thigh, measurements were developed to calculate the fat / skin volume of the thigh. In addition, a linear measurement was taken perpendicularly through the fat / skin layers of the mid-thigh (MSST). Separate volume measurements of fat and skin layers were not possible at 19 weeks as only one linear feature was visible at the skin border, (Figure 2.45). Fat and skin could not be distinguished as two separate layers, even with sufficient magnification of the image on-screen and so the volume measurement devised of for fat / skin layers included them as one structure. By tracing around the outermost layer of the thigh for each slice, the thigh volume could be calculated and then by tracing around the inside of the linear fat / skin layer the muscle and bone component could be calculated as a volume. By subtracting the MV / FV component from the total TV, the fat / skin volume (SV) could be derived.

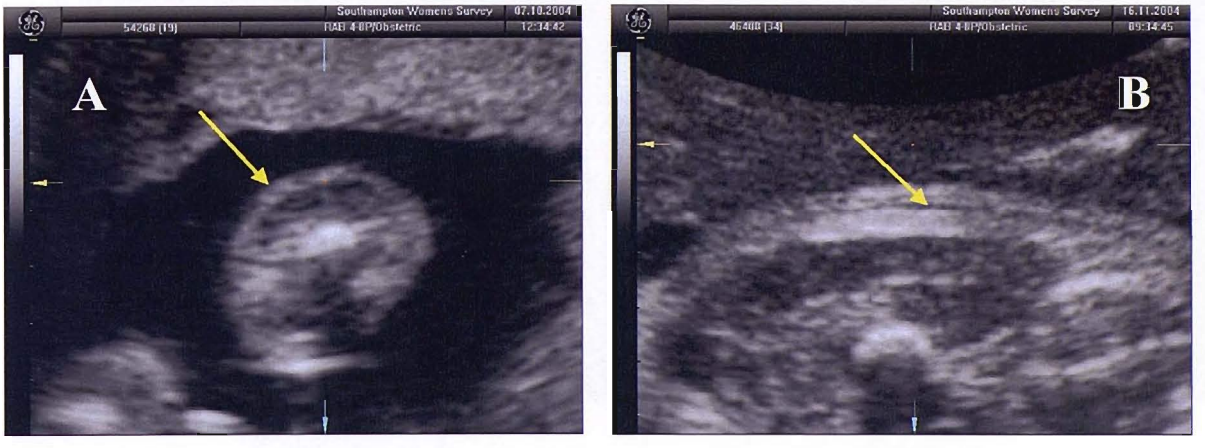


*Figure 2.45: Fat and skin layers at 19 and 34 weeks. **A** shows the fat / skin layer at 19 weeks which can be seen as a continuous linear feature (arrowed). **B** shows the fat / skin layer at 34 weeks in which outer skin layer and subcutaneous fat are divided by a darker layer of connective tissue (arrowed).*

At 19 weeks there is no distinct subcutaneous fat layer around the fetal thigh. It has been observed previously that there would be little fat anywhere on the fetus at this stage, as fat stores are primarily accrued from 28 weeks onwards. By 34 weeks the fetus exhibits a layer of subcutaneous fat around the abdomen and limbs and on ultrasound imaging there becomes 2 distinct layers of skin and subcutaneous fat, seen as white layers, separated by a darker line of connective tissue. Thus both tissues can be distinguished by this gestation, (Figure 2.45 B). As both layers, if there had been any fat at the 19 week stage, had been considered as one layer, they were measured as a combined volume at 34 weeks, termed the 'fat / skin' layer. Further work at other intervening gestations may be necessary to identify at which gestations fat and skin layers appear as separate structures.

2.7.5 Maternal body composition

The amniotic fluid contained within the pregnant uterine gestation sac, creates an ideal medium through which to visualise the fetus. Structures can be clearly defined when surrounded by fluid. However, the ultrasound beam travelling from the transducer face has to traverse maternal tissues before encountering the uterine contents. The passage of the ultrasound beam is least attenuated if the maternal abdomen is lean. Greater abdominal wall thickness, greater muscle mass or greater number of fat cells (adiposity) within the layer, reduces the quality of the ultrasound image, (Figure 2.46). Whilst no participant was excluded from the study because of increased adiposity, the resultant images were considered carefully for quality. Some of the volume data sets were excluded or measured again at this stage for reproducibility. Any scan that showed a discrepancy of 5% or more between duplicate measurements was excluded, as these results were too variable use safely.

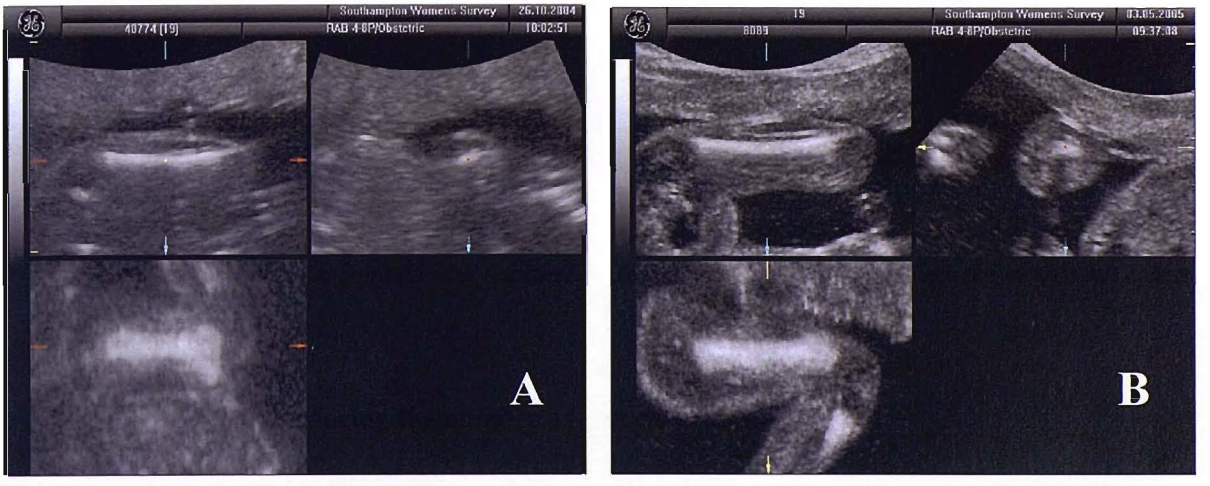


*Figure 2.46: Loss of resolution with adiposity. **A** shows the loss of image resolution at 19 weeks when the mother has a greater abdominal wall thickness. **B** shows the image produced when the mother has a lean abdominal wall.*

2.7.6 Reducing the number of core measurements

After the preliminary studies had been completed, it was decided that some measurements were of little value for the proposed research. Predominantly, measurements taken on the reconstructed C plane were dropped (FPAP, FDAP and FMSAP), as these were subject to greatest measurement variation and difficulty of execution on a reconstructed image. The antero-posterior measurements of proximal (FPAP) and distal (FDAP) shaft were seen to be less valuable than their cross-sectional values and were also variable in reproduction. These were also dropped, as was the FMSTS which suffered shadowing and flaring even though it was taken on the A plane. Ultrasound imaging is subject to this phenomenon especially where a linear structure that is perpendicular to the ultrasound wave-front, is of a dense medium such as bone.

The linear MSST measurement was also dropped as this measurement, although trouble-free to produce, was not considered to be such a descriptive measure as fat / skin volume. In a previous study, (Bernstein, 2004) where a 2D linear measurement of thigh fat / skin layers taken on a thigh CSA, was not found to be as valuable as taking a CSA at that level and suffered from 27% inter-operator measurement discrepancy. Thus MSST was discarded in favour of more useful measurements.

The measurements of MTCSA and MTMCSA were also dropped from future collection. Whilst they were easily acquired, again, they did not have as good descriptive potential as the volume measurements which could be calculated. Thus the 8 core measurements remaining were:

Thigh volume	TV
Fat / skin volume	SV
Muscle volume	MV
Femur volume	FV
Femur length	FL
Femur proximal cross-sectional area	Proximal CSA
Femur mid-shaft cross-sectional area	Mid-shaft CSA
Femur distal cross-sectional area	Distal CSA

2.8 Movement on DXA scans and analysis problems

Movement was also a problem for the neonatal DXA scans. Although the infant was swaddled and ideally asleep during the scan, random and unexpected movements did occur during the scanning process. The total body neonatal DXA scan takes about 6–10 minutes to complete and even a resting infant can move unpredictably during sleep. The scan was not attempted if the infant could not be pacified, but if the infant awoke during the examination then restful coaxing was employed to keep movement to a minimum. Once the total body scan had been completed and stored, any images containing blurred or missing data were excluded from the analysis. During analysis it was apparent that some scans had suffered from infant movement and would not provide an accurate reading of BA, BMD and BMC, so it was decided that two operators (PM and NH) would simultaneously evaluate the scans which showed movement and decide whether they were to be included or excluded. The encouraging results from the first 55 scans ensured the continuance of DXA data collection and with a larger number for analysis sex differences could be assessed.

2.9 Methods of statistical analysis to be employed

For the statistical analysis of the scan data, a specialist package was used. Originally this was STATA 8.0 (2004), but as analysis at later stages in the research was performed this was replaced by STATA 9.2 (STATA Statistical Software Release 9.2, 2006, STATA Corporation, College Station, Texas, USA). This allowed regression techniques to adjust for duration of gestation 19 and 34 week scans and at birth. All measurements were taken 3 times and the mean used for the analyses. When quoting statistically significant numbers a 'p' value of less than 0.05 was considered statistically significant and Pearson correlations were used to show the strength of the association, expressed as 'r' values. Paired *t- tests* were also employed for technique comparisons or to test the difference between two sets of values, normally distributed along the x-axis and these 't scores' were used to give an additional measure of the strength and direction of the associations.

To aid the speed and accuracy of measurement technique, a specialist package known as Sonoview® 2000 was used on a computer unconnected to the ultrasound system. The data was transferred to compact disks then onto the computer for storage and measurement at a later date.

The method for volume acquisition was suggested by the manufacturers of the KretzGE ultrasound system. However, this multi-slice technique, or *multi-planar mode*, has only been reported as useful for bone in Chang's study of the fetal humerus, (Chang *et al.,* 2003). By using it on the femur, the technique needed to be adapted to take into account the dense nature of bone at 34 weeks and the irregularities of the growing bone ends, something that is not so apparent in the humerus. Thigh volume, fat / skin volume, muscle volume and femur volume calculations were made using the 3D multi-plane facility, using 3 mm increments along the femur, muscle and thigh at 19 weeks, and 5 mm at 34 weeks. A larger number of slices gave a better result, although this increased processing time, as observed by others, (Farrell *et al.,* 2001; Nelson *et al.*, 2002). This was a painstaking technique, but gave reproducible results.

2.9.1 Reference population

The SWS cohort was used as it provided an already characterised set of recruits attending the SWS Ultrasound Unit for their pregnancy scans. This sampling may already have biases within the group as these women agreed to take part in an involved series of interviews, measurements, family data collection, blood tests and scans, devised for a nutrition study of women before and during pregnancy. Those who declined to take part in the survey may be in the group whose offspring could exhibit musculo-skeletal anomalies or a predisposition to impaired fetal development, which may be associated with a group of less motivated or nutritionally aware cohort of women. Some SWS studies have shown that individuals who have lower educational attainment are less likely to

eat a balanced 'prudent' diet, (Robinson *et al.,* 2004). The SWS cohort has been described in publications which suggest that the gamut of individuals contained within the group is representative of Southampton population, (Inskip *et al.,* 2005).

Standard European measurement charts were used for measurements at each scan and in particular femur charts were used to compare the range of sizes with the normal ranges devised by Chitty and Altman in 2002. Gestational age was plotted against femur length in the SWS cohort and the distribution was similar with the Chitty charts. This suggests that findings from the SWS cohort may be applicable to the general population.

2.10 Requirement to perform laboratory tests

After initial techniques were developed an *in vitro* system was designed, scanning with the same equipment that had been used on live participants. This aided testing for accuracy of volume acquisition and subsequent measurement generation using the stored volumes allowing reproducibility. Ultimately, this aided the recognition of reproducible core fetal measurements, their value and variation and assisted in confirming or refuting the preliminary studies.

Chapter 3 – *In Vitro* Evaluation of Techniques for Fetal Musculo-Skeletal Measurements

Introduction

One of the major aims of this study was to validate the techniques employed in the acquisition of volume data sets for reproducibility and also to validate measurement reproducibility of linear trace and volume dimensions taken on the stored digital 3DUS volumes. This validation has not been attempted to date in the published literature.

When performing an ultrasound examination, optimal equipment settings and standardisation of operation should provide a repeatable, reliable technique, which is important even for the most experienced technician. During the technique development stage of this study it was necessary to identify measurement variability, assess the degree of variability, and refine the technique so that reproducibility could be achieved. In this way any measurements found to be inconsistent could be excluded.

Each fetus and mother at scan, presented a variety of situations where there may have been more or less adipose tissue in the maternal abdomen, inaccessible position of the fetus, extreme fetal activity or great distance between the transducer and the ROI. All of these situations afforded opportunity to adapt acquisition technique and which affected image quality. It was therefore sensible to assess acquisition and measurement techniques using less variable circumstances, in a controlled way, so as to highlight the most or least effective variables, measurements and methods.

In Vitro Evaluation

It was decided that performing acquisition and measurements in a laboratory situation (*in vitro)* using the same equipment that would be used on the SWS cohort, would help to assess measurement accuracy, variability and reproducibility. These exact same tests would not be possible *in vivo*, but they would be useful in explaining discrepancies. It was decided that by using a test-tank containing rods of steel and Perspex, and using fresh animal bone within the tank, scan acquisition using the KretzGE ultrasound system would be possible and measurement on the stored volumes tested. This testing would aid with the accuracy and reproducibility of the technique.

3.1 Rationale for evaluation

It was apparent that there was great variation in some of the measurements obtained in the first period of scanning, particularly those taken on the reconstructed planes. These were more difficult to achieve, more demanding to maintain consistency and if used for research, their validity should be questioned. The system's performance was checked by the manufacturers to ensure its integrity and further evaluation by phantom testing was considered. Commercial phantoms are very expensive and not designed for research purposes, and so a less elaborate but more specific device was constructed comprising organic material to mimic bone.

3.2 Test equipment production

In commercial phantoms the test objects such as pins and rods are embedded in a gel or solution that has the same acoustic properties as mammalian tissue. It was not possible to create a phantom matching the human uterine environment so a test-tank filled with water, containing rods and bones of varying proportions, was devised for this experiment. Ultrasound has an acoustic velocity of 1540 m/s when travelling through mammalian tissue. To mimic tissue the test-tank was filled with filtered water maintained at 24°C, which gave an acoustic speed of 1485.4 m/s. This was the closest speed achievable *in vitro* and has been authenticated in published phantom studies, (Duck, 1990; Lubbers and Graaf, 1998; Berg *et al.,* 2000; Dudley *et al.*, 2002). PM adopted this method as a fetal core temperature, normally of 37°C, could not be achieved or maintained in the scan room.

The Medical Physics and Bioengineering Department of Southampton General Hospital (SGH), manufactured a test-tank from 1 cm sheets of Perspex, riveted together and sealed with silicone gel at the seams to produce a water-tight open-topped box, (Figure 3.1). A square 'port' was cut into one side, sealed with a clear plastic membrane, against which the transducer face was placed. The box was lined with a soundproofing layer to absorb scattered ultrasound. This sealed tank was then filled with water and left to settle with a long mercury thermometer secured into one of the corners. Ceramic holders were place at the side margins of the tank away from the port opening, between which the rods and bones were suspended. Distances in cm were marked along the sides of the tank away from the side containing the port, so that accurate placement of the rods was possible. The open top of the tank was covered with a cloth to prevent dust falling into the water and to prevent turbulence from the air-conditioning unit from rippling the water. The air-conditioner was set to 24°C to maintain a constant temperature in the room and once dust and air bubbles had settled and the thermometer in the water was reading a constant temperature then testing commenced.

Figure 3.1: Ultrasound test-tank. This consists of a Perspex water bath into which water and rods or bones can be placed then scanned through the side port. The pink tank lining is a sound absorbing medium.

3.3 Test 1 and Test 2: Rods and bones

For Test 1, 6 rolled steel rods and 2 of extruded Perspex, (various diameters) were measured in diameter using calibrated electronic callipers, (Figure 3.2). The mid-point of each rod was marked so that measurements were taken in the same place each time. Additionally, 2 fresh fetal lamb femora were acquired from the Biological Research Facility (SGH). These were excised at post-mortem, from Welsh Mountain Sheep fetuses at 123 days of gestation. At this stage the femora were approximately 6–7 cm in length, comparable in size to a human 34-week fetal femur. These bones were bleached and frozen at –20°C. Additionally, 4 lamb femora were commercially obtained from 6-month-old lambs, which were slaughtered 5 days prior to collection and kept frozen at –20°C. The bones were thawed at room temperature for 6 hours prior to testing. Fresh bone was used in this experiment, as the acoustic velocity of ultrasound in fresh bone is closer to bone *in utero* and the velocity of ultrasound is approximately 10% higher if the bone is dry, (Lang, 1970; Duck, 1990).

Figure 3.2: White Perspex and steel rods used in the test-tank. N.B. the variety of diameters.

The narrowest diameter steel rod was submerged in the water, suspended on ceramic holders 10 cm from the scanning port. Coupling gel was applied to the transducer, which was then held against the port, (Figure 3.3). Once the water had settled a 3D sweep was made, using the settings for obstetric scanning, with the rod in the middle of the ROI.

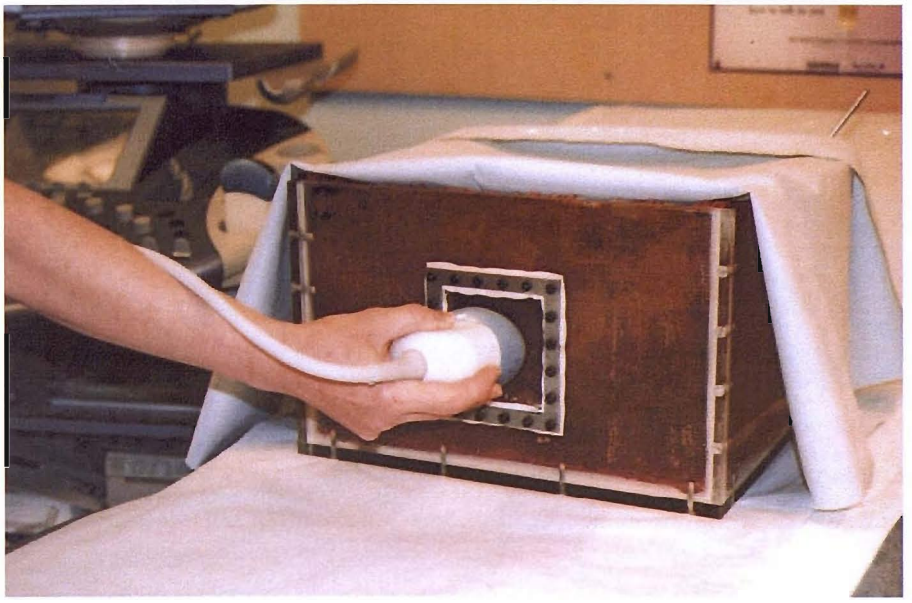


Figure 3.3: The 3D transducer held on the scanning port of the test-tank. The tank is covered to prevent dust falling in. The temperature is monitored by a thermometer (right far corner).

The images were frozen on-screen and stored on the system's hard-drive for measurement later, (Figures 3.4 and 3.5). The experiment was then repeated with the bones, resting on a 2 cm aqueous gel pad within the water bath, as the ceramic holders interfered with the scan image. Care was taken to maintain the bones in the correct anatomical orientation in which they had been measured at the mid-shaft point.

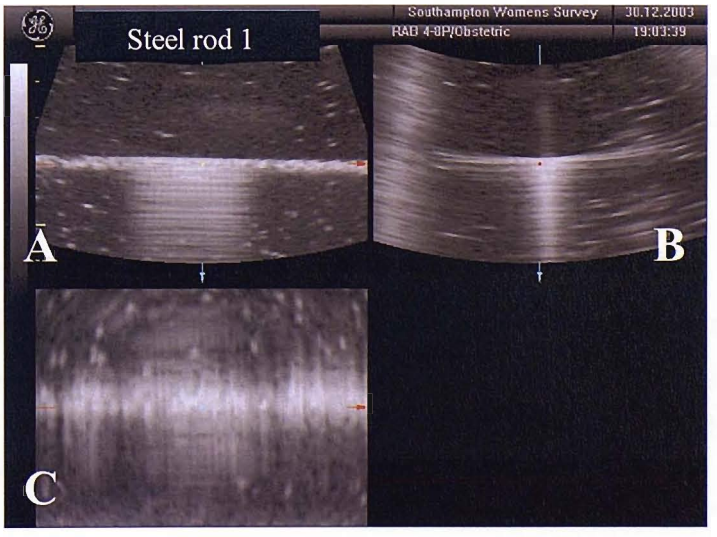


Figure 3.4: Steel rod 1 on the stored image. Note the noisy image produced by the dense steel.

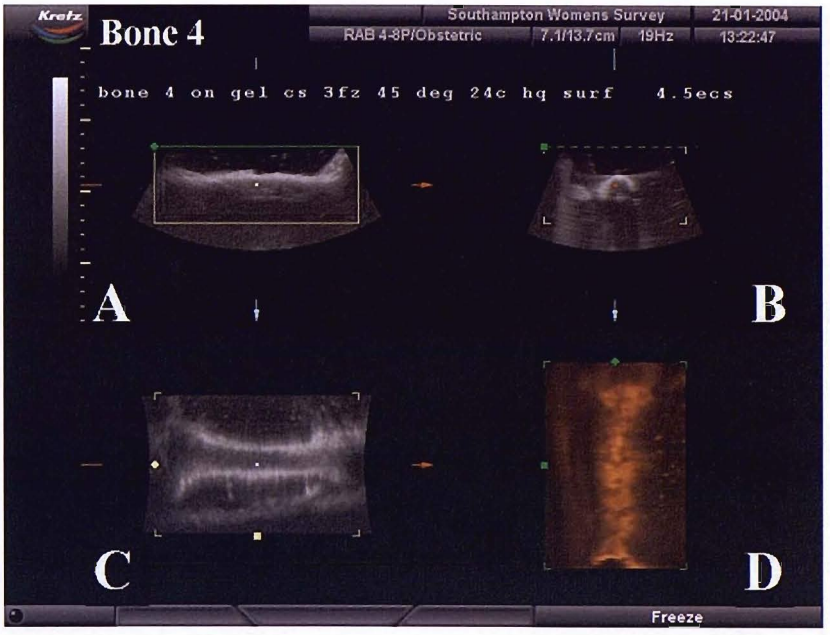


Figure 3.5: Bone 4 on the stored image. A fetal sheep bone suspended in water in the test-tank. Small echoes seen within the water represent gas and particles, most noticeable on the surface-rendered plane D.

After all of the rods and bones had been scanned, the volumes were stored onto the system's hard-drive for evaluation at a later stage. Different settings were used to obtain the best image and all of these volumes were stored. The test-tank was emptied and all equipment and surfaces were treated with disinfectant. The cleaned, dried, labelled and bagged bones were returned to the freezer, ensuring each bag was labelled clearly and sealed. Three weeks later, the test was repeated so technique and repeatability could be evaluated; this second testing was called Test 2.

3.4 Test 3: String tests for axial and lateral resolution

It was noted that on all of the images, the reconstructed C plane contained images of poorest resolution. To assess whether these discrepancies were inherent with the machine, measurement or material dependent, 'string tests' were performed using a Perspex and nylon line matrix manufactured by the Department of Medical Physics and Bioengineering (SGH), (Figure 3.6). The nylon threads were positioned 26 mm apart in horizontal and vertical directions. This was checked with electronic callipers prior to commencing this test. The test-tank was set up as previously described but without ceramic or gel holders. The matrix was submerged in the water and positioned on a side edge on the floor of the test-tank, with the Perspex face-plate parallel to the side of the tank containing the scan window.

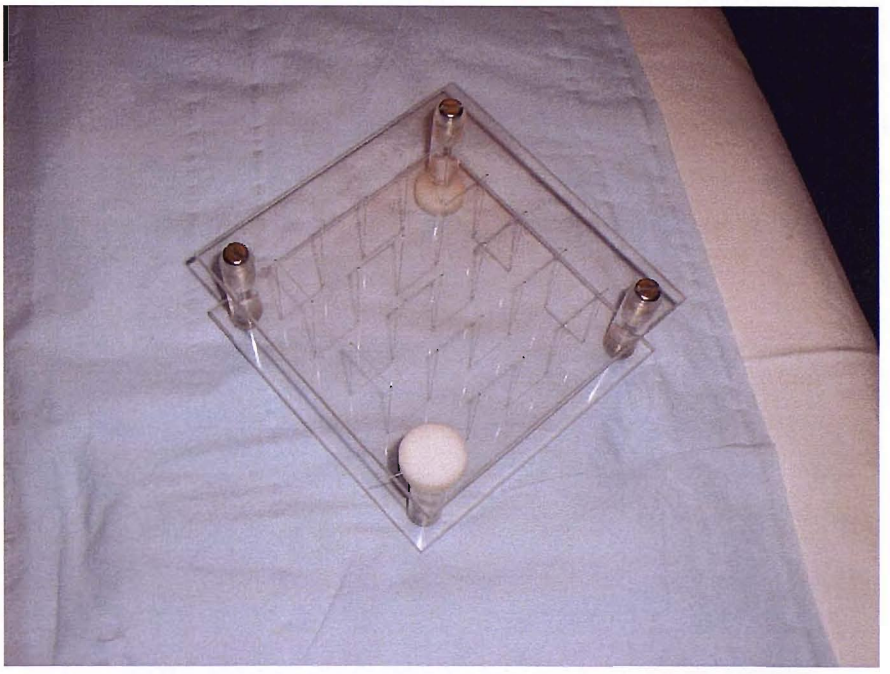


Figure 3.6: String matrix with nylon threads 26 mm apart in both horizontal and vertical directions.

By submerging this matrix into the test-tank, the nylon threads are seen as a series of dots when scanned, (Figure 3.7). For the purpose of this test the matrix was positioned 5 cm from the port face.

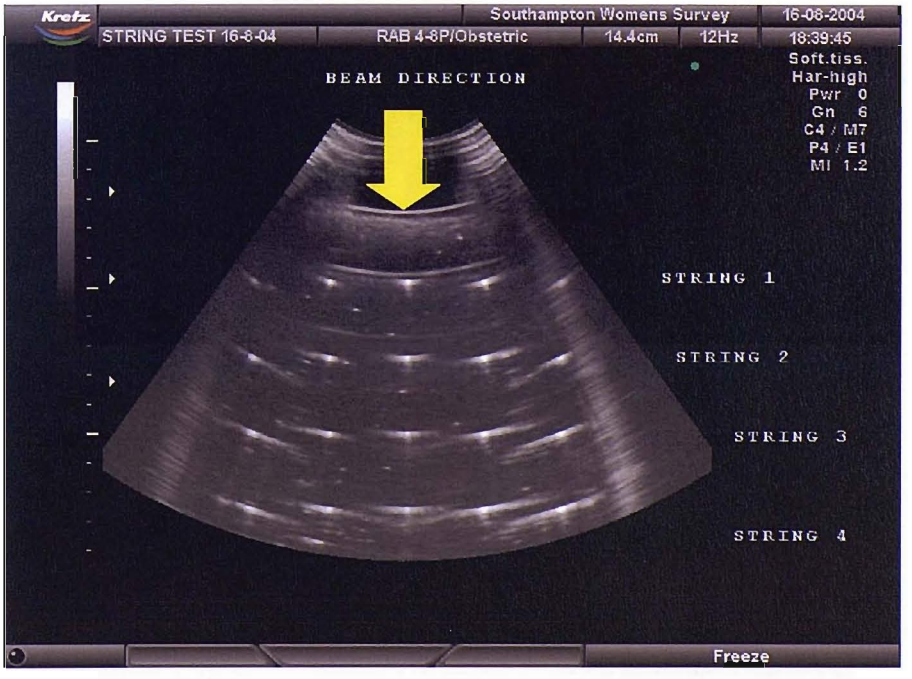


Figure 3.7: The string matrix on a scan image. The threads end-on showing as dots.

The 2D ultrasound images were frozen on-screen and measurements taken by electronic callipers, which were then compared with actual known distances. Measurements were made vertically on stored image in the *axial* direction, which is parallel to the direction of wave-front propagation, and in the *lateral plane*, perpendicular to the wave-front, to test if calliper readings were affected by the distance from the central axis of the beam, or distance from the transducer, (Figures 3.8 and 3.9).

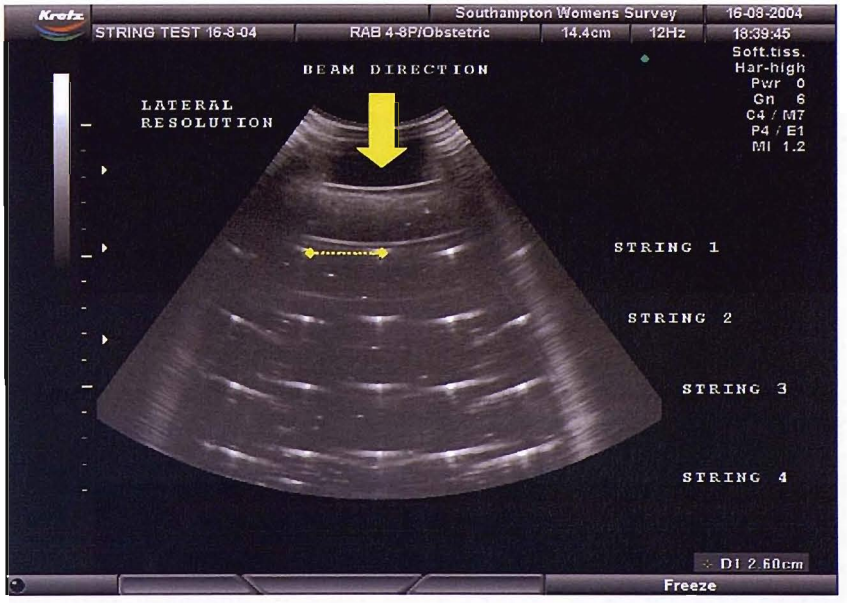


Figure 3.8: Lateral resolution: measurements taken perpendicular to the direction of the beam.

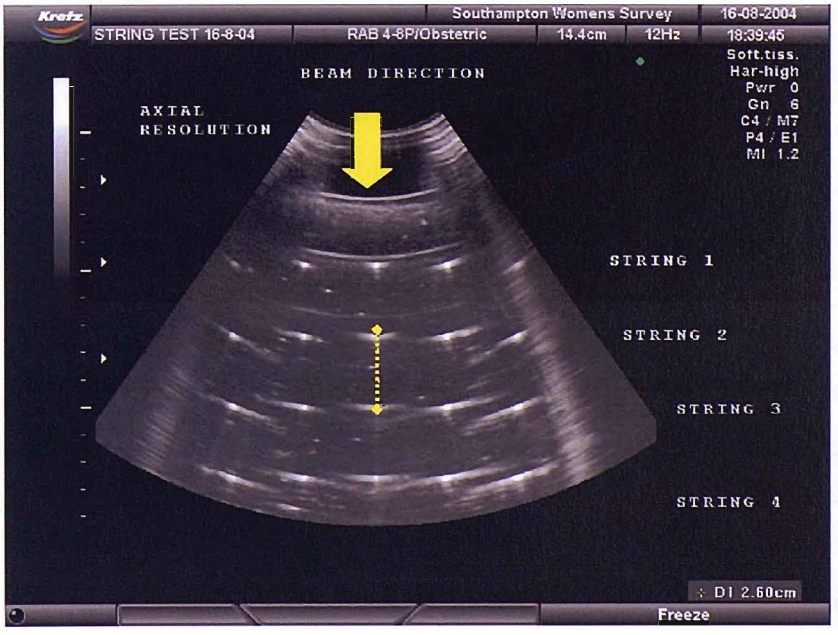


Figure 3.9: Axial resolution: measurements taken parallel to the direction of the beam.

3.5 Test 4: Distance tests for resolution

The reconstructed C plane images were poor in resolution on screen, the resolution becoming worse if the target image was in the far field of the ROI. A Perspex rod with an 8 mm diameter was suspended in the water-bath, parallel to the port. 3D volume acquisitions were taken with the rod at 5 cm, 10 cm, 12 cm and 15 cm from the transducer to test if the resolution decreased with distance, (Figure 3.10). On-screen measurements were then taken from the resultant images from the C plane.

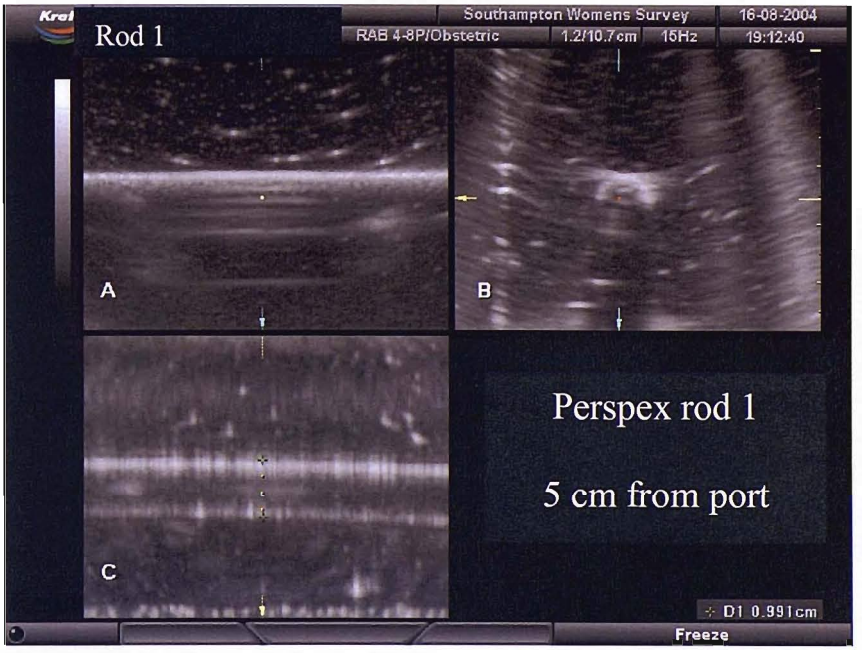


Figure 3.10: A Perspex rod of 8 mm diameter, 5 cm from the port; scan appearances.

3.6 Results and discussion

There were limitations in this period of testing as bone volumes could not be derived using the test tank. With there being a greater discrepancy between bone and water than would be experienced *in vivo* with bone and soft tissue, the increased shadowing and flaring made it impossible to visualise the entire length of the bones being used. This was especially apparent in the larger bones which contained more mineral content. By the time lambs are 6 months old, the metaphyseal ends have expanded greatly, forming the articulations of the hip and knee and these are portions are very dense, hence the accelerated artefact. At this large size it was also impossible to include the entire bone in the ROI of the scan field. However, linear and trace measurements were possible and each measurement was taken 3 times with the mean value of the triplet being used in the analyses.

3.6.1 Rods and bones

The first experiment using rods and bones (Test 1) within the test tank involved measuring each material at its mid-point with electronic callipers, before the rod or bone was scanned and measurements taken on-screen at the same point. A triplet of measurements was taken each time. Test 2 repeated Test 1, performed 3 weeks later to assess reproducibility; the results from both tests are shown below in Table 3.1.

TEST 1 (cm)						
	Measured by callipers TS	Measured from scan image TS	% difference between calliper and scan TS	Measured by callipers in AP	Measured from scan image AP	% difference between calliper and scan AP
steel rod 1	0.316	0.338	7.0	0.316	*	
steel rod 2	0.395	0.358	–9.34	0.395	*	
steel rod 3	0.475	0.437	–8.0	0.475	*	
steel rod 4	0.632	0.534	–15.5	0.632	1.031	63.1
steel rod 5	0.95	0.777	–18.2	0.95	1.767	86.0
steel rod 6	1.268	0.867	–31.6	1.268	1.76	38.8
Perspex rod 1	0.802	0.555	–30.8	0.802	1.06	32.2
Perspex rod 2	2.246	1.33	–40.8	2.246	2.63	17.1
bone 1	0.914	0.616	–32.6	0.895	1.09	21.8
bone 2	0.87	0.622	–28.5	0.868	0.954	9.9
bone 3	1.7	1.7	0.00	1.786	2.12	18.7
bone 4	1.865	1.61	–13.7	1.868	2.23	19.4
bone 5	2.046	1.85	–9.9	2.145	2.74	27.7
bone 6	2.277	2.01	–11.7	2.105	2.4	14.0

TEST 2 (cm)						
	Measured by callipers TS	Measured from scan image TS	% difference between calliper and scan TS	Measured by callipers AP	Measured from scan image AP	% difference between calliper and scan AP
steel rod 1	0.316	0.326	3.2	0.316	1.058	234.8
steel rod 2	0.395	0.387	–2.0	0.395	*	
steel rod 3	0.475	0.495	4.2	0.475	0.663	39.6
steel rod 4	0.632	0.46	–27.2	0.632	*	
steel rod 5	0.95	0.681	–28.3	0.95	1.34	41.1
steel rod 6	1.268	0.833	–34.3	1.268	1.22	–3.8
Perspex rod 1	0.802	0.611	–23.8	0.802	1.3	62.1
Perspex rod 2	2.246	1.34	–40.3	2.246	2.39	6.4
bone 1	0.863	0.422	–51.1	0.849	1.43	68.4
bone 2	0.857	0.823	–4.0	0.855	1.14	33.3
bone 3	1.76	1.73	–1.7	1.846	2.28	23.5
bone 4	1.7	1.69	–0.6	1.85	2.11	14.1
bone 5	1.99	1.82	–8.5	2.158	2.45	13.5
bone 6	2.013	1.94	–3.6	2.082	2.45	17.7

*Table 3.1: Results of Test 1 and Test 2; mean diameters of rods and bones. N.B. * suboptimal views.*

Test 2 recreated the original trial and verified the intra-operator variability within the measurements taken, (4.6% SD for linear measurements). This revealed the technique was reproducible, but the measurements were variable, especially in the denser rods and bones. In both Test 1 and Test 2 volume measurements of the shorter bones and rods were attempted, but due to the dense nature of the materials used, intense echoic boundaries and loss of detail in shadowing made it impossible to place the callipers accurately.

In the transverse plane (measured as plane A) the measurements from scan were systematically smaller than actual values, (Figure 3.11). This phenomenon results from the wave front striking the rod or bone directly at 90°, encouraging maximum reflection to the transducer, high speed refraction and scatter of the ongoing beam, which in turn leads to a loss of information in the image of furthermost edge of the bone or rod. Thus on-screen, these denser materials displayed bright edges where the ultrasound beam initially contacts, with little of the beam transmitting through. This foreshortens the structure making it difficult to place the callipers accurately when measuring on the frozen image. The overall effect is to create smaller than actual size measurements. To quantify this, the percentage difference between the calliper measurements and the scan measurements was examined, (Table 3.2). It can be seen that in the TS plane in Test 1 all of the scan values were consistently smaller than the calliper measurements, denoted by the minus sign before each percentage. For the combined materials the mean decrease in the measurements taken was –15.0% in Test 1 and –12.1% in Test 2.

Percentage difference in measurement between calliper and scan				
	TS		**AP**	
	Test 1	**Test 2**	**Test 1**	**Test 2**
Steel rods	–12.6	–13.4	62.6	77.9
Perspex rods	–15.4	–11.9	16.1	31.1
Fetal lamb bones	–20.4	–18.9	16.8	41.7
Juvenile lamb bones	–11.8	–4.2	20.4	15.1
Combined materials	–15.0	–12.1	29.0	41.5

Table 3.2: Measurement differences between calliper and scan dimensions (in %); TS and AP planes for both Test1 and Test 2.

Conversely the AP measurements on-screen in the C plane in both tests were systematically greater than those taken with callipers; 29% increase in the combined materials for Test 1 and 41.5% for Test 2. This was mainly due to the poor image quality displayed in the reconstructed C plane, which made calliper placement difficult. From the above table it can be seen that steel rods gave the greatest mean percentage increase in scan measurement as there was significant scattering artefact produced from the ultrasound striking such a dense material. This scatter degraded the

image to an extent where the noise levels made the image difficult to resolve. Thus many measurements were overestimated. Perspex rods and fetal lamb bones revealed similar results in Test 1 (16.1% and 16.8% respectively).

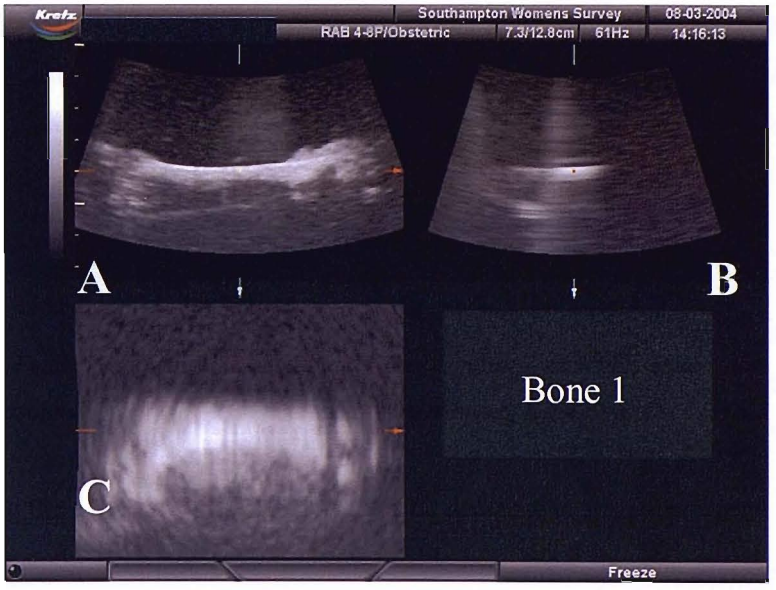


Figure 3.11: Fetal sheep femur in the test-tank. This demonstrates highly echoic bone edge where the primary ultrasound beam strikes. Additionally there is loss of definition of the far side of the bone in shadow.

By repeating the experiment the same systematic decrease in scan measurements in the AP plane and increase in the TS plane was observed, (Table 3.2). What was also noted was the discrepancy in the measurement results from one test to another. The in-between measurement variability within the measurement triplets had been seen to be small, but there was variation in the mean values from Test 1 to Test 2. On further inspection it was seen that with the exception of the steel rods, the remainder of the measurements, although systematically smaller, were closer to the measurements taken with callipers, bringing the combined materials mean to –12.1% from –15.0%. In the AP plane the scan measurements, with the exception of the juvenile lamb bones, were still consistently greater than those taken by calliper and this difference increased from Test 1 to Test 2, (29.0% to 41.5%).

It can be speculated that measurement accuracy improved in the TS plane as a result of technique familiarity on the part of the operator. The randomness of the AP measurement results may be explained by degradation of the organic materials used by prolonged freezing at –20°C between tests or by the unpredictable nature of ultrasound in dense materials as reported by other researchers, (Goldstein, 2000a). The reconstruction process involved with the C plane probably added to the image degradation, which in turn made calliper placement on-screen inaccurate.

3.6.2 String tests for axial and lateral resolution

Test 3 (string test) showed that on-screen, axial and lateral measurements on non-reconstructed planes, showed minimal discrepancy, (Table 3.3). In the near field, both in the centre and at the adjacent row of threads either side matched 26mm. In the far field (10–15cm from the transducer), both lateral and axial measurements were slightly larger (1–2%). This indicated that the ultrasound system measurement was accurate in 2D, especially in the axial direction; parallel to the direction of beam propagation.

The small degree of measurement discrepancy in the far field may partly be due to the width of the beam, which is wider at depth within the image. This affects lateral resolution, which deteriorates with distance from beam source, (Dudley *et al.*, 1998). Thus, measurements taken beyond optimal focal depth (around 10–12 cm for this system, comparable to string 3) will be greater. In this trial the measurements were 4% greater than actual size in the far field.

TEST 3: Raw data (mm)					
Axial resolution test		scan 1	scan 2	scan 3	mean
Central row	vertical strings 1 to 2	26	26	26	26.0
	vertical strings 2 to 3	26	26	26	26.0
	vertical strings 3 to 4	26.4	25.6	26	26.0
Row to left of centre	vertical strings 1 to 2	26.4	26	26	26.1
	vertical strings 2 to 3	26	26	26	26.0
	vertical strings 3 to 4	26	26	26	26.0
Lateral resolution test		scan 1	scan 2	scan 3	mean
Left to centre row	horizontal on string 1	26	26	26	26.0
	horizontal on string 2	26.4	26.4	26	26.3
	horizontal on string 3	26.8	26.4	26.4	26.5
	horizontal on string 4	27.2	26.8	26.8	26.9

Table 3.2: The string test raw data for axial and lateral resolution. Each value is the mean of 3 measurement triplets. Horizontal strings 1, 2, 3 and 4 were a, b, c and d distance (in cm) from the transducer, respectively.

Test 4 was performed to ascertain if there was any degeneration of resolution in the image at distance from the transducer.

3.6.3 Distance tests for resolution

In the reconstructed planes, all linear measurements were systematically larger than actual size, (Table 3.3). It was shown in Test 3 that the system measures accurately, so the discrepancies must have occurred during the reconstruction process. The virtual cuboid of acquired volume data will not be strictly uniform in shape, but 'wedged' from near to far field, side to side and front to back, with the voxels furthermost from the transducer being larger than those closest in the near field, (Figure 3.12). This has the effect of distorting the image further away from the transducer. When the data are reconstructed, the edge boundaries are 'smeared' which can appear to enlarge the object on screen and makes calliper placement difficult, as the operator has to decide where the boundaries actually lie. The human eye compounds this by over-estimating where the boundary sits and so callipers may be placed several pixels distant from the correct location.

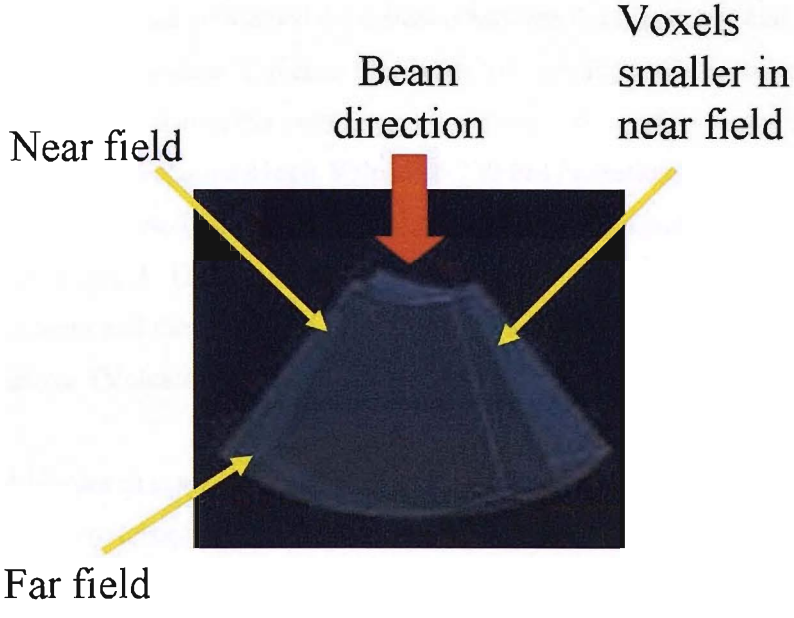


Figure 3.12: Virtual volume of 3D ultrasound acquisition data.

In practice, the further away from the transducer the target was placed, the greater the degree of distortion, leading to greater measurement size. In obstetrics, the best images are taken in the near field, with the transducer approximately 5 to 10 cm from the fetus. Table 3.4 shows that even at 5 cm with this test, there was a 22% increase in the on-screen measurement compared with the known size. This test used a Perspex rod so that there would be less refraction, flare and shadowing in the image than if a steel rod or dense bone was used. The rod was placed at different distances from the transducer and it can be seen that at 12 cm, a 78% increase in measurement was produced, which was unacceptable.

TEST 4: Distance test for resolution (cm)		
Distance from transducer	Perpsex rod diameter	Measured - C plane
5	0.803	0.985 (+22%)
10	0.803	1.183 (+47%)
12	0.803	1.430 (+78%)
15	0.803	Sub-optimal

Table 3.4: Distance test for resolution using a Perspex rod. Mean values (cm) used.

Discussion of *In Vitro* Results

The test-tank produced was basic in design, but it exposed inaccuracies when volume data sets were reconstructed. The tests proved that a repeatable technique had been achieved, but measurement discrepancies arose from inherent failings within the reconstruction process. The major cause of poor resolution concerned the distance between the transducer and target, predominantly in the reconstructed C plane. This impacted on calliper placement, influencing variability and inaccuracies within the measurements obtained. At the time of testing, the system's manufacturers (KretzGE) recognised their Voluson® 730 had limitations in its imaging capabilities. They agreed resolution in the reconstructed plane C was considerably lower than that of the primary scan plane A. The system's resolution was particularly poor for low-contrast targets in reconstructed planes and these images also contained significant system artefacts parallel to the face of the transducer, (Voluson® 730 Operation Manual).

Discrepancies also arose in scaling, as the actual volume of data acquired was not an exact cuboid matrix but comprised voxels of smaller near-field elements and larger far-field data that were not cuboid in shape. Thus measurements on reconstructed planes at lower locations in the reconstructed C plane image are not useful for calculating 'real' measurements of the target. As the degree of discrepancy in the C plane was so great it was decided that FPAP, FDAP and FMSAP should be abandoned for the remainder of the analysis, but a small trial of these measurements would attempted *in vivo* to confirm the lack of reliability. Discarding these measurements was disappointing as the C plane lies 90° to the longitudinal aspect of the femur and thigh, and in practical terms this excludes measurements made in the coronal aspect, a plane not easily achievable by free-hand scanning. From these results, it was clear that measurements taken on the reconstructed planes, especially the coronal / horizontal C plane should not be used unless the ROI can be recreated at an exact depth each time and measurement correction applied. In practical terms this was not possible. To verify whether these features affected scanning in the clinical situation, similar tests were then performed using living subjects. It was therefore necessary to commence data collection of measurements *in vivo* using SWS participants.

Chapter 4 – *In Vivo* Evaluation of Techniques for Fetal Musculo-Skeletal Measurements

Introduction

The accuracy of acquisition and measurement technique had been addressed *in vitro* and it was therefore necessary to assess the precision of these new techniques in the clinical setting, where the measurements could be used to ascertain biological differences between fetuses. Practical issues, such as movement elimination whilst taking a volume acquisition, were assessed, together with placement of the transducer over the area of interest for the best image quality. Angle of sweep, time of sweep, resting state of the fetus, depth and position of focus, image quality settings, maternal position and breathing and the taking of multiple sweeps on a variety of different settings were varied to gain experience and hone technique for volume acquisition.

In Vivo Evaluation

Once satisfactory settings and techniques had been attained, the examination of measurement reproducibility on volume images taken on living fetuses was considered. New difficulties arose when volume measurements were attempted. Bone and soft tissue have very different appearances on ultrasound images and it was decided that measurement variability should take into account which tissues were being scrutinised.

4.1 Initial trials

The first 70 recruited pregnancies had 3DUS volume data acquired from their scans and triplets of measurements taken from the frozen stored images of all 15 core parameters in the uppermost fetal thigh. Additionally, the first 29 fetuses had length and volume measurements of both femora at 19 weeks. Paired t-tests showed there was no significant difference between the dimensions of either left or right femur ($p = 0.42$, $t = 0.84$ for length and $p = 0.41$, $t = 0.93$ for volume). Thereafter, only the most easily accessible femur, lying perpendicular to the long axis of the ultrasound beam was used.

To ensure the study group was representative of the population from which it was selected femur length was plotted against gestational age and the results compared with well-documented charts used in fetal biometry. At 19 weeks there was significant correlation between femur length and gestational age ($p<0.001$, $r = 0.12$). At 34 weeks the correlation was not as significant, but this was comparable to a prominent UK study, where it was weakly

correlated in late gestation (p = 0.27, r = 0.06), (Chitty *et al.*, 2002). Collection of SWS recruitment continued and once 130 sets of scans at both 19 and 34 weeks had been obtained, intra-operator measurement variability was tested on trace, linear and volume measurements.

4.2 Intra-operator measurement variability

Measurement variability within the triplets of measurements taken was explored. Linear, trace and volume measurements were assessed after the first 130 subjects had been recruited and the results are tabulated below:

Intra-operator variability– Analysis of first 130 of each type of measurement			
	Core measurement	Standard deviation (SD)	n
Between linear measurement triplets			
19 weeks	Femur length	0.015	*130*
34 weeks	Femur length	0.014	*130*
Between trace measurement triplets			
19 weeks	Mid-shaft CSA	0.005	*130*
34 weeks	Mid-shaft CSA	0.014	*130*
Between volume measurement triplets			
19 weeks	Femur volume	0.008	*130*
34 weeks	Femur volume	0.046	*130*

Table 4.1: Tabulation showing the results of intra-operator variability; linear, trace and volume measurements for the first 130 subjects.

The difference in measurement of linear measurement (femur length) was similar at 19 and 34 weeks (SD of 0.15 and 0.14 respectively). However, traced measurements were liable to suffer from intra-operator variability and so mid-shaft CSA and volume measurements constructed from multiple traces, showed a greater range of intra-operator variability. At 19 weeks the SD of measurement for mid-shaft CSA was 0.005, which rose to 0.14 for the 34 week traces. As larger areas require greater distances to be traced, greater variation was produced at 34 weeks. The femur volume measurements showed a larger variation than the trace and linear measurements, with an SD of 0.008 at 19 weeks, which rose to 0.46 at 34 weeks. This was not surprising, especially in bone, as there was more difficulty in placing callipers on bone edges than on soft tissue boundaries in late gestation, when there has been increased bone mineral accrual. This measurement was more variable because each slice was being traced around and the total

volume calculation was constructed from 15–20 slices, the software adding the slices and intervening volumes together for the final total. The results of these first analyses of intra-operator variability suggested a reproducible technique *in vivo* for linear, trace and volume measurements had been developed for bone in the first sample of subjects.

4.3 Between-subject measurement variation

After intra-operator reproducibility had been tested, it was necessary to ascertain if the techniques were sensitive enough to highlight differences between subjects. Analysis of the first 130 SWS subjects who had scans at both 19 and 34 weeks was performed. The mean of each scan triplet of measurements for femur length, mid-shaft CSA and femur volume was taken and the between-subject SD was calculated. The results are shown in Table 4.2 below.

Between-subject variation – Analysis of first 130 subjects			
	Core measurement	Standard deviation (SD)	n
Between-subject linear measurement variation			
19 weeks	Femur length	0.221	*130*
34 weeks	Femur length	0.284	*130*
Between-subject trace measurement variation			
19 weeks	Mid-shaft CSA	0.056	*130*
34 weeks	Mid-shaft CSA	0.214	*130*
Between-subject volume measurement variation			
19 weeks	Femur volume	0.12	*130*
34 weeks	Femur volume	0.57	*130*

Table 4.2: Tabulation showing the results of intra-operator variability; linear, trace and volume measurements for the first 130 subjects.

From Table 4.2 it can be seen that femur length measurements taken showed a between-subject SD of 0.221 at 19 weeks, which demonstrated a greater variation in measurement between subjects than produced in intra-operator studies; which was 0.015. This suggests that the biological variation between fetuses was greater than the measurement error variation within individual fetuses. The between-subject variation for femur length showed a between-subject SD of 0.284 at 34 weeks, which again indicated a wider range of biological differences than at 19 weeks, as would be expected. For trace measurement mid-shaft CSA the pattern was similar

with an SD of 0.056 at 19 weeks, rising to 0.214 at 34 weeks. Similarly for femur volume the SD was 0.12 at 19 weeks and 0.57 at 34 weeks. These indicated a greater biological difference between each fetus, the range being greater at 34 weeks when there was a greater spread of fetal size. Measurement error in relation to biological variability in the full sample of 517 fetuses is considered further in Chapter 5 – Results 1, including coefficients of variation.

The fetal size differences seen here can be compared to well-documented charts for estimated fetal weight using scan measurements. The standard obstetric ultrasound charts used for Caucasians incorporated into the software of many European ultrasound machines show that at 19 weeks the range of expected weights within between the 5th to 95th centile range is 266 to 348g; a variation of 82g, whereas at 34 weeks the range is between 1963 and 1655g; a variation of 692g, (Chitty *et al.*, 2002).

4.4 Initial analysis of fetal variables

Since it had been established that acquisition and techniques were reproducible and accurate, an initial batch of analysis was considered using fetal variables collected using the methods already described. One of the core aims of the study was to investigate fetal size, growth and body composition using pre-natal measurements and so it was necessary to test the techniques acquired. The initial trial utilised the first 134 SWS babies to be delivered, so their birthweights would be available. The correlations of thigh volumes and birthweights are tabulated below:

Association of thigh volume and birthweight – First analysis				
		p	r	n
19 weeks	boys	**<0.001**	**0.21**	*63*
	girls	**0.002**	**0.44**	*66*
34 weeks	boys	**<0.001**	**0.60**	*59*
	girls	**<0.001**	**0.43**	*60*

Table 4.3: Associations between thigh volumes and birthweight.

From Table 4.3 it can be seen that thigh volume showed a strong association with birthweight at 19 weeks, the effect being stronger in boys ($p < 0.001$, $r = 0.21$) than in girls ($p = 0.002$, $r = 0.44$). The association remained in both sexes at 34 weeks ($p < 0.001$), with the strength of the association still being stronger in boys. This predictive association was also seen in the thigh volume study of Chang and colleagues (2003) which supports the results obtained here. With these results to hand, data collection continued and analysis of other variables commenced,

where it was seen that femur volume and birthweight in both sexes showed no significant association ($p = 0.1$ at 19 weeks and 0.27 at 34 weeks). Thigh volume and MTCSA measurements were associated, as expected for both sexes at 19 and 34 weeks ($p < 0.001$ for both gestations). Also femur length and mid-shaft CSA were significantly associated for both sexes at both gestations ($p < 0.001$).

Statistical power calculations were performed to derive a minimum number of subjects to be scanned for the best analysis. A figure of 280 subjects was found to be sufficient. However, this figure was derived at the commencement of data collection, before it was deemed necessary to analyse data within boys and girls separately. It was therefore decided that a minimum of 200 boys and 200 girls would be needed for analysis to strengthen the power of the study and it was deemed feasible that this many recruits could be brought into the study over the following year.

Discussion of *In Vivo* Results

After reviewing the stored scans, it was decided that some of the earlier scan volume acquisitions should be revisited, as scanning and measurement technique been developed and adjusted. Some images were found to be sub-optimal because of incorrectly set TGC or contrast and were subsequently dropped if the image quality could not be improved in the post-processing part of the analysis. Some measurements taken on smaller structures such as mid-shaft CSA and MSST, because of their size, were difficult to perform. By expanding the B plane on which they were taken, to the full size of the screen, calliper placement became easier and so more of these measurements could be included.

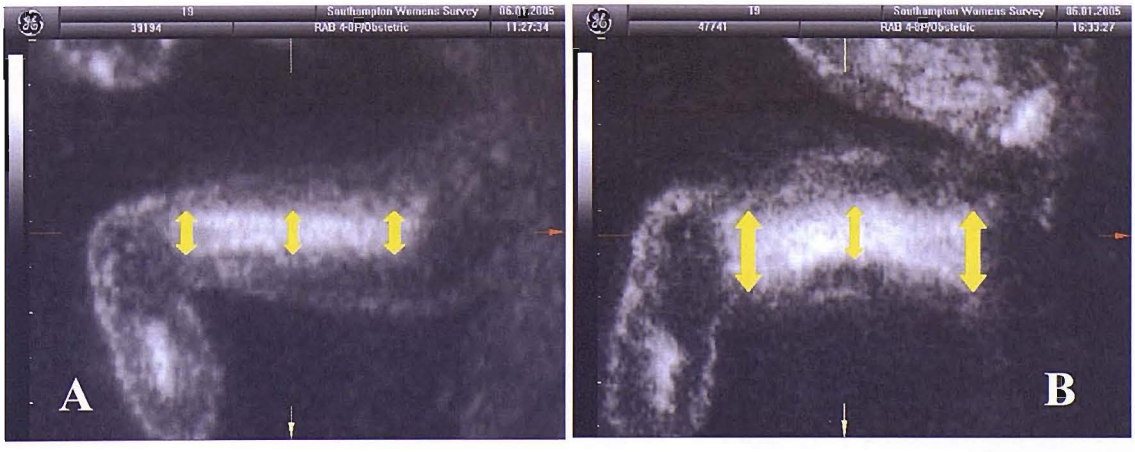


Figure 4.1: Scan images of the fetal femur at 19 weeks. The usual parallel-sided femur (A) and the occasional 'splayed' ends of the femur (B). AP measurements (in yellow) show difference in size between the metaphyseal ends and the mid-shaft region; this is more pronounced in B.

It was noticed that proximal, mid-shaft and distal shaft diameters were significantly larger in AP (front to back) direction than transverse diameter (side to side) ($p < 0.001$). This may have been reflection of anatomical fact, as electronic calliper measurements taken on the sheep femur confirmed an actual increase in AP dimension with regard to the TS diameter. Additionally, from post-mortem and archaeological specimens it has been seen in human fetal bones, (author's personal observation; Scheuer and Black, 2000, p383). It was noticed that in some fetuses the difference between the mid-shaft AP dimension and the metaphyseal end AP dimension was more pronounced, as shown in Figure 4.1.

Compounding this effect it was clear from the evidence of the *in vitro* test-tank experiments, measurements taken in the AP plane viewed on the C plane were systematically larger. The C plane was known to have inherent problems with scaling, resolution and focal distance. The AP dimensions of the proximal and distal femoral metaphyseal ends (FPAP and FDAP) were also difficult to measure accurately as again these were visualised on the C plane, formed from reconstructed data.

Tracing around the shaft CSA was more consistent and an initial plot of these values showed that at 19 and 34 weeks, the proximal CSA was usually greater than distal CSA. The difference was greater at 34 weeks as the femur is developing its secondary ossification centre in preparation for the complex construction of the proximal femur and hip joint.

At 19 weeks it appeared usual for the proximal CSA to be equal to or larger than the distal CSA. However, 15% of the femora at 34 weeks included in this analysis showed an equal to larger distal CSA which did not fit the anticipated pattern. To examine this further both proximal and distal CSA measurements were plotted against each other and the results are shown in Figure 4.2. This scatter plot shows proximal CSA plotted against distal CSA for each subject. If the values were equal then the point lies on the *line of equality* drawn through the cluster of points. If the proximal CSA was greater than distal, then the point lies below the line of equality. It can be seen from the plots that the majority of subjects had a proximal CSA greater than their distal CSA. Those with a greater distal CSA than proximal CSA may be individuals showing an anatomical variation, or as hypothesised here, may be fetuses of mothers whose circulating vitamin D was decreased, causing an abnormality of bone development similar to that seen in rickets. Insufficiency of vitamin D results in increased metaphyseal CSA in long bones and can be detected on radiographs, (Figure 4.3).

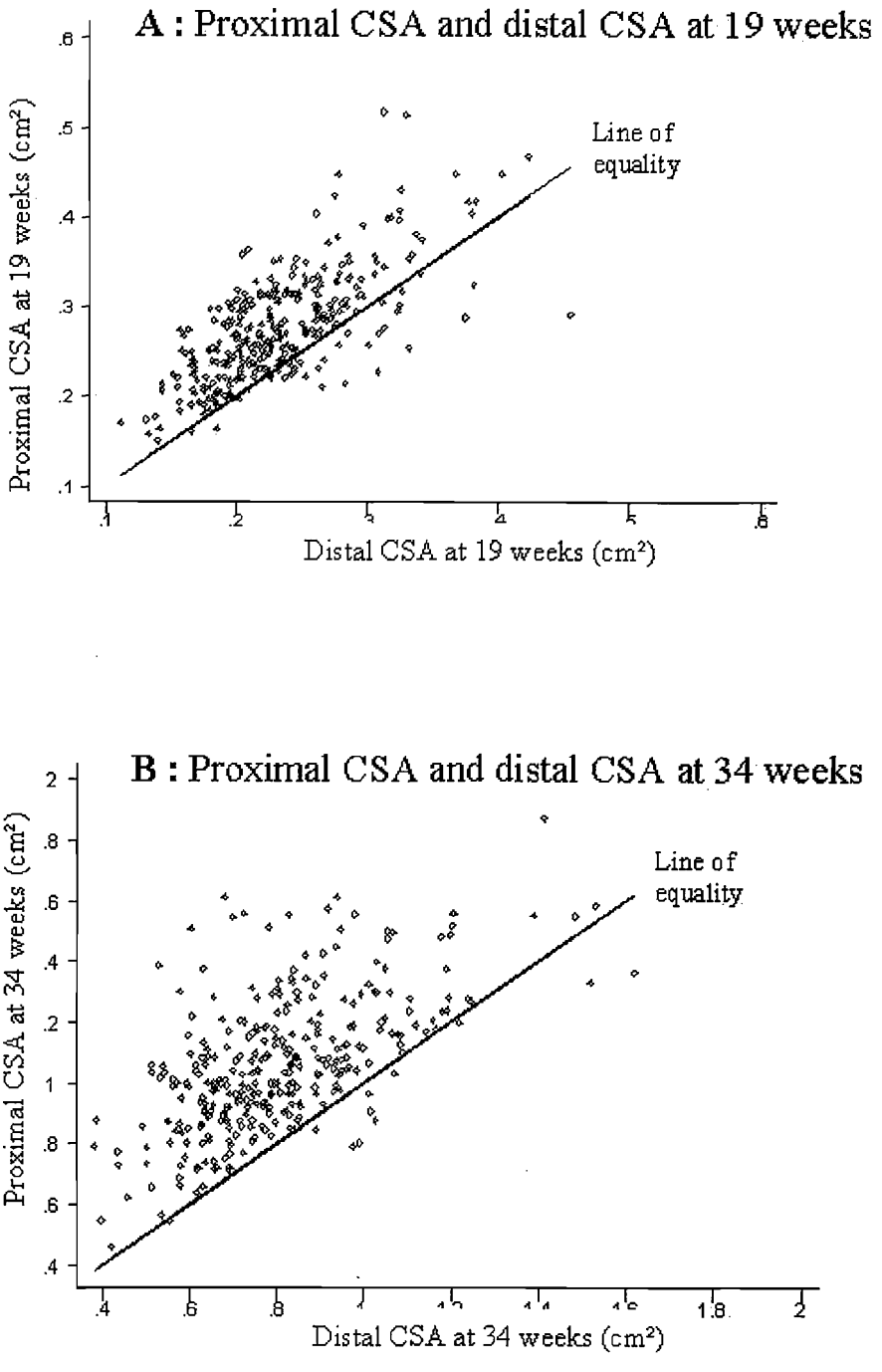


Figure 4.2: Proximal CSA plotted against distal CSA. A shows plotting for the first 134 subjects at 19 weeks. The points lying below the line of equality represent femora with a larger distal CSA. B shows the plot at 34 weeks.

Further investigation of this finding was needed to see if this was a true physiological marker or a normal variation and as the CSA measurements of proximal, mid-shaft and distal femur had proved more reliable and descriptive than the linear diameters, it was decided to continue to collect CSA data in preference in the next phase of the recruitment.

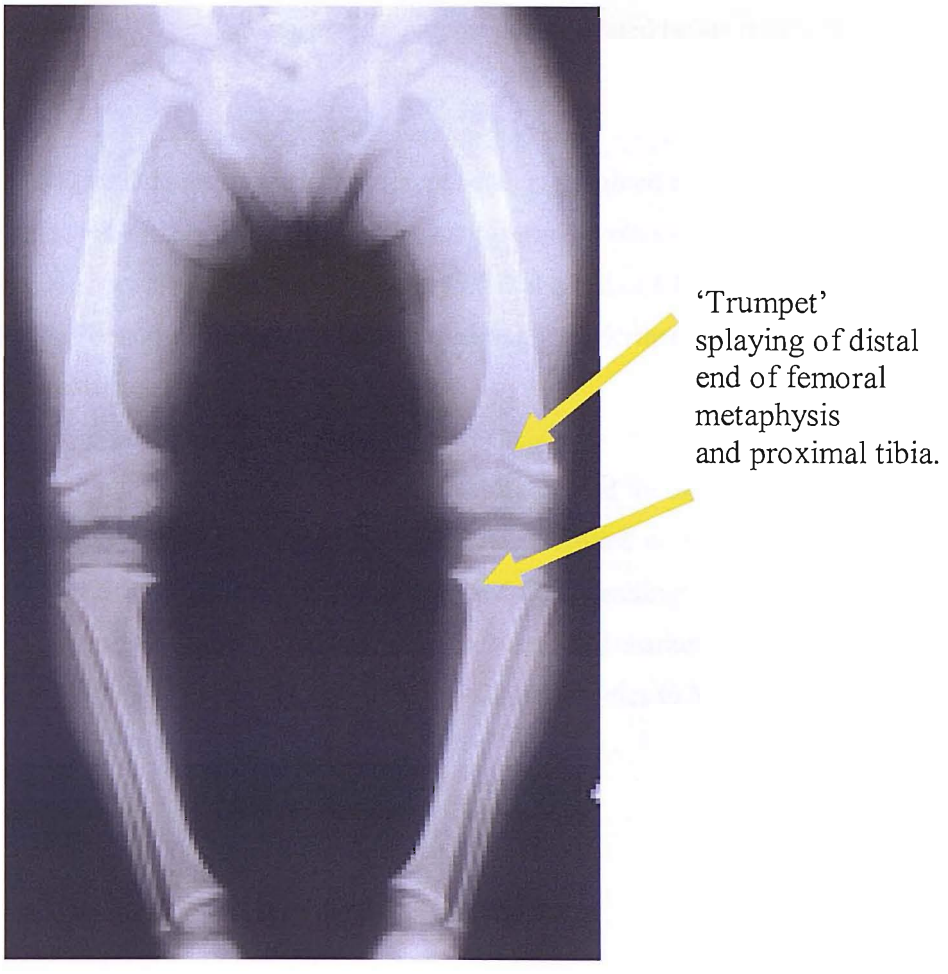


Figure 4.3: Radiograph of a 2 year old with rickets (vitamin D deficiency). Note the 'trumpet-like' ends of the shafts of the long bones particularly in the femora and tibiae.

Measurements made on the soft tissues of the fetal thigh had not proved as difficult as those made on bone as the skin to amniotic fluid boundary and the fat / skin to muscle boundary could be clearly defined. This made it possible to perform evaluations of fetal body composition which could be correlated with maternal data. In this way maternal influences on fetal body composition were explored.

Some of the earliest scans had not included enough of the soft tissue for thigh, muscle and fat / skin volumes, as the ROI or angle of sweep had not been optimised, but the useable data of the bone was still included in the analyses. Care was thereafter taken to ensure all of the thigh tissues were included on volume acquisitions.

At this primary stage, volume technique had also been streamlined, with the optimal settings for acquisition programmed into the KretzGE system. Knowledge of time and movement issues

made acquisition time faster and more consistent and so image demonstrated better resolution and ease of interpretation.

It was concluded that the difficult and inconsistent measurements highlighted in the initial trials, had been proven to be unsound by the *in vitro* and *in vivo* analysis and were subsequently no longer collected, leaving the core 8 measurements as described at the end of Chapter 3. In the final analysis of the finished cohort, these measurements were analysed further to assess the reproducibility of each measurement in its own right.

The data collection then continued until a sufficient number was acquired for analysis, totalling 525. Data from the initial SWS pre-pregnant interviews were also analysed in isolation and together with the results of the ultrasound scans, birthweight and DXA readings of the babies. Additionally, maternal vitamin D concentration was correlated with fetal characteristics. The results appear in Chapter 5 – Relations of Fetal and Maternal Characteristics to Musculo-Skeletal Development.

Chapter 5 – Relations of Fetal and Maternal Characteristics to Musculo-Skeletal Development

Introduction

To understand fetal growth and development, attention must be paid to maternal influences such as her own body mass index (BMI), age, height, birthweight, whether she has ever smoked, her vitamin D status during pregnancy, and pre-pregnant anthropometry. In this chapter the final fetal cohort data of 517 fetuses, their mothers' data and the relationship between the two sets of results are presented. In addition, intra-operator variability and between-subject variation for the different types of measurements was repeated with the larger cohort, to ascertain if the measurements to be used in the analyses were as sound as those generated by preliminary *in vivo* studies. These measurements were femur length (cm) as a representation of linear measurement, mid-shaft CSA (cm^2) representing trace area measurements, femur volume (cm^3) representing bone volume calculation and thigh volume (cm^3), representing volumes taken on soft tissue. In Table 5.1 the results are presented for both 19 and 34 weeks, as each gestation has different issues that influence measurement error. Also, images of bone and soft tissue had their own challenges and so these two tissues are taken separately in the analysis.

Results 1 – Confirmation of Intra-operator and Between-subject Measurement Variability

From Table 5.1 it can be seen that the intra-operator coefficient of variation (CV) between each triplet of linear measurements (femur length measured in cm) at 19 weeks was 0.6% over the 3 measurements, compared with 0.4% at 34 weeks. These small values suggest a reliable technique was employed. This contrasts with the between-subject CV for the same linear measurement of 7.2% at 19 weeks and 4.4% at 34 weeks. This shows that there is a wide size range in the fetuses; the intra-class correlation method (ICC) suggests that 98.5% of the measurement variation is due to biological variation of the fetuses and not measurement error (Table 5.1). ICC represents the proportion of the total variance (100%) that is between-subject variance.

Trace measurements are more problematic as they are performed using operator skill in placing the trace on the correct anatomical location at every point in the trace. Linear measurements are reliant upon operator skill when placing a calliper point at each end of the femoral diaphysis. This allows a reproducible calculation, less dependent on operator ability. The trace measurements of the femoral mid-shaft analysed here shows that at 19 weeks the intra-operator CV was 5.6%; falling to 3.8% at

34 weeks, when the bone edges were more distinct. The between-subject CV for 19 and 34 weeks were 19.6% and 20.1% respectively. Again this shows a greater range of biological variation as compared to variability produced by the measurement method. The ICC for trace measurements at 19 weeks was 63.9%, suggesting that 36.1% of variability arises from measurement error. This was probably due to the distance between the electronic cursors produced by the ultrasound system and software being too large for the small cross-sectional area of the femoral shaft being measured. At 34 weeks, the CSA of the femur is larger and easier to trace around with the electronic callipers and so the ICC rose to 95.5%, suggesting that only 4.5% of the variability reflects measurement error.

	Intra-operator variability			**Between-subject variation**			**ICC**
19 weeks	**range***	**median***	**SD (CV**)**	**range**	**mean**	**SD (CV)**	**%**
Femur length (cm)	0 – 0.153	0.027	0.017 (0.6%)	2.51 – 3.80	3.05	0.221 (7.2%)	98.5
Mid-shaft CSA (cm^2)	0 – 0.053	0.013	0.007 (5.6%)	0.061 – 0.223	0.138	0.027 (19.6%)	63.9
Femur volume (cm^3)	0 – 0.107	0.033	0.017 (3.4%)	0.240 – 0.907	0.520	0.103 (19.9%)	68.3
Thigh volume (cm^3)	0 – 1.087	0.093	0.072 (0.9%)	3.91 – 14.58	7.55	1.53 (20.4%)	98.7
	Intra-operator variability			**Between-subject variation**			**ICC**
34 weeks	**range***	**median***	**SD (CV**)**	**range**	**mean**	**SD (CV)**	**%**
Femur length (cm)	0 – 0.233	0.033	0.023 (0.4%)	5.68 – 7.21	6.44	0.284 (4.4%)	98.5
Mid-shaft CSA (cm^2)	0 – 0.133	0.020	0.012 (3.8%)	0.177 – 0.577	0.341	0.068 (20.1%)	95.5
Femur volume (cm^3)	0 – 0.493	0.073	0.048 (1.7%)	1.71 – 4.26	2.86	0.519 (18.2%)	97.7
Thigh volume (cm^3)	0 – 12.88	0.487	0.542 (0.5%)	57.8 – 150.8	98.3	15.81 (16.1%)	99.9

Table 5.1: Intra-operator variability and between-subject variation. The range and median* values relate to the sum of the absolute difference between triplicate measurements and their mean. The CV** was calculated using Martin Bland's root mean square method.*

Volume calculations were separated into bone and soft tissue analyses as bone was difficult to image with its reflection and shadowing, making calliper placement problematic. This was especially significant at 19 weeks when the structures were smaller. The soft tissue boundaries within the fetal thigh were easier to distinguish at 34 weeks and so made measurement more reproducible. From Table 5.1 it can be seen that femur volume at 19 weeks showed 3.4% intra-operator variability over the measurement triplet, compared with a 19.9% variation between subjects. The ICC was 68.3% which meant that 31.7% of measurement error was produced by the operator. At 34 weeks the intra-operator variability over the measurement triplet was 1.7%, perhaps resulting from better resolution of the mineralised bone ends at this later gestation. The between-subject variation between fetal femur lengths was 18.2% and the ICC was 97.7%, indicating that only 2.3% of the total measurement inconsistency was due to operator variability.

The intra-operator variability at 19 weeks over the measurement triplet was 0.9% and 0.5% at 34 weeks, suggesting a sound technique was being used. The variation in sizes between fetuses was 20.4% at 19 weeks and 16.1% at 34 weeks. The ICC for 19 weeks was 98.7% showing 1.3% operator inconsistency. This was reduced at 34 weeks to 0.1% operator variability.

These results show that linear measurements are reproducible with minimal operator-variability and they provided a sensitive method of demonstrating differences between subjects. However, the CSA traces were disappointing as at 19 weeks the limitations of the equipment produced an ICC of 63.9%. This was also true of femur volume at 19 weeks, which by the nature of the volume calculation technique relies on operator traces to assimilate the volume. The discrepancy was almost certainly a result of the calliper spacing not being optimum for small structures. Thigh volume at 19 weeks showed a low measurement inconsistency, as thighs at 19 weeks are usually surrounded by amniotic fluid making the skin surface easy to identify. At 34 weeks the fetus is usually flexed and the skin surface can sometimes be difficult to visualise, but overall linear, trace, bone volume and soft tissue volumes produced low measurement error.

The measurement techniques were shown to be reproducible making this modality a useful tool for assessing differences between subjects. The measurements obtained were now ready to be used for the study of their relationships with maternal variables as well as dimensions obtained form the thigh tissues. Presented here are the fetal summary statistics obtained from the 517 fetuses. Scan measurements, DXA readings and birthweight have been used in the analyses.

Results 2 – Descriptive Data of Fetal Characteristics and Gender Influences

5.1 Description of cohort and exclusions

The entire SWS cohort consists of 12,500 women; 1246 became pregnant and presented for their 19 week anomaly scan during the research recruitment period. Between October 2002 and July 2005, PM recruited as many women as possible from those who attended their scan appointment, but from a sub-set whose GPs had given consent for them to be included in the section of the SWS where a neonatal DXA scan would be offered. In total 525 pregnant women were selected for musculo-skeletal imaging and all but 8 were in included in the analyses (Table 5.2).

Cohort recruitment statistics		
	Reason for exclusion	n
Recruited		*525*
Excluded	1 multiple abnormalities	*1*
	1 heart defect operated on at birth	*1*
	1 stillborn at 39 weeks	*1*
	1 maternal Crohn's disease – offspring severely growth retarded	*1*
	2 withdrew from study	*2*
	1 Down syndrome	*1*
	1 omphalocoele - surgically corrected after birth	*1*
	Total for analysis	*517*

Table 5.2: Cohort recruitment numbers and reasons for exclusion.

The ethnic groups represented in the cohort were predominantly Caucasian (including northern and southern European, North American, South American, South African, Australasian), 1 Afro-Caribbean, 2 black African, 2 Chinese, 10 Asians and 1 Eastern Mediterranean. Some studies have shown that ethnicity may influence biometric measurements. For example, Shipp and co-workers (2001) found smaller than expected femur length measurements amongst Asian babies and larger than expected femur lengths in Afro-Caribbean fetuses compared with white Caucasians. Also Iranian fetuses have been found to have smaller head geometry and shorter femur length measurements in comparison with Western studies, ($p<0.05$), (Beigi and ZarrinKoub, 2000). Conversely, no differences have been reported for the humerus, (Zelop *et al.*, 2003). Studies in Asia using a fetal femur length to neonatal crown-heel ratio have also shown that as a combined index these two measurements were on average 1.1 cm shorter in Malay and Chinese populations

(Lim *et al.*, 2000). However, in this study cohort only 16 pregnancies (3.1%) were not of white Caucasian origin and so blended ethnicity here was not a significant issue.

5.2 Fetal summary statistics

The 517 studied pregnancies produced 266 boys and 251 girls (51.5% and 48.6% respectively) and their gestational data is tabulated in Table 5.3. Within this group, 491 had both 19 and 34 week scans and 15 fetuses were scanned at 19 weeks alone. Of these, 8 delivered before the 34 week scan appointment and 3 missed the opportunity to be scanned due to other commitments. A further 4 datasets were rejected for sub-optimal images. All datasets were checked for normal distribution, before ranges, means and standard deviations were calculated. Paired t-tests were also performed to test for sex differences between the measurements.

5.3 Gestational data

From Table 5.3 it can be seen that there was a small range of gestational ages at each scan, delivery and DXA scan. A regression model was used to adjust for the variation in gestation between fetuses at each event, so all results could be analysed at a single constructed point in time. The target gestation for each scan was 19 weeks and 3 days and 34 weeks and 4 days. It was not always possible to arrange the mother's scan appointments to coincide with the exact gestation due to weekends, holidays, scan machine availability or other commitments, so a small range within which each scan could be performed was tolerated (Table 5.3). The mean for number of days for the 19 week scan to be performed was 137 days, which was 1 day different to the desired gestation of 19 weeks and 3 days (136 days). The target gestation of 34 weeks and 4 days matched the mean obtained for the 493 scans performed in the 34 week visit.

Babies born before 37 weeks of gestation are considered premature and within this cohort 29 (6%) were identified as premature. Babies born after 42 weeks are considered 'post-mature' and 20 (5%) of such babies were seen within the 517 cohort. The remaining 468 babies (89%) were within the usual gestational range and the ranges for the entire group and the each sex are presented in Table 5.3. This indicates that there were no differences between mean gestational age at delivery between boys and girls, but the range of birth gestations show that some of the boys delivered earlier than their female counterparts (174–300 days for boys and 188–300 days for girls). To test if there was any significant difference in the range of gestation at birth between boys and girls, a non-parametric (Mann–Whitney test) was performed after the converting the values to z scores. It revealed that there was no significance difference in gestational birth range between the two groups ($p = 0.2$, $t = 1.3$).

	Scan gestation data (combined sexes)				
	n	range (days)	mean (days)	range (weeks + days)	mean (weeks + days)
19 week scan	*515*	127 – 146	137	18+1 – 20+6	19+4
34 week scan	*493*	231 – 248	242	33+0 – 35+5	34+4
	Birth gestation				
	n	range (days)	mean (days)	range (weeks + days)	mean (weeks + days)
both sexes	*517*	173 – 300	280	24+5 – 42+6	40+0
boys	*266*	174 – 300	283	24+6 – 42+6	40+4
girls	*251*	188 – 300	282	26+6 – 42+6	40+2
	DXA gestation				
	n	range (weeks + days)	mean (weeks + days)	time from delivery to DXA (days)	mean (days)
both sexes	*285*	35+6 – 44+1	41+2	1 – 16	7.8
boys	*145*	35+6 – 44+1	41+2	1 – 16	7.8
girls	*140*	35+6 – 44+1	41+2	1 – 16	7.8

Table 5.3: Tabulation of scan gestation, gestation at delivery and gestation at DXA scan; for the combined group and both individual sexes and the time elapsed from delivery to DXA scan.

The parents of all the babies within the 517 cohort, with the exception of those who were premature (29), were invited to return to the SWS within 14 days of birth, for the infant to undergo a DXA scan to assess bone density. Of this group 295 returned for scan (57%) and the parents gave written consent for the scan to take place. During 3 of the scans the infant could not be pacified sufficiently to allow the scan to be completed and so these were dropped from the analysis. Three further scans were abandoned due to a retrospective finding that the equipment settings were not optimal after a technical fault had been rectified. This fault did not endanger the fetuses being scanned as it concerned detection thresholds within the unit and not radiation production. Further quality assurance tests concluded that the equipment had been calibrated properly and scans after this period were included in the study. A further 4 scans were dropped from the analysis as they were considered poor quality. This rendered 285 DXA scans suitable for analysis and this number represented 55% of the entire cohort, (Table 5.3). Ideally, the DXA should be performed soon after birth as it gives information about bone mineral accrual at the end of pregnancy, before post-natal nutrition has begun to have an effect on the infant. Scanning the day after delivery whilst the

mother and baby were still in hospital was possible in many cases. The average time that DXA scans were performed was 7.8 days after delivery with the maximum being 16 days. At 2 weeks of age and older, the baby can be difficult to scan as he or she becomes more alert and responsive to their surroundings and movement during the scan becomes a serious issue. From previous studies it has also been shown that after 14 days of delivery, milk being fed to the baby begins to have an impact on neo-natal bone development and so it is important to capture the bone data before this occurs, as this will be more accurate representation of bone mineral acquisition during pregnancy.

5.4 2D and 3D ultrasound scan data

The summary scan data presented here outlines the 8 core measurements taken at scan from stored scan volumes at both 19 (Table 5.4) and 34 (Table 5.5) weeks. Each measurement was taken 3 times and the mean value used in the analyses. The minimum, maximum and mean of each range for the entire group and each sex is presented. The standard deviation (SD) value is an indication of how close to the mean the spread of values lies (the mean +/– 2 standard deviations indicates 95% of the range) and this shows that at 19 weeks structures such as the soft tissue volumes have more variation in size range. The SD for thigh volume is 1.34 as compared to the SD for femur volume which is 0.1 for combined groups, indicating a smaller spread of values for bone. The other bone measurements also lie within a smaller range with a SD of 0.2 and below. When divided by sex it can be seen that for all volumes boys' measurements have a greater SD from the mean than girls. However, the remaining bone biometrics were equal in value for both sexes. Thus the deviation from the mean is greatest in soft tissues.

At 34 weeks this pattern is more pronounced (Table 5.4), with bone measurements, including volume, showing a small SD for both sexes. The greatest spread of measurement is for thigh volume, which shows an overall SD of 15.29. At 34 weeks for the fat / skin components there is a reversal in the pattern of greater variability in boys; 9.07 for girls and 8.67 in boys.

Summary scan data						
19 weeks		n	mean	min	max	SD
Thigh volume (cm^3)	boys	*220*	6.64	2.83	11.91	1.41
	girls	*211*	6.26	2.99	9.17	1.24
	both	*431*	6.44	2.83	11.91	1.34
Fat / skin volume (cm^3)	boys	*219*	1.87	0.35	4.10	0.63
	girls	*208*	1.75	0.51	3.34	0.54
	both	*427*	1.81	0.35	4.11	0.59
Muscle volume (cm^3)	boys	*221*	4.27	1.37	7.53	1.06
	girls	*208*	4.00	1.77	6.91	0.95
	both	*429*	4.14	1.37	7.23	1.02
Femur volume (cm^3)	boys	*248*	0.49	0.26	0.80	0.10
	girls	*236*	0.47	0.24	0.74	0.09
	both	*484*	0.48	0.24	0.81	0.10
Femur length (cm)	boys	*261*	2.92	2.49	3.43	0.20
	girls	*247*	2.91	2.41	3.54	0.20
	both	*502*	2.92	2.40	3.54	0.20
Proximal CSA (cm^2)	boys	*252*	0.30	0.12	0.51	0.06
	girls	*236*	0.26	0.15	0.46	0.06
	both	*488*	0.27	0.12	0.51	0.06
Mid-shaft CSA (cm^2)	boys	*255*	0.13	0.08	0.21	0.03
	girls	*245*	0.13	0.06	0.20	0.03
	both	*500*	0.13	0.06	0.21	0.03
Distal CSA (cm^2)	boys	*244*	0.23	0.14	0.38	0.05
	girls	*235*	0.22	0.11	0.42	0.05
	both	*479*	0.23	0.11	0.42	0.05

Table 5.4: The ranges, means and standard deviations of scan measurements taken at 19 weeks; (n = 517).

Summary scan data						
34 weeks		n	mean	min	max	SD
Thigh volume (cm^3)	boys	*223*	94.48	59.54	145.98	15.40
	girls	*205*	91.54	51.36	129.69	15.06
	both	*428*	93.07	51.36	145.98	15.29
Fat / skin volume (cm^3)	boys	*222*	39.13	20.07	66.13	8.67
	girls	*201*	38.52	21.58	67.52	9.07
	both	*427*	38.83	20.07	67.52	8.86
Muscle volume (cm^3)	boys	*223*	52.62	33.08	77.91	8.64
	girls	*206*	50.35	27.39	75.59	8.07
	both	*429*	51.53	27.39	77.91	8.44
Femur volume (cm^3)	boys	*247*	2.84	1.66	4.11	0.52
	girls	*236*	2.75	1.74	4.19	0.51
	both	*483*	2.80	1.66	4.19	0.52
Femur length (cm)	boys	*250*	6.35	5.58	7.07	0.29
	girls	*241*	6.37	5.79	7.22	0.27
	both	*491*	6.36	5.58	7.22	0.28
Proximal CSA (cm^2)	boys	*240*	1.03	0.56	1.62	0.21
	girls	*230*	1.00	0.48	1.62	0.20
	both	*470*	1.01	0.48	1.62	0.21
Mid-shaft CSA (cm^2)	boys	*249*	0.34	0.20	0.57	0.07
	girls	*240*	0.34	0.17	0.54	0.07
	both	*489*	0.34	0.17	0.57	0.07
Distal CSA (cm^2)	boys	*241*	0.79	0.40	1.56	0.19
	girls	*227*	0.77	0.40	1.64	0.18
	both	*468*	0.78	0.40	1.64	0.19

Table 5.5: The ranges, means and standard deviations of scan measurements taken at 34 weeks; (n = 517).

Overall analysis of the measurements revealed that some dimensions were different for boys and girls and so paired t-tests were performed to quantify if these differences were significant. The results of these tests are shown in Table 5.6.

5.5 Gender differences in fetal thigh measurements

The sex difference results shown in Table 5.6 demonstrate that at 19 weeks the volume measurements are greater in boys than girls. In addition it can be seen that the gender difference is significant for thigh volume ($p = 0.001$, $t = 3.24$), fat / skin volume ($p = 0.04$, $t = 2.06$), muscle volume ($p = 0.005$, $t = 2.85$) and femur volume ($p = 0.01$, $t = 2.55$), with the boys again being larger than the girls. However, femur length and CSA measurements show no significant difference between the sexes, (Table 5.6).

At 34 weeks, it can be seen from Table 5.7 that the volume of the fat / skin layer no longer shows a significant difference between the sexes ($p = 0.48$, $t = 0.71$). Additionally, the femur volumes in boys are greater than girls, but this is not quite significant ($p = 0.07$, $t = 1.84$). However, at this later gestation, total thigh volume is greater in boys ($p = 0.05$, $t = 1.99$) and the muscle volume is significantly greater in boys ($p = 0.005$, $t = 2.80$). Femur length and femur shaft cross-sectional areas show no significant difference between the sexes at 34 week, (Table 5.6).

	Fetal scan measurement sex differences					
Fetal measurement : 19 weeks		mean	95% CI	n	paired t- test	
					t	p
Thigh volume (cm^3)	boys	6.64	6.45 – 6.83	*220*	**3.24**	**0.001**
	girls	6.22	6.06 – 6.39	*211*		
Fat / skin volume (cm^3)	boys	1.87	1.78 – 1.95	*219*	**2.06**	**0.04**
	girls	1.75	1.68 – 1.82	*208*		
Muscle volume (cm^3)	boys	4.27	4.13 – 4.41	*221*	**2.85**	**0.005**
	girls	3.40	3.87 – 4.13	*208*		
Femur volume (cm^3)	boys	0.49	0.47 – 0.50	*248*	**2.55**	**0.01**
	girls	0.46	0.45 – 0.48	*236*		
Femur length (cm)	boys	2.92	2.89 – 2.95	*261*	0.83	0.41
	girls	2.91	2.88 – 2.93	*247*		
Proximal CSA (cm^2)	boys	0.270	0.263 – 0.277	*252*	1.20	0.23
	girls	0.270	0.256 – 0.271	*236*		
Mid-shaft CSA (cm^2)	boys	0.131	0.128 – 0.134	*255*	0.80	0.42
	girls	0.129	0.126 – 0.133	*245*		
Distal CSA (cm^2)	boys	0.231	0.224 – 0.237	*244*	1.69	0.09
	girls	0.223	0.216 – 0.229	*235*		

Table 5.6: Paired t-test results on the core scan measurements at 19 weeks.

	Fetal scan measurement sex differences					
Fetal measurement : 34 weeks		mean	95% CI	n	paired t-test	
					t	p
Thigh volume (cm^3)	boys	94.48	92.45 – 96.51	*223*	**1.99**	**0.05**
	girls	91.54	89.46 – 93.61	*205*		
Fat / skin volume (cm^3)	boys	39.13	37.98 – 40.27	*222*	0.71	0.48
	girls	38.52	37.27 – 39.77	*205*		
Muscle volume (cm^3)	boys	52.62	51.48 – 53.76	*223*	**2.80**	**0.005**
	girls	50.34	49.24 – 51.45	*206*		
Femur volume (cm^3)	boys	2.84	2.77 – 2.90	*247*	1.84	0.07
	girls	2.75	2.69 – 2.82	*236*		
Femur length (cm)	boys	6.35	6.32 – 6.39	*250*	–0.85	0.40
	girls	6.37	6.34 – 6.41	*241*		
Proximal CSA (cm^2)	boys	1.03	1.00 – 1.06	*240*	1.63	0.11
	girls	1.00	0.97 – 1.03	*230*		
Mid-shaft CSA (cm^2)	boys	0.34	0.33 – 0.35	*249*	–0.40	0.69
	girls	0.34	0.33 – 0.35	*240*		
Distal CSA (cm^2)	boys	0.80	0.77 – 0.82	*241*	1.25	0.21
	girls	0.77	0.75 – 0.80	*227*		

Table 5.7: Paired t-test results on the core scan measurements at 34 weeks.

Further analysis of the fetal thigh tissues was undertaken so ascertain if there were any differences in the proportions of bone, muscle and fat / skin between the sexes at both 19 and 34 weeks. The proportions were calculated as percentages and paired t-tests were used to highlight any differences between male and female offspring. The results are presented in Table 5.8 as percentages of the total thigh volume (100%) at 19 weeks and 34 weeks gestation and the associated t-tests show that although there were differences in proportions of the tissues within the thigh, these differences were not statistically significant.

From Table 5.8 it can be seen that at 19 weeks thigh volume comprised 26.8% of fat / skin tissue in boys. This volume was 28.3% in girls, but the difference between the sexes was not statistically significant ($p = 0.55$, $t = -0.60$). Fat begins to be accrued by the fetus after 28 weeks and so by 34 weeks the proportion of thigh that was fat / skin tissue rose to 41.1% in boys and 41.7% in girls. Again this difference was not significant ($p = 0.26$, $t = -1.13$).

	Proportions of fetal thigh tissues						
19 weeks		min (%)	max (%)	mean (%)	n	paired t- test	
						t	p
Fat / skin	boys	6.3	45.8	26.8	*219*	–0.60	0.55
	girls	8.0	45.1	28.3	*208*		
Muscle	boys	45.2	84.6	65.2	*221*	0.87	0.39
	girls	44.5	82.4	63.9	*208*		
Bone	boys	4.0	15.2	7.4	*222*	–0.47	0.64
	girls	4.9	13.3	7.4	*211*		
	Proportions of fetal thigh tissues						
34 weeks		min (%)	max (%)	mean (%)	n	paired t- test	
						t	p
Fat / skin	boys	28.3	51.1	41.1	*222*	–1.13	0.26
	girls	28.8	52.2	41.7	*205*		
Muscle	boys	44.4	68.3	55.9	*222*	1.19	0.24
	girls	44.9	67.7	55.1	*205*		
Bone	boys	1.9	4.8	3.0	*223*	–0.31	0.76
	girls	1.8	4.7	3.1	*205*		

Table 5.8: The proportion of fetal thigh that is bone, muscle and fat / skin layers.

Muscle accrual had a different pattern. At 19 weeks the proportion of the total thigh which was muscle was 65.2% in boys and 63.9% in girls, but by 34 weeks that proportion became 55.9% in boys and 55.1% in girls. Although this appeared to be a decrease in muscle tissue as a proportion of the total thigh volume, in real terms the muscle mass had increased, but there had been an even greater increase in the fat / skin layer as the fetus commenced accrual of subcutaneous fat stores. It can be seen from the table that 7.4% of the thigh in both sexes comprised bone at 19 weeks, which decreased to approximately 3% of the thigh volume by 34 weeks. This is because muscle is accrued and fat is deposited under the skin, which changed the proportions.

The histograms in Figures 5.1 and 5.2 demonstrate the difference in thigh tissue between the sexes. At 19 weeks paired t-tests show that there were statistically significant differences between the quantities of bone in boys' thighs to girls ($p = 0.01$, $t = 2.55$). In addition this was true for muscle ($p = 0.005$, $t = 2.85$) and fat / skin volumes ($p = 0.04$, $t = 2.06$).

By 34 weeks the gender differences showed a different pattern. From the histogram in Figure 5.2 it can be seen that there continued to be a significant difference in muscle volume between boys and girls ($p = 0.005$, $t = 2.80$), but the difference in bone volume had decreased ($p = 0.07$, $t = 2.84$).

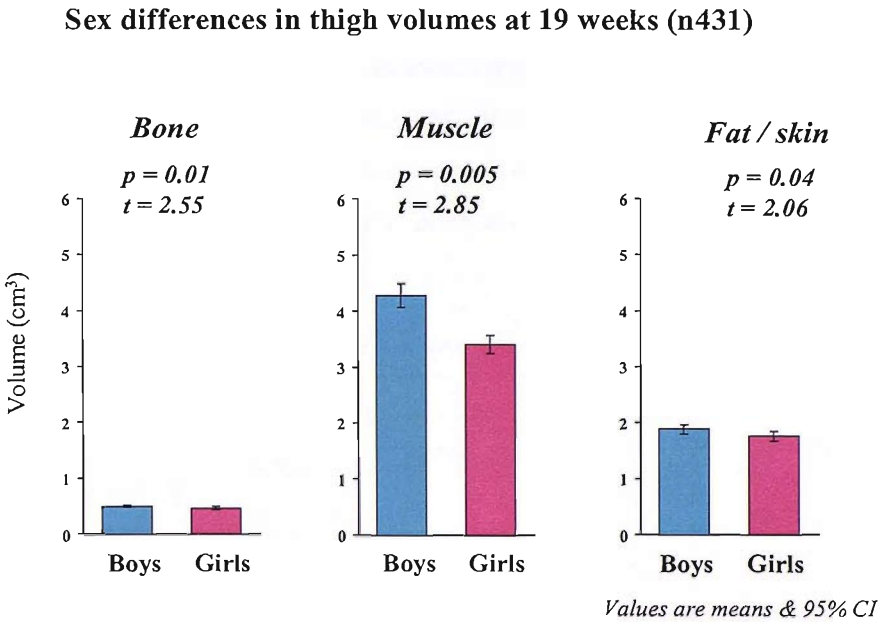


Figure 5.1: Histograms demonstrating gender differences in tissue volumes at 19 weeks.

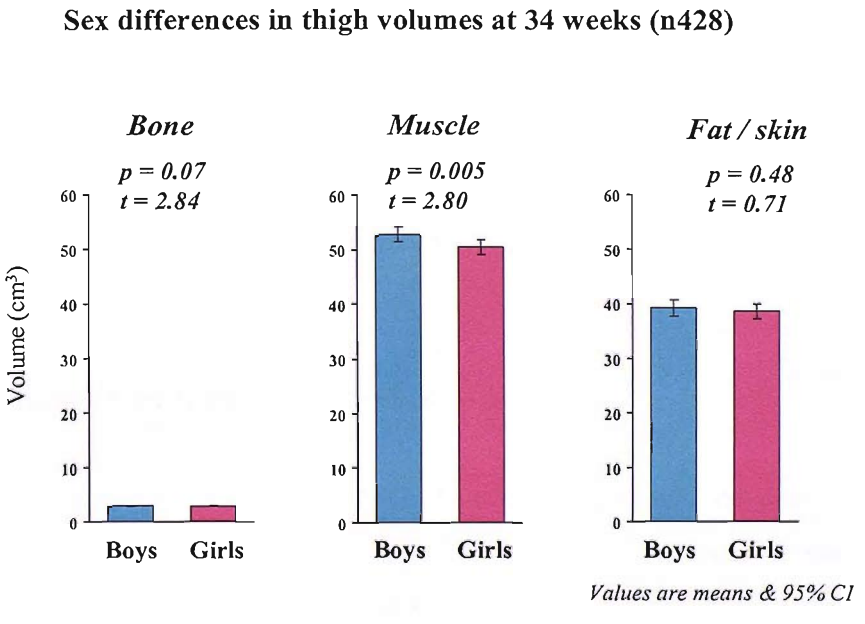


Figure 5.2: Gender differences in body composition at 34 weeks.

Additionally, there was now no significant difference between fat / skin volumes in boys and girls ($p = 0.48$, $t = 0.71$). These results suggest gender differences in the fetal body composition at different trimesters of pregnancy.

5.6 DXA measurements of neonatal bone

Of the entire 517 cohort, 295 of the neonates returned for a DXA scan. After excluding 10 scans for technical reasons, 285 were suitable for analysis; 55% of the cohort. The total body DXA scan generated measurements of bone mineral content (measured in g) bone mineral density (expressed in g/cm^2) and total bone area (measured in cm^2). Summary statistics are tabulated:

Neonatal DXA sex differences						
DXA readings		**mean**	**95% CI**	**n**	**paired *t*- test**	
					t	**p**
Neonatal bone mineral content (g)	boys	64.69	61.94 – 67.45	*145*	**2.80**	**0.006**
	girls	59.65	57.40 – 61.89	*140*		
Neonatal bone mineral density (g/cm^2)	boys	0.53	0.53 – 0.54	*145*	1.48	0.14
	girls	0.53	0.52 – 0.53	*140*		
Neonatal total bone area (cm^2)	boys	121.26	116.75 – 125.77	*145*	**2.79**	**0.006**
	girls	113.01	109.33 – 116.69	*140*		

Table 5.9: Summary statistics for neonatal DXA measurements; t-tests for gender differences in right-hand columns.

Table 5.9 shows that in neonatal boys, bone mineral content was on average 5g greater than in girls which was statistically significant ($p = 0.006$, $t = 2.80$). Neonatal bone mineral density showed no difference between genders ($p = 0.14$, $t = 1.48$), whereas total neonatal bone area readings indicated that boys had an average of 8.25 cm^2 greater bone area than girls, which was also statistically significant ($p = 0.006$, $t = 2.79$). These results were then correlated against the core fetal scan measurements as shown in Tables 5.10 and 5.11.

5.6.1 Neonatal bone mineral content (BMC)

From the results in Table 5.10 it can be seen that at 19-week thigh volume was positively associated with neonatal bone mineral content ($p = 0.002$, $r = 0.20$). However, this relationship only remained in boys when the group was divided by sex ($p = 0.02$, $r = 0.22$ for boys only). At 34 weeks thigh volume was positively associated with bone mineral content ($p<0.001$, $r = 0.39$); the relationship remained when the group was divided by gender ($p<0.001$, $r = 0.49$ for boys and $p = 0.005$, $r = 0.26$ for girls). Fat / skin volume at 19 weeks was not associated with bone mineral content, but greater bone mineral content was predicted by greater fat / skin volumes at 34 weeks ($p<0.001$, $r = 0.28$). At 19 weeks muscle volume was significantly associated with neonatal bone mineral content ($p <0.001$, $r = 0.22$) and this strong association was maintained at 34 weeks ($p<0.001$, $r = 0.43$).

	Neonatal bone mineral content								
	both sexes			boys			girls		
Fetal measurement: 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.20**	**0.002**	*238*	**0.22**	**0.02**	*124*	0.11	0.24	*114*
Fat / skin volume (cm^3)	0.08	0.26	*234*	0.15	0.10	*123*	–0.09	0.34	*111*
Muscle volume (cm^3)	**0.22**	**<0.001**	*236*	**0.20**	**0.02**	*125*	**0.20**	**0.03**	*111*
Femur volume (cm^3)	0.06	0.33	*268*	0.05	0.55	*137*	0.01	0.87	*131*
Femur length (cm)	0.09	0.15	*281*	0.01	0.86	*143*	**0.17**	**0.04**	*138*
Proximal CSA (cm^2)	**0.12**	**0.05**	*270*	0.12	0.17	*139*	0.10	0.24	*131*
Mid-shaft CSA (cm^2)	0.09	0.13	*276*	0.11	0.19	*139*	0.05	0.58	*137*
Distal CSA (cm^2)	0.04	0.53	*264*	–0.02	0.85	*136*	0.09	0.34	*128*

	Neonatal bone mineral density								
	both sexes			boys			girls		
Fetal measurement: 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.07	0.31	*238*	0.11	0.22	*124*	–0.01	0.88	*114*
Fat / skin volume (cm^3)	–0.03	0.68	*2346*	0.06	0.52	*123*	–0.16	0.09	*111*
Muscle volume (cm^3)	0.11	0.09	*236*	0.11	0.23	*125*	0.09	0.33	*111*
Femur volume (cm^3)	–0.03	0.65	*268*	–0.04	0.61	*137*	–0.03	0.70	*131*
Femur length (cm)	0.04	0.51	*281*	–0.12	0.16	*143*	**0.19**	**0.03**	*138*
Proximal CSA (cm^2)	0.05	0.46	*270*	0.04	0.61	*139*	0.04	0.66	*131*
Mid-shaft CSA (cm^2)	0.09	0.16	*276*	0.05	0.59	*139*	0.12	0.16	*137*
Distal CSA (cm^2)	–0.01	0.85	*264*	–0.03	0.73	*136*	–0.01	0.96	*128*

	Neonatal total bone area								
	both sexes			boys			girls		
Fetal measurement: 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.21**	**0.001**	*238*	**0.23**	**0.01**	*124*	0.13	0.18	*114*
Fat / skin volume (cm^3)	0.10	0.13	*234*	0.17	0.07	*123*	–0.06	0.51	*111*
Muscle volume (cm^3)	**0.22**	**<0.001**	*236*	**0.21**	**0.02**	*125*	**0.20**	**0.03**	*111*
Femur volume (cm^3)	0.08	0.20	*268*	0.07	0.42	*137*	0.03	0.69	*131*
Femur length (cm)	0.09	0.14	*281*	0.04	0.62	*143*	0.14	0.09	*138*
Proximal CSA (cm^2)	**0.13**	**0.03**	*270*	0.13	0.13	*139*	0.12	0.17	*131*
Mid-shaft CSA (cm^2)	0.08	0.20	*276*	0.11	0.19	*139*	0.02	0.82	*137*
Distal CSA (cm^2)	0.05	0.43	*264*	–0.01	0.88	*136*	0.10	0.25	*128*

Table 5.10: BMC, BMD and bone area and their relationships scan measurements at 19 weeks.

	Neonatal bone mineral content								
	both sexes			boys			girls		
Fetal measurement: 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.39**	**<0.001**	*245*	**0.49**	**<0.001**	*129*	**0.26**	**0.005**	*116*
Fat / skin volume (cm^3)	**0.28**	**<0.001**	*244*	**0.42**	**<0.001**	*145*	0.13	0.16	*116*
Muscle volume (cm^3)	**0.43**	**<0.001**	*246*	**0.49**	**<0.001**	*129*	**0.32**	**<0.001**	*117*
Femur volume (cm^3)	**0.23**	**<0.001**	*274*	**0.30**	**<0.001**	*138*	0.13	0.14	*136*
Femur length (cm)	**0.24**	**<0.001**	*276*	**0.28**	**<0.001**	*139*	**0.23**	**0.008**	*137*
Proximal CSA (cm^2)	0.11	0.07	*263*	**0.20**	**0.02**	*133*	–0.03	0.75	*130*
Mid-shaft CSA (cm^2)	**0.19**	**0.002**	*275*	**0.25**	**0.003**	*139*	0.16	0.07	*136*
Distal CSA (cm^2)	0.15	0.13	*264*	**0.21**	**0.02**	*135*	0.05	0.58	*129*
	Neonatal bone mineral density								
	both sexes			boys			girls		
Fetal measurement: 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.10	0.13	*245*	0.12	0.17	*129*	0.06	0.53	*116*
Fat / skin volume (cm^3)	0.01	0.84	*244*	0.10	0.27	*128*	–0.06	0.49	*116*
Muscle volume (cm^3)	**0.18**	**0.005**	*246*	**0.17**	**0.05**	*129*	0.17	0.06	*117*
Femur volume (cm^3)	**0.12**	**0.05**	*274*	0.14	0.11	*138*	0.10	0.27	*136*
Femur length (cm)	0.05	0.37	*276*	-0.04	0.60	*139*	**0.17**	**0.05**	*137*
Proximal CSA (cm^2)	–0.001	1.00	*263*	0.05	0.59	*133*	–0.07	0.42	*130*
Mid-shaft CSA (cm^2)	0.10	0.09	*275*	**0.21**	**0.01**	*139*	0.02	0.81	*136*
Distal CSA (cm^2)	0.06	0.31	*264*	0.11	0.20	*135*	–0.01	0.91	*129*
	Neonatal total bone area								
	both sexes			boys			girls		
Fetal measurement: 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.43**	**<0.001**	*245*	**0.53**	**<0.001**	*129*	**0.28**	**0.003**	*116*
Fat / skin volume (cm^3)	**0.31**	**<0.001**	*244*	**0.45**	**<0.001**	*128*	0.16	0.09	*116*
Muscle volume (cm^3)	**0.45**	**<0.001**	*246*	**0.52**	**<0.001**	*129*	**0.32**	**<0.001**	*117*
Femur volume (cm^3)	**0.24**	**<0.001**	*274*	**0.31**	**<0.001**	*138*	0.13	0.14	*136*
Femur length (cm)	**0.26**	**<0.001**	*276*	**0.34**	**<0.001**	*139*	**0.21**	**0.01**	*137*
Proximal CSA (cm^2)	**0.13**	**0.03**	*263*	**0.22**	**0.01**	*133*	–0.001	0.95	*130*
Mid-shaft CSA (cm^2)	**0.19**	**0.002**	*275*	**0.24**	**0.005**	*139*	**0.18**	**0.04**	*136*
Distal CSA (cm^2)	**0.16**	**0.008**	*264*	**0.21**	**0.02**	*135*	0.07	0.18	*129*

Table 5.11: BMC, BMD and bone area and their relationships with scan measurements at 34 weeks.

At 19 weeks it was surprising to see that femur volume was not predictive of neonatal bone mineral content ($p = 0.33$, $r = 0.06$), but at 34 weeks femur volume was strongly associated with neonatal

bone mineral content ($p<0.001$, $r = 0.23$). This association was only true for boys when divided by sex ($p<0.001$, $r = 0.30$ for boys and $p = 0.14$, $r = 0.13$ for girls). At 19 weeks femur length was associated with bone mineral content in girls only ($p = 0.04$, $r = 0.17$), but at 34 weeks femur length had significant positive association with neonatal bone mineral content ($p<0.001$, $r = 0.28$ for boys and $p = 0.008$, $r = 0.23$ for girls). Additionally, at 19 weeks the proximal CSA was also significantly associated with bone mineral content ($p = 0.05$, $r = 0.12$). At 34 weeks there was a weak association between proximal CSA and neonatal bone mineral content, but was only significant in boys ($p = 0.02$, $r = 0.20$). There were no associations between mid-shaft CSA and bone mineral content or between distal CSA and bone mineral content at 19 weeks. At 34 weeks the mid-shaft CSA was associated with neonatal bone mineral content ($p = 0.002$, $r = 0.19$), but only in boys when divided by sex ($p = 0.01$, $r = 0.21$).

5.6.2 Neonatal bone mineral density (BMD)

At 19 weeks there was only one significant association seen between the bone portion of the fetal thigh and bone mineral density. Greater femur length in girls was associated with greater neonatal bone mineral density ($p = 0.03$, $r = 0.19$), but there was no such association with boys. This relationship continued at 34 weeks, again solely in girls ($p = 0.05$, $r = 0.17$). As can be seen from Table 5.11, at 34 weeks fetal muscle volume was positively associated with neonatal bone mineral density ($p = 0.005$, $r = 0.18$), but this association was only seen in boys when the group was divided ($p = 0.05$, $r = 0.17$).

Femur volume at 34 weeks was significantly associated with bone mineral density for the entire study group ($p = 0.05$, $r = 0.12$), but this association was not apparent when the group was divided by sex. Having a larger femur mid-shaft CSA was also associated with increased bone mineral density at 34 weeks, but only in boys ($p = 0.01$, $r = 0.21$).

5.6.3 Neonatal bone area (BA)

From Tables 5.10 and 5.11 it can be seen that thigh volume at 19 weeks was predictive of higher neonatal bone area ($p = 0.001$, $r = 0.21$), which remained significant only in boys when the group was divided by sex ($p = 0.01$, $r = 0.23$). This was not the case at 34 weeks when neonatal bone area was strongly associated with thigh volume in both sexes ($p<0.001$, $r = 0.43$ combined). The association remained strong when the group was divided by sex.

Fat / skin volume at 19 weeks was not associated with neonatal bone area, but at 34 weeks there was a strong association for both sexes combined ($p<0.001$, $r = 0.45$). The relationship between greater fetal fat / skin volume at 34 week and neonatal bone area was strong in boys alone ($p<0.001$, $r = 0.45$), but in girls there was no association ($p = 0.09$, $r = 0.16$). Greater fetal thigh muscle volume was strongly associated with neonatal bone area at 19 weeks for combined and individual sex groups ($p<0.001$, $r = 0.22$ combined). This association was highly significant at 34 weeks for combined and single sex groups (all $p<0.001$).

Femur volume was not associated with bone area at 19 weeks, but by 34 weeks there was a significant positive association for the entire group ($p <0.001$, $r = 0.24$), showing that babies with a larger bone area had large femur volumes. This association was seen in boys ($p <0.001$, $r = 0.31$), but was not apparent in girls ($p = 0.14$, $r = 0.13$). Figure 5.3 shows the association between quarters of the distribution of neonatal BMC and femur volume at 19 and 34 weeks.

There was no association between femur length at 19 weeks and subsequent neonatal bone area. However, at 34 weeks femur length was highly predictive of greater neonatal bone area ($p<0.001$, $r = 0.26$ for the combined group). These relations are shown in Figure 5.4.

All CSA measurements at 19 weeks, with the exception of proximal CSA showed no association with neonatal bone area. Proximal CSA was significantly associated with bone area for both sexes combined ($p = 0.03$, $r = 0.13$), but not when analysed separately in boys and girls. At 34 weeks there were positive associations for the entire group ($p = 0.03$, $r = 0.13$), which remained significant in boys ($p = 0.01$, $r = 0.22$). Interestingly, there was no association between proximal CSA and bone area for girls ($p = 0.95$, $r = -0.001$).

Mid-shaft CSA at 19 weeks was not associated with neonatal bone area. In contrast, fetal mid-shaft CSA at 34 weeks was positively associated with neonatal bone area for the entire group ($p = 0.002$, $r = 0.19$). This strong association remained when the group was divided by sex ($p = 0.005$, $r = 0.24$ for boys and $p = 0.04$, $r = 0.18$ for girls). Distal CSA at 19 weeks was not associated with neonatal bone area, but at 34 weeks distal CSA showed a positive association with neonatal bone area in the entire group ($p = 0.008$, $r = 0.16$). This association was also seen in the boys ($p = 0.02$, $r = 0.21$), but not in the girls ($p = 0.18$, $r = 0.07$).

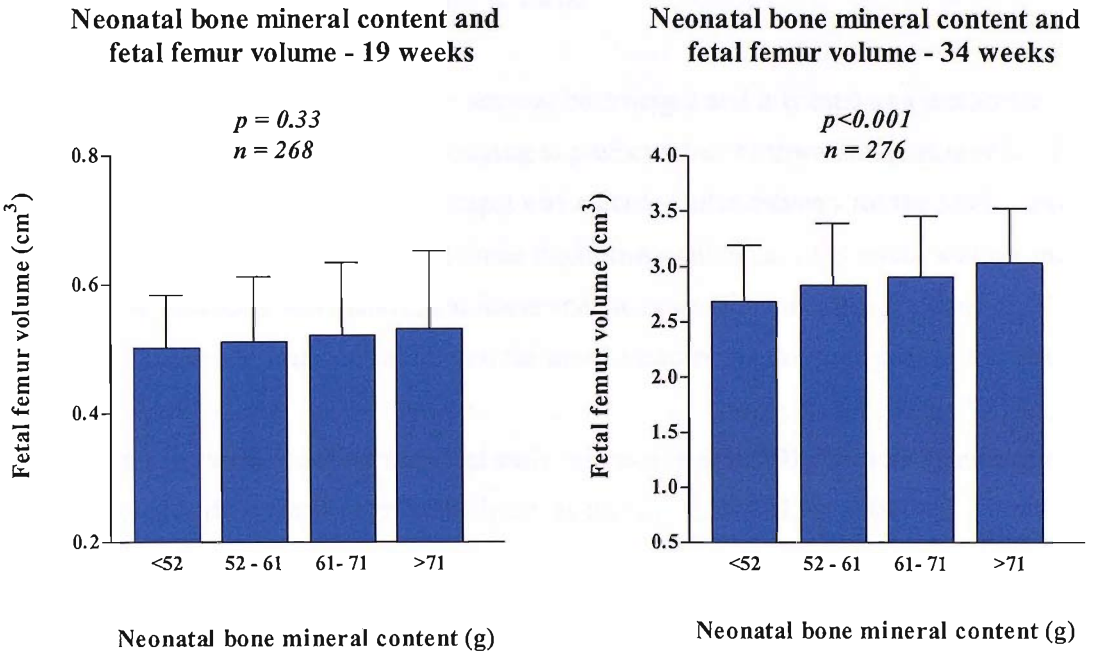


Figure 5.3: The relationship between fetal femur volume and neonatal BMC. Left: Femur volume and BMC at 19 weeks. Right: Femur volume and BMC at 34 weeks. Values are means and 95% CI.

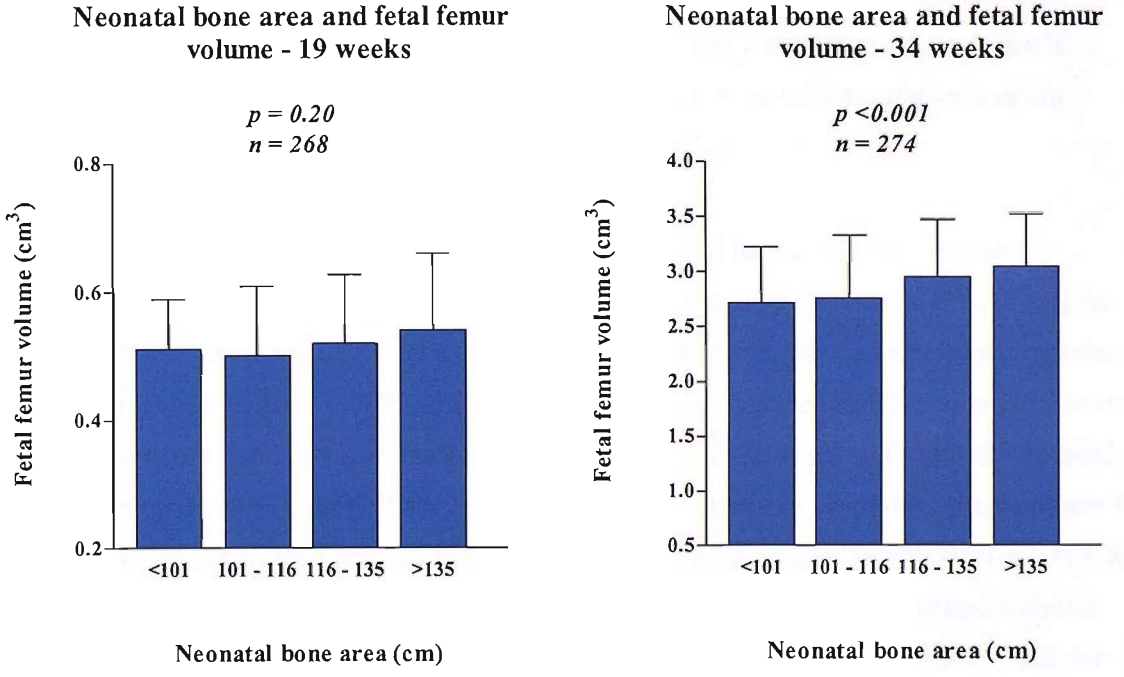


Figure 5.4: The relationship between fetal femur volume and neonatal bone area. Left: Femur volume and bone area at 19 weeks. Right: Femur volume and bone area at 34 weeks. Values are means and 95% CI.

5.7 Relationships with infant weight at birth

Many obstetric studies place great importance on birthweight and it is used as a measure of fetal outcome. Some studies use ultrasound imaging to predict infant birthweight, (Liang *et al.,* 1997; Chang *et al.,* 1997b). For comparison, weight was recorded after delivery for the SWS cohort, using calibrated electronic scales. In two cases the birthweight of an SWS infant was not included in the analysis; one baby was delivered at home and the other at another hospital outside of the region. The birthweight was correlated with the novel measurements developed for this study.

From the summary table it can be seen that male infants averaged 191g heavier than females (Table 5.12), a difference that was strongly statistically significant ($p<0.001$, $t = 4.04$).

Summary for birthweight (g)					
	n	mean	min	max	SD
both sexes	*515*	3440	700	4870	462.27
boys	*264*	3533	700	4870	465.75
girls	*251*	3342	920	4860	444.54

Table 5.12: Summary statistics for birthweight.

The 8 core scan measurements and the DXA readings were then correlated with the infant's birthweight, to see if there were any predictive values in the ante-natal measurements or any associations between size at birth and bone readings (Table 5.13).

From Table 5.13 it can be seen that thigh, muscle fat / skin and femur volumes were highly predictive of infant weight at birth. However, the association was lost for femur volume in girls when divided by sex ($p = 0.10$, $r = 0.11$). Femur length at 19 weeks was also strongly associated with birthweight ($p<0.001$, $r = 0.16$), but this association only remained significant in girls when divided by sex ($p = 0.003$, $r = 0.19$ for girls and $p = 0.06$, $r = 0.12$ for boys). Proximal and distal CSA were not associated with birthweight for the group as a whole or divided by sex, but there was a significant association between mid-shaft CSA and birthweight in boys ($p = 0.005$, $r = 0.18$). At 34 weeks all of the core measurements were significantly associated with birthweight with the exception of the distal and proximal CSA, but this was only seen in girls ($p = 0.43$, $r = 0.05$ for proximal CSA and $p = 0.28$, $r = 0.07$ for distal CSA) .

When the infants' birthweights were correlated with DXA readings it can be seen from the table that all of the variables were positively associated with birthweight ($p<0.001$) for both sexes and the combined group.

	Birthweight								
	both sexes			**boys**			**girls**		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.34**	**<0.001**	*430*	**0.30**	**<0.001**	*221*	**0.36**	**<0.001**	*211*
Fat / skin volume (cm^3)	**0.18**	**<0.001**	*426*	**0.19**	**0.005**	*220*	**0.14**	**0.04**	*208*
Muscle volume (cm^3)	**0.33**	**<0.001**	*428*	**0.27**	**<0.001**	*220*	**0.38**	**<0.001**	*208*
Femur volume (cm^3)	**0.18**	**<0.001**	*482*	**0.18**	**0.006**	*246*	0.11	0.10	*236*
Femur length (cm)	**0.16**	**<0.001**	*506*	0.12	0.06	*259*	**0.19**	**0.003**	*247*
Proximal CSA (cm^2)	0.06	0.21	*486*	0.10	0.12	*250*	–0.002	0.97	*236*
Mid-shaft CSA (cm^2)	**0.14**	**0.001**	*498*	**0.18**	**0.005**	*253*	0.10	0.12	*245*
Distal CSA (cm^2)	0.06	0.20	*477*	0.04	0.57	*242*	0.06	0.39	*235*

	Birthweight								
	both sexes			**boys**			**girls**		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.72**	**<0.001**	*428*	**0.71**	**<0.001**	*223*	**0.71**	**<0.001**	*205*
Fat / skin volume (cm^3)	**0.62**	**<0.001**	*427*	**0.62**	**<0.001**	*222*	**0.62**	**<0.001**	*205*
Muscle volume (cm^3)	**0.64**	**<0.001**	*429*	**0.64**	**<0.001**	*223*	**0.62**	**<0.001**	*206*
Femur volume (cm^3)	**0.29**	**<0.001**	*483*	**0.31**	**<0.001**	*247*	**0.24**	**<0.001**	*236*
Femur length (cm)	**0.44**	**<0.001**	*491*	**0.51**	**<0.001**	*264*	**0.39**	**<0.001**	*241*
Proximal CSA (cm^2)	**0.13**	**0.005**	*470*	**0.18**	**0.006**	*240*	0.05	0.43	*230*
Mid-shaft CSA (cm^2)	**0.19**	**<0.001**	*489*	**0.20**	**0.001**	*249*	**0.20**	**0.002**	*240*
Distal CSA (cm^2)	**0.13**	**0.007**	*468*	**0.16**	**0.02**	*241*	0.07	0.28	*227*

	Birthweight								
	both sexes			**boys**			**girls**		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	**0.75**	**<0.001**	*285*	**0.78**	**<0.001**	*145*	**0.71**	**<0.001**	*140*
Bone mineral density	**0.31**	**<0.001**	*285*	**0.34**	**<0.001**	*145*	**0.29**	**<0.001**	*140*
Bone area	**0.78**	**<0.001**	*285*	**0.79**	**<0.001**	*145*	**0.73**	**<0.001**	*140*

Table 5.13: Core fetal scan measurement, birthweight and DXA results.

5.8 Fetal femoral metaphyseal splaying

From the preliminary study measuring AP and TS dimensions of the mid-femoral shaft and proximal and distal portions of the shaft in 130 fetuses showed that the bone increases at a greater rate in length than in AP or TS dimension. Due to the variability of these measurements taken on reconstructed planes and the difficulties experienced when imaging bone, measurements of these anatomical planes were dropped in favour of more reproducible CSA measurements at the proximal, mid-shaft and distal portions of the femoral metaphyses. However, both types of measurement showed that there was variability between fetuses in the geometric shape of the femoral metaphyses.

From the initial 130 scans it was seen that although there was a five-fold increase in femur volume between 19 and 34 weeks, much of this increase concentrated around the metaphyseal ends. The central portion of the shaft showed a doubling of mid-shaft CSA, whereas diaphyseal expansion increased 297% in the distal CSA and 264% in proximal CSA.

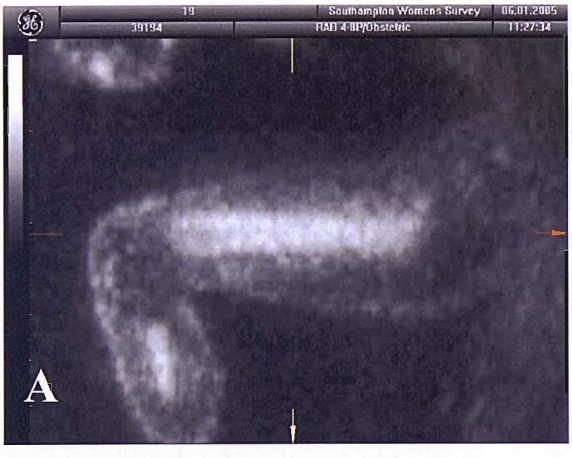


Figure 5.5A: Parallel-sided femur at 19 weeks.

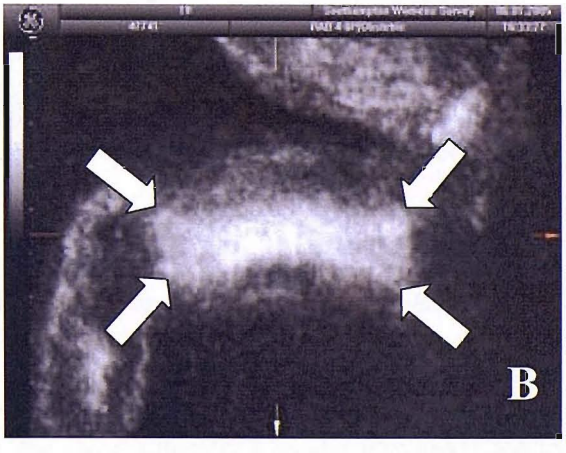


Figure 5.5B: Splayed metaphyseal ends (arrowed).

The femoral metaphyses in a fetus appears as a linear structure when imaged using ultrasound. At 19 weeks the homogeneous echo of the femur is seen as a relatively parallel-sided structure, as seen in Figure 5.5A. However, in some fetuses the growing ends of the metaphyses appear splayed and 'trumpet-shaped' as in Figure 5.5B. In this study it was noticed that 10% of the femora scanned had this appearance. On further investigation it was seen that this appearance was associated with an increase in the CSA of the metaphyseal ends (proximal CSA and distal CSA).

During fetal development the CSA of both metaphyseal ends are equal in size, but in this study, it was seen that distal CSA of the shaft, at the knee-joint, was greater than the proximal CSA at the hip joint. In children and adults, it is usual for the proximal portion of the femoral shaft to be more

robust than its distal counterpart, as this is the end of the bone which is involved with the hip joint. In fetuses the larger proximal metaphyseal dimension can be seen by ultrasound by 32 weeks.

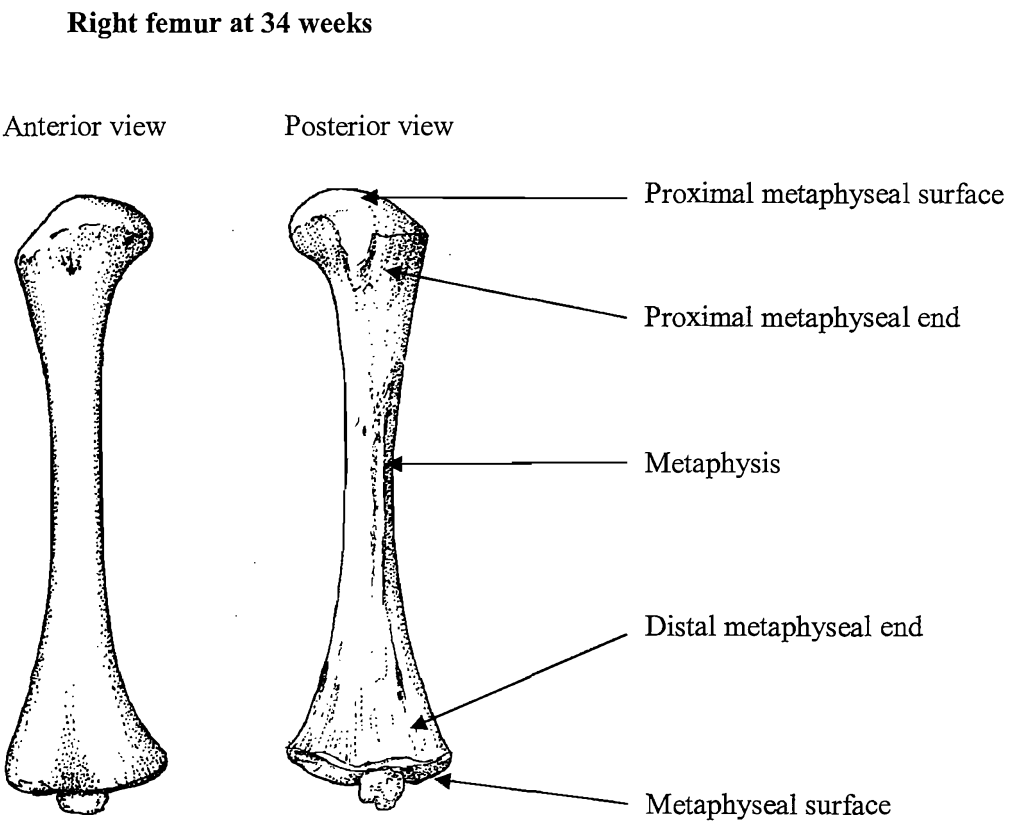


Figure 5.6: Authors' drawing of a post-mortem 34-week fetal femur specimen. The bone is right-sided and both anterior and posterior views are displayed. Epiphyses not shown.

There is normal metaphyseal splaying by 34 weeks, but there is usually a greater proximal CSA to distal CSA. In Figure 5.6 above it appears that the distal portion is larger than the proximal, but in the usual configuration, the proximal CSA is seen to greater than the distal, as the distal end is usually ovoid in shape when viewed end-on. Further investigation of this variation was performed to reveal if this was an anatomical variation or an effect of maternal environment and the results of this will be presented in Results 4, section 5.17.1.

5.9 Growth velocities of fetal characteristics

Absolute values of dimensions of the fetal thigh showed differences between sexes at 19 and 34 weeks and so to develop this the growth velocity of each parameter was examined for sex differences. Each measurement used was converted to a *z* score, which is a dimensionless quantity derived by subtracting the mean value of a spread from the individual score, then dividing the

difference by the standard deviation of the distribution. In this way a *z* score is able to indicate how many units a characteristic such as growth velocity is above or below the mean. This allows direct comparison of different values to be made. The difference between each core measurement for each subject at 19 weeks was compared to the *z* score of the corresponding measurement at 34 weeks and the difference in these scores was expressed as a percentage increase. T-tests were then performed to highlight any gender differences and in this way variation in growth velocity between the sexes was determined in addition to variation of growth velocity between different tissues. The results are tabulated in Table 5.14.

	Fetal thigh tissue % increase from 19 to 34 weeks						
		min (%)	max (%)	mean (%)	*n*	*t*- test	
						t	p
Thigh volume (cm^3)	both	7.9	27.6	15.0	*368*	–0.73	0.47
	boys	7.9	27.6	14.9	*191*		
	girls	9.3	27.1	15.2	*177*		
Fat / skin volume (cm^3)	both	8.5	103.5	23.9	*364*	–0.80	0.43
	boys	8.5	103.5	23.7	*189*		
	girls	9.0	52.9	24.1	*175*		
Muscle volume (cm^3)	both	6.6	33.6	13.1	*366*	0.23	0.82
	boys	6.6	33.6	13.1	*190*		
	girls	7.6	26.4	13.2	*176*		
Femur volume (cm^2)	both	3.2	13.0	6.1	*454*	–0.49	0.62
	boys	3.2	13.0	6.1	*231*		
	girls	3.2	11.2	6.1	*223*		
Femur length (cm)	both	1.8	2.7	2.2	*482*	–1.00	0.32
	boys	1.9	2.5	2.2	*245*		
	girls	1.8	2.7	2.2	*237*		
Proximal CSA (cm^2)	both	1.5	8.0	3.9	*446*	0.58	0.56
	boys	1.8	7.6	4.0	*228*		
	girls	1.5	8.0	3.9	*218*		
Mid-shaft CSA (cm^2)	both	1.2	5.0	2.7	*472*	–0.79	0.43
	boys	1.3	4.6	2.7	*238*		
	girls	1.2	5.0	2.7	*234*		
Distal CSA (cm^2)	both	1.5	8.1	3.6	*434*	–0.26	0.79
	boys	1.6	7.8	3.6	*220*		
	girls	1.5	8.1	3.7	*214*		

Table 5.14: Growth velocities of scan variables; 19 to 34 weeks. These are shown as % increase. T-tests were also performed to show any sex differences.

The results were then combined with maternal characteristics to examine maternal influences on fetal growth velocity. The findings are shown in Results 4.

From the table it can be seen that thigh volume increases between 7.9 and 27.6% from 19 to 34 weeks, with a mean of 15% for the entire group. The mean increase was marginally higher in girls (0.33%) which was not statistically significant ($p = 0.47$, $t = -0.73$). When the thigh tissues are separated fat / skin volume increase between 19 and 34 weeks also showed no significant difference between the sexes ($p = 0.43$, $t = -0.80$). This was also the case for muscle volume where the mean increase of 13.1% was constant for boys and girls. For femur volume the mean % increase from 19 to 34 weeks was equal for both sexes at 6.1%. For femur length the % increase for both groups ranged from 1.8 to 2.7% with a mean of 2.2% for all groups, which was the smallest increase for any of the measurements. The proximal CSA area showed a mean % increase of 3.9% for all groups and the distal CSA showed a mean increase of 3.6% for the group. The range for each of these groups was from 1.2 to 3.9%. This increase at the growing ends of the bone was greater than the mid-shaft CSA which showed a range of increase from 1.2 to 5%, with a mean of 2.7%. Thus the ends of the metaphysis are showed more growth in cross-sectional dimensions than the main part of the shaft.

The growth velocity of core measurements showed subtle differences between different areas of the femur for the entire group. Growth velocity for all parameters between 19 and 34 weeks showed no difference between sexes. To examine whether faster growth was related to weight at delivery, the core measurement growth velocities were correlated with fetal birthweight and the findings are shown in Table 5.15.

	Weight at delivery								
	both sexes			**boys**			**girls**		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	**0.38**	**<0.001**	*368*	**0.40**	**<0.001**	*191*	**0.36**	**<0.001**	*177*
Fat / skin volume	**0.36**	**<0.001**	*364*	**0.36**	**<0.001**	*189*	**0.38**	**<0.001**	*175*
Muscle volume	**0.31**	**<0.001**	*366*	**0.34**	**<0.001**	*190*	**0.27**	**0.004**	*176*
Femur volume	**0.10**	**0.04**	*454*	0.09	0.16	*231*	0.11	0.12	*223*
Femur length	**0.27**	**<0.001**	*482*	**0.37**	**<0.001**	*245*	**0.18**	**0.005**	*237*
Proximal CSA	0.06	0.21	*446*	0.06	0.41	*228*	0.06	0.41	*218*
Mid-shaft CSA	0.03	0.52	*472*	0.01	0.86	*238*	0.06	0.34	*234*
Distal CSA	0.06	0.19	*434*	0.12	0.08	*220*	0.006	0.93	*214*

Table 5.15: Growth velocity core measurements and birthweight.

From Table 5.15 it can be seen that faster growth velocity between 19 and 34 weeks for thigh volume was strongly associated with birthweight in both sexes, as thigh composition was representative of the tissues involved in total body mass ($p<0.001$, $r = 0.38$). This also applied to fat / skin volume ($p<0.001$, $r = 0.36$) and muscle volume ($p<0.001$, $r = 0.31$) and remained significant when divided by sex. Although significantly associated for femur volume, the strength of this association was not as strong for the soft tissue components ($p = 0.04$, $r = 0.10$ for femur volume). The strong relationship between growth velocity of the femur and weight at delivery disappeared in both sexes when the group was divided. The growth velocity of femur length was also strongly associated with weight at delivery ($p<0.001$, $r = 0.27$) and remained significantly associated when divided by sex although slightly weaker in girls ($p<0.01$, $r = 0.37$ for boys and $p = 0.005$, $r = 0.18$ for girls). The remaining growth velocity bone parameters (proximal, mid-shaft and distal CSA) were not associated with weight at delivery.

5.10 Growth velocity of bone and bone mineral accrual

To further assess the accrual of bone and mineral content in the fetal femur, the growth velocity between 19 and 34 weeks was calculated for femur volume and length and then correlated with bone mineral content at DXA. The left-sided histogram in Figure 5.7 shows femur volume growth velocity according to quarters of the distribution of BMC; there was a significant positive association. There was a similar relation for femur length growth velocity seen, in the right-sided histogram in Figure 5.7. Thus it could be inferred that growth velocity of femur volume and length were good predictors of neonatal BMC.

The next series of analyses involved the combination of fetal and maternal characteristics. This involved the core fetal scan measurements and DXA readings in conjunction with maternal pre-pregnant characteristics and some characteristics ascertained during pregnancy. The next section, Results 3, describes the maternal variables used before combined analysis with fetal parameters.

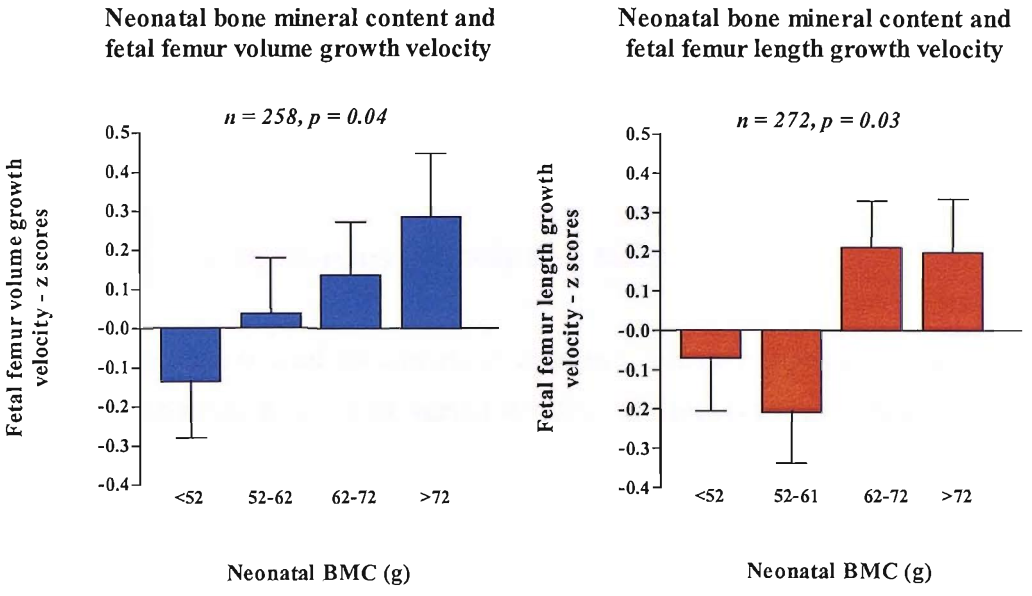


Figure 5.7: The relationship between fetal femur growth velocity and DXA readings. Left: Femur volume and BMC. Figure 5.7 Femur length and neonatal BMC. The distribution has been divided into quarters of the distribution. Values are means and 95% CI.

Results 3 – Descriptive Data of Maternal Characteristics

5.10 Maternal summary statistics

The Southampton Women's Survey is a large population study involving thousands of subjects, who were measured by research nurses in their own homes before pregnancy. The anthropometric data presented here represents the subset of 517 mothers in this study. The age of recruitment for the pre-pregnant phase of the SWS was 20–34 years and within this subset, the age range of the mothers at delivery was between 22 to 40 years, with a mean age of 31. The women recruited for the SWS were of reproductive age and some had already had children (parous). This meant that for some the baby they carried through the research period was their first or second and in some cases an addition to a large family.

Maternal birthweight was asked at the pre-pregnant interview and, where possible, their mothers' recollection of birthweight was used to corroborate this data. Many mothers had been delivered at Princess Anne Hospital, Southampton, where most of this research was undertaken and so access to their birth records was also possible. Researchers from the SWS were able to verify many of the birthweights obtained at interview. Where documentary evidence was not available the best

estimate from the mothers and their mothers' was used. In total 488 maternal birthweights were accurately recorded and ranged from 908g to 4909g with a mean of 3235g. Of the women, 137 were smokers and 380 were non-smokers when interviewed prior to pregnancy. The numbers of those who were smoking during their pregnancy was not available for this analysis.

5.11 Maternal anthropometry and body mass index

Anthropometric data was collected by the research nurses at the pre-pregnant interview. The measurement techniques for height, sum of skin-fold thicknesses and arm muscle area were standardised to ensure that every participant was measured in the same manner, using the same technique and equipment. The protocols followed by the SWS Research Nurses for the taking of anthropometric data are included in the Appendix IV.

Body mass index (BMI) was calculated by weight / (height ^{2}). The range of BMI for this cohort is shown in Table 5.16 together with maternal summary statistics, smoking status, parity and vitamin D concentrations taken from their blood at the 34 week scan appointment.

Maternal summary statistics					
		n	min	max	mean
Age at delivery (years)		*517*	22	40	31
Own birthweight (g)		*488*	908	4909	3235
Height (cm)		*515*	139	186	164
Sum of skin-fold thickness –SSF (mm)		*511*	23.6	168.6	68.4
Arm muscle area – AMA (cm^2)		*512*	15.1	80.7	34.5
Body mass index (BMI) (kg/m^2)		*515*	18.2	39.9	24.5
34 week vitamin D concentration (nmol/L)		*424*	8	180	65.63
		n			
Smoking status :	non-smokers	*380*			
	smokers	*137*			
Parity :	nulliparous (first child)	*307*			
	multiparous 1 (has one other child)	*144*			
	multiparous 2 (has 2+ other children)	*66*			

Table 5.16: Summary statistics for maternal variables. This includes pre-pregnant and pregnancy data.

To evaluate the musculo-skeletal development of the fetus, their mothers' blood was taken by a research nurse at the 34 week scan appointment. Of the 517 women in the cohort 424 (82%) consented to have blood taken. From the table it can be seen that the vitamin D concentrations ranged from 8 to 180 nmol/L, with an average concentration of 65.63 nmol/L.

Results 4 – Relations of Maternal Body Composition and Characteristics to Fetal Thigh Volumes

One of the aims of this research was to use the 3D ultrasound technology to research maternal influences on fetal growth and size. By scanning the fetus with ultrasound and DXA, then correlating the results with known measurements and characteristics from the mother, a picture of maternal influences may be drawn. Pre-pregnant data from the mother such as body mass index (BMI), arm muscle area (AMA), sum of skin-fold thicknesses (SSF) and smoking status have been correlated with scan data from her fetus. In addition her age, height, her own birthweight, how many children she has had (parity) has been studied in relation to her fetus. Finally, influences during pregnancy have been assessed for their influence on the fetus and these are the amount of circulating vitamin D in the mother in late gestation, obtained by blood tests. The variables to be used are outlined below:

Pre-pregnant maternal variables.

Maternal height
Maternal BMI
Maternal SSF
Maternal AMA

Maternal influences on fetal size and growth.

Age
Maternal birthweight
Parity
Maternal smoking status
Maternal vitamin D status

Each maternal variable was correlated with the 8 core fetal scan measurements and neonatal DXA results; the results are shown in Tables commencing with Table 5.17.

5.12 Maternal height and fetal scan measurements

Maternal height was a variable selected reflecting bone length which would be compared to the bone dimensions of the fetus. The protocol for precise measurement is included in Appendix V.

	Maternal height								
	both sexes			boys			girls		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.07	0.18	*429*	0.03	0.64	*219*	0.12	0.10	*210*
Fat / skin volume (cm^3)	0.04	0.37	*425*	0.04	0.57	*218*	0.06	0.43	*207*
Muscle volume (cm^3)	0.06	0.22	*427*	0.01	0.85	*220*	0.13	0.07	*207*
Femur volume (cm^3)	0.05	0.31	*482*	0.07	0.28	*247*	0.03	0.67	*235*
Femur length (cm)	0.73	0.10	*506*	0.06	0.31	*260*	0.19	0.08	*246*
Proximal CSA (cm^2)	0.02	0.66	*486*	0.02	0.76	*251*	0.03	0.71	*235*
Mid-shaft CSA (cm^2)	0.04	0.33	*498*	0.12	0.06	*254*	–0.04	0.58	*244*
Distal CSA (cm^2)	–0.001	0.98	*477*	–0.07	0.28	*243*	0.07	0.29	*234*

	Maternal height								
	both sexes			boys			girls		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.18**	**<0.001**	*426*	**0.18**	**0.01**	*222*	**0.18**	**0.008**	*204*
Fat / skin volume (cm^3)	**0.13**	**0.008**	*425*	**0.14**	**0.05**	*221*	0.12	0.08	*204*
Muscle volume (cm^3)	**0.18**	**<0.001**	*427*	**0.17**	**0.01**	*222*	**0.19**	**0.006**	*205*
Femur volume (cm^3)	**0.13**	**0.005**	*481*	0.08	0.23	*246*	**0.19**	**0.004**	*235*
Femur length (cm)	**0.20**	**<0.001**	*489*	**0.19**	**0.003**	*249*	**0.21**	**0.001**	*240*
Proximal CSA (cm^2)	0.07	0.12	*468*	0.11	0.10	*239*	0.04	0.59	*229*
Mid-shaft CSA (cm^2)	0.02	0.70	*487*	0.03	0.63	*248*	0.003	0.96	*239*
Distal CSA (cm^2)	0.004	0.94	*466*	0.01	0.94	*240*	0.002	0.98	*226*

	Maternal height								
	both sexes			boys			girls		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	0.06	0.29	*285*	0.07	0.38	*145*	0.08	0.38	*140*
Bone mineral density	–0.01	0.94	*285*	0.06	0.48	*145*	–0.06	0.49	*140*
Bone area	0.07	0.24	*285*	0.07	0.43	*145*	0.10	0.24	*140*

Table 5.17: Maternal height and core fetal measurements; correlated at 19 and 34 weeks. Maternal height was measured in cm.

From the data in Table 5.17, it can be seen that at 19 weeks there was no association between maternal height and any of the core fetal measurements. However, by 34 weeks, the mother's height was significantly positively associated with thigh volume in both sexes ($p<0.001$, $r = 0.18$) and each sex ($p = 0.01$, $r = 0.18$ for boys and $p = 0.008$, $r = 0.18$ for girls). Maternal height was also positively associated with fetal fat / skin volumes at 34 weeks ($p = 0.008$, $r = 0.13$); after division by sex the relationship was significant for boys ($p = 0.05$, $r = 0.14$) and did not reach significance in the girls ($p = 0.08$, $r = 0.12$). The mother's height was also positively associated the amount of fetal thigh muscle at 34 weeks ($p <0.001$, $r = 0.18$); ($p = 0.01$, $r = 0.17$ for boys and $p = 0.006$, $r = 0.19$ for girls). Maternal height was also positively associated with femur length 34 weeks ($p<0.001$, $r = 0.19$); the association remained significant in separate analyses in boys and girls ($p = 0.003$, $r = 0.19$ and $p = 0.001$, $r = 0.21$, respectively). There were no significant associations between maternal height and CSA measurements at 19 or 34 weeks. Additionally, maternal height was not predictive of neonatal bone mineral content, bone mineral density or total bone area.

5.13 Maternal body mass index and fetal scan measurements

As an overall measure of maternal body composition maternal body mass index was used. This was then correlated with the core fetal scan measurements and DXA readings as shown in Table 5.18. Body mass index (BMI) is a measure of fat and lean mass based on height and weight that applies to both adult men and women. In the SWS, a BMI was derived from maternal height and weight prior to pregnancy and was calculated by taking the weight of the individual in kilograms (kg) and dividing it by the height (m) squared ($BMI = kg / m^2$). The SWS mothers ranged from 18 (under-nourished) to 42 (morbidly obese).

Maternal BMI was weakly associated with thigh volume in boys at 19 weeks ($p = 0.03$, $r = 0.15$), but was not true for the combined group or girls on their own. By 34 weeks the situation was very different; maternal BMI was strongly associated with fetal thigh volume ($p<0.001$, $r = 0.19$). This relationship was significant for boys when the group was divided by sex ($p<0.001$, $r = 0.27$), but was not associated in girls ($p = 0.25$, $r = 0.08$).

Maternal BMI and proximal CSA were inversely associated at 19 weeks ($p = 0.03$, $r = -0.10$); this association remained in girls when divided by sex ($p<0.001$, $r = -0.25$). There were no other associations between maternal BMI and fetal volumes at 19 weeks, however maternal BMI was strongly associated with fat / skin volumes by 34 weeks in the combined group and in boys alone ($p<0.001$, $r = 0.20$ and $p<0.001$, $r = 0.28$, respectively). The relationship in girls did not reach significance ($p = 0.09$, $r = 0.12$).

Maternal BMI was positively associated with muscle volume at 34 weeks ($p = 0.002$, $r = 0.15$). This relationship was seen in boys as a separate group ($p<0.001$, $r = 0.26$), but not for girls. Maternal BMI was negatively associated with femur volume in girls ($p = 0.02$, $r = -0.16$), which was unexpected and the trend in the group as a whole was in a negative direction, although not reaching significance ($p = 0.16$, $r = -0.07$).

	Maternal BMI								
	both sexes			**boys**			**girls**		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.08	0.09	*429*	**0.15**	**0.03**	*219*	–0.01	0.86	*210*
Fat / skin volume (cm^3)	0.03	0.58	*425*	0.11	0.09	*218*	–0.09	0.22	*207*
Muscle volume (cm^3)	0.07	0.13	*427*	0.09	0.20	*220*	0.04	0.55	*207*
Femur volume (cm^3)	–0.005	0.91	*482*	0.06	0.32	*247*	–0.09	0.17	*235*
Femur length (cm)	0.01	0.85	*506*	0.05	0.44	*260*	–0.04	0.52	*246*
Proximal CSA (cm^2)	**–0.10**	**0.03**	*486*	0.03	0.62	*251*	**–0.25**	**<0.001**	*235*
Mid-shaft CSA (cm^2)	0.03	0.54	*498*	0.08	0.24	*254*	–0.02	0.76	*244*
Distal CSA (cm^2)	0.01	0.76	*477*	0.10	0.12	*243*	–0.09	0.17	*234*

	Maternal BMI								
	both sexes			**boys**			**girls**		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.19**	**<0.001**	*426*	**0.27**	**<0.001**	*222*	0.08	0.25	*204*
Fat / skin volume (cm^3)	**0.20**	**<0.001**	*425*	**0.28**	**<0.001**	*221*	0.12	0.09	*204*
Muscle volume (cm^3)	**0.15**	**0.002**	*427*	**0.26**	**<0.001**	*222*	0.02	0.79	*205*
Femur volume (cm^3)	–0.07	0.16	*481*	0.01	0.89	*246*	**–0.16**	**0.02**	*235*
Femur length (cm)	0.07	0.13	*489*	0.11	0.09	*249*	0.01	0.87	*240*
Proximal CSA (cm^2)	–0.08	0.10	*468*	–0.01	0.84	*239*	**–0.15**	**0.03**	*229*
Mid-shaft CSA (cm^2)	–0.08	0.08	*487*	0.09	0.80	*248*	**–0.26**	**<0.001**	*239*
Distal CSA (cm^2)	–0.03	0.46	*466*	–0.002	0.98	*240*	–0.08	0.24	*226*

	Maternal BMI								
	both sexes			**boys**			**girls**		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	0.08	0.17	*285*	**0.20**	**0.01**	*145*	–0.06	0.48	*140*
Bone mineral density	0.03	0.61	*285*	0.10	0.23	*145*	–0.04	0.68	*140*
Bone area	0.09	0.14	*285*	**0.20**	**0.02**	*145*	–0.04	0.64	*140*

Table 5.18: Maternal BMI and fetal core measurement; at 19 and 34 weeks.

Another surprising association between BMI and fetal measurement was the negative association of BMI and proximal CSA at 34 weeks in girls ($p = 0.03$, $r = -0.15$). The trend across the groups was also negative, but not significant. This negative trend was also seen between maternal BMI and mid-shaft CSA in girls at 34 weeks ($p<0.001$, $r = -0.26$).

Maternal BMI was positively associated in boys with both neonatal bone mineral content ($p = 0.01$, $r = 0.20$) and neonatal bone area ($p = 0.02$, $r = 0.20$). There was a negative trend with all three DXA parameters (BMC, BMD and BA) in girls.

5.14 Maternal skin-fold thickness and fetal scan measurements

The assessment of body composition, that is how much fat, lean tissue and bone a person has, is essential in evaluating their nutritional status. There is evidence that, for example, a mother's ability to nourish her fetus is influenced by her level of fatness before she becomes pregnant. There are numerous methods for measuring the amount of fat someone has, but many require expensive equipment and cannot be used in people's homes. Skin-fold thickness measurements are simple and non-invasive and require relatively inexpensive equipment. The sum of skin-fold thicknesses (SSF) was a pre-pregnant maternal variable chosen as a representative of a maternal fat / skin layers that could be compared with the fat / skin layers of her offspring. It is derived from the sum of triceps, biceps, sub-scapular and supra-iliac skin-fold measurements made using callipers and is expressed in mm. The protocol for measuring the sum of skin-fold thicknesses is included in Appendix V.

Maternal SSF was not associated with fetal tissue volumes (Table 5.19), with the exception of a negative association between SSF and proximal CSA at 19 weeks in girls ($p = 0.001$, $r = -0.21$). However by 34 weeks the greater the SSF in the mother prior to pregnancy, the greater the thigh volume in her fetus ($p<0.001$, $r = 0.22$). This association was rather stronger in boys than in girls ($p<0.001$, $r = 0.28$ for boys and $p = 0.05$, $r = 0.14$ for girls).

SSF was selected as it was a variable which could be compared with the fat / skin volume of her fetus and although there was no relationship at 19 weeks between maternal and fetal skin measures. This may be in part due to age at which this was tested in the fetus. Subcutaneous fat stores do not accumulate in the fetus until 28 weeks and so this layer at 19 weeks is small in comparison with the other thigh tissues. However, by 34 weeks there was an association between mothers with greater SSF and fetal fat / skin volumes ($p<0.001$, $r = 0.23$). Again this relationship was stronger in boys ($p<0.001$, $r = 0.30$) than in girls ($p = 0.03$, $r = 0.15$).

From the table it can be seen that at 34 weeks there was a strong association between maternal SSF and muscle volume (p = 0.001, r = 0.16). When divided by sex there was no association in girls but an association in boys (p <0.001, r = 0.24).

	Maternal sum of skin-fold thicknesses								
	both sexes			**boys**			**girls**		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.05	0.30	*426*	0.12	0.07	*217*	–0.04	0.56	*209*
Fat / skin volume (cm^3)	–0.01	0.81	*422*	0.08	0.27	*216*	–0.12	0.10	*206*
Muscle volume (cm^3)	0.05	0.30	*424*	0.07	0.31	*218*	0.02	0.78	*206*
Femur volume (cm^3)	–0.005	0.91	*479*	0.05	0.44	*245*	–0.07	0.28	*234*
Femur length (cm)	0.02	0.66	*502*	0.04	0.48	*257*	–0.02	0.79	*245*
Proximal CSA (cm^2)	–0.07	0.14	*483*	0.06	0.38	*249*	**–0.21**	**0.001**	*234*
Mid-shaft CSA (cm^2)	–0.006	0.90	*494*	0.04	0.51	*251*	–0.06	0.39	*243*
Distal CSA (cm^2)	0.001	0.98	*474*	0.07	0.27	*241*	–0.08	0.23	*233*

	Maternal sum of skin-fold thicknesses								
	both sexes			**boys**			**girls**		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.22**	**<0.001**	*424*	**0.28**	**<0.001**	*220*	**0.14**	**0.05**	*204*
Fat / skin volume (cm^3)	**0.23**	**<0.001**	*423*	**0.30**	**<0.001**	*219*	**0.15**	**0.03**	*204*
Muscle volume (cm^3)	**0.16**	**0.001**	*425*	**0.24**	**<0.001**	*220*	0.06	0.39	*205*
Femur volume (cm^3)	–0.007	0.89	*478*	0.08	0.24	*244*	–0.10	0.13	*234*
Femur length (cm)	**0.11**	**0.01**	*486*	0.10	0.10	*247*	0.12	0.07	*239*
Proximal CSA (cm^2)	–0.06	0.21	*465*	–0.01	0.87	*237*	–0.12	0.07	*228*
Mid-shaft CSA (cm^2)	–0.06	0.20	*484*	0.08	0.20	*246*	**–0.22**	**<0.001**	*238*
Distal CSA (cm^2)	–0.02	0.68	*463*	0.001	0.98	*238*	–0.05	0.49	*225*

	Maternal sum of skin-fold thicknesses								
	both sexes			**boys**			**girls**		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	0.08	0.16	*283*	**0.18**	**0.03**	*143*	–0.01	0.93	*140*
Bone mineral density	0.06	0.33	*283*	0.12	0.14	*143*	0.02	0.84	*140*
Bone area	0.08	0.16	*283*	**0.17**	**0.04**	*143*	0.004	0.97	*140*

Table 5.19: Maternal sum of skin-fold thicknesses and core fetal scan measurements; at 19 and 34 weeks. Maternal SSF was measured in mm.

Increased maternal SSF was associated with greater femur length at 34 weeks ($p = 0.01$, $r = 0.11$), but this association weakened when divided the group was by gender ($p = 0.07$, $r = 012$ for girls and $p = 0.10$, $r = 0.01$ for boys). Interestingly, maternal SSF had a strong inverse relationship mid-shaft CSA at 34 weeks in girls alone ($p < 0.001$, $r = -0.22$) which was absent in boys and thus not significant for the group as a whole.

From Table 5.19 it can be seen that there was a positive association between SSF and bone mineral content in boys ($p = 0.03$, $r = 0.18$), but not for girls. Additionally, increased maternal SSF was associated with increased bone area in boys ($p = 0.04$, $r = 0.17$). There was no association between maternal SSF and neonatal bone mineral density for either boys or girls.

5.15 Maternal arm muscle area and fetal scan measurements

The measurement of arm muscle area (AMA) has been devised to assess adult muscle mass using upper arm measurements. It is derived from the triceps skin-fold measurement (mm) and mid-arm circumference (mm). In practice this derived measurement tends to overestimate arm muscle area when compared with CT evaluation of the same tissues. To correct for this an equation developed by Heymsfield and co-workers, was applied. This involved subtracting the bone component of the upper arm from the figure obtained and this correction factor was utilised for the AMA measurements. The derived value is expressed as cm^2 and the protocol for this measurement and the equation by which it is derived in shown in Appendix V. AMA was then correlated with the core fetal scan measurements and DXA readings. The results are shown in Table 5.20.

Maternal AMA and fetal thigh volume were significantly associated at 19 weeks, (Table 5.20) ($p = 0.02$, $r = 0.11$). There was also a weak relationship between maternal AMA and muscle volume in girls at 19 weeks ($p = 0.08$, $r = 0.12$), which was slightly stronger but did not reach significance in the combined group ($p = 0.06$, $r = 0.09$). Maternal AMA and thigh volume were strongly associated at 34 weeks ($p < 0.001$, $r = 0.17$). After dividing by sex, the significance was lost in girls ($p = 0.09$, $r = 0.12$) but retained in boys ($p = 0.004$, $r = 0.19$). Increased maternal AMA was strongly associated with increased fat / skin volumes at 34 weeks (Table 5.21) ($p = 0.007$, $r = 0.13$), but once this was divided by sex the relationship was only significant in boys ($p = 0.02$, $r = 0.16$). It was anticipated that greater maternal AMA would be associated with greater muscle volume in the offspring and at 34 weeks this proved to be the case ($p < 0.001$, $r = 0.18$). However, this association was only true in boys once the group had been divided by sex ($p = 0.004$, $r = 0.19$ for boys and $p = 0.07$, $r = 0.13$ for girls). As can be seen from Table 5.20 maternal AMA was not associated with bone volume or femur dimensions at 19 or 34 weeks, or neonatal DXA readings.

	Maternal arm muscle area								
	both sexes			boys			girls		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.11**	**0.02**	*427*	0.08	0.27	*218*	0.12	0.08	*209*
Fat / skin volume (cm^3)	0.09	0.08	*423*	0.08	0.22	*217*	0.06	0.37	*206*
Muscle volume (cm^3)	0.09	0.06	*425*	0.04	0.55	*219*	0.12	0.08	*206*
Femur volume (cm^3)	0.01	0.84	*480*	0.04	0.56	*246*	–0.04	0.54	*234*
Femur length (cm)	0.02	0.68	*503*	0.06	0.36	*258*	–0.02	0.71	*245*
Proximal CSA (cm^2)	–0.05	0.32	*483*	0.002	0.97	*241*	–0.08	0.23	*234*
Mid-shaft CSA (cm^2)	–0.007	0.88	*495*	–0.01	0.89	*252*	–0.01	0.88	*243*
Distal CSA (cm^2)	0.03	0.52	*474*	–0.02	0.78	*249*	0.05	0.49	*233*

	Maternal arm muscle area								
	both sexes			boys			girls		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	**0.17**	**<0.001**	*426*	**0.19**	**0.004**	*222*	0.12	0.09	*204*
Fat / skin volume (cm^3)	**0.13**	**0.007**	*425*	**0.16**	**0.02**	*221*	0.09	0.18	*204*
Muscle volume (cm^3)	**0.18**	**<0.001**	*427*	**0.19**	**0.004**	*222*	0.13	0.07	*205*
Femur volume (cm^3)	–0.05	0.27	*479*	–0.03	0.61	*245*	–0.08	0.20	*234*
Femur length (cm)	0.03	0.48	*487*	0.06	0.33	*248*	0.003	0.97	*239*
Proximal CSA (cm^2)	–0.02	0.60	*467*	0.01	0.86	*239*	–0.08	0.25	*228*
Mid-shaft CSA (cm^2)	0.02	0.69	*485*	0.09	0.16	*247*	–0.06	0.38	*238*
Distal CSA (cm^2)	–0.04	0.42	*465*	–0.04	0.57	*240*	–0.05	0.48	*225*

	Maternal arm muscle area								
	both sexes			boys			girls		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	0.05	0.45	*285*	0.10	0.22	*145*	–0.03	0.71	*140*
Bone mineral density	0.02	0.76	*285*	0.07	0.42	*145*	–0.04	0.67	*140*
Bone area	0.04	0.48	*285*	0.10	0.24	*145*	–0.03	0.70	*140*

Table 5.20: Maternal arm muscle area and core fetal scan measurements; 19 and 34 weeks. Maternal AMA was measured in cm^2.

5.16 Maternal influences on fetal size and growth

Measured maternal parameters as already described, were taken at the pre-pregnant interview by research nurses. These were measures of adult physical dimensions and body composition. This next section outlines some maternal characteristics other than those measured before pregnancy;

the mother's age at delivery, her own birthweight, how many children she delivered prior to the pregnancy being studied and if she had smoked before this pregnancy.

5.16.1 Maternal age at delivery and fetal scan measurements

	Maternal age								
	both sexes			boys			girls		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.03	0.55	*431*	–0.03	0.65	*220*	0.10	0.14	*211*
Fat / skin volume (cm^3)	0.05	0.27	*427*	0.004	0.95	*219*	0.12	0.08	*208*
Muscle volume (cm^3)	0.03	0.61	*429*	–0.03	0.65	*221*	0.09	0.18	*208*
Femur volume (cm^3)	–0.02	0.65	*484*	–0.10	0.11	*248*	0.08	0.20	*236*
Femur length (cm)	0.04	0.41	*508*	0.02	0.81	*261*	0.06	0.35	*247*
Proximal CSA (cm^2)	<0.001	1.00	*488*	–0.01	0.83	*252*	0.01	0.83	*236*
Mid-shaft CSA (cm^2)	–0.02	0.65	*500*	–0.06	0.36	*255*	0.02	0.75	*245*
Distal CSA (cm^2)	–0.04	0.35	*479*	**–0.13**	**0.04**	*244*	0.05	0.42	*235*
	Maternal age								
	both sexes			**boys**			**girls**		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.01	0.82	*428*	–0.05	0.50	*223*	0.07	0.31	*205*
Fat / skin volume (cm^3)	0.01	0.81	*427*	–0.04	0.55	*222*	0.07	0.34	*205*
Muscle volume (cm^3)	0.01	0.78	*429*	–0.03	0.72	*223*	0.05	0.47	*206*
Femur volume (cm^3)	–0.02	0.60	*483*	–0.10	0.11	*247*	0.06	0.38	*236*
Femur length (cm)	0.01	0.83	*491*	0.03	0.64	*250*	–0.01	0.85	*241*
Proximal CSA (cm^2)	0.03	0.48	*470*	–0.04	0.55	*240*	0.11	0.10	*230*
Mid-shaft CSA (cm^2)	–0.04	0.36	*489*	0.03	0.70	*249*	–0.12	0.07	*240*
Distal CSA (cm^2)	–0.02	0.65	*468*	–0.07	0.31	*241*	0.03	0.66	*227*
	Maternal age								
	both sexes			**boys**			**girls**		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	–0.09	0.12	*285*	–0.13	0.11	*145*	–0.03	0.72	*140*
Bone mineral density	–0.09	0.15	*285*	–0.08	0.35	*145*	–0.09	0.29	*140*
Bone area	–0.08	0.19	*285*	–0.13	0.12	*145*	–0.003	0.97	*140*

Table 5.21: Maternal age and core fetal scan measurements; 19 and 34 weeks.

The 517 mothers ranged in age at delivery from 22 to 40, with a mean age of 31. Table 5.21 shows there were no associations between maternal age at delivery and fetal scan measurements, with the

exception of an inverse association with proximal CSA in boys at 19 weeks ($p = 0.04$, $r = -0.13$), suggesting that as mother's age increased the proximal CSA became smaller. Additionally maternal age had no association with neonatal DXA results.

5.16.2 Maternal birthweight and fetal scan measurements

Maternal birthweight was ascertained from the women themselves, from their mothers' or from hospital records from Princess Anne Hospital, where many of the women had themselves been delivered. There were 450 maternal birthweights recorded and 515 of their offspring had recorded weight at delivery. There were 448 instances where both maternal and neonatal birthweights could be compared and the mean birthweight of the mothers was 204g smaller than their offspring. The relationship between a mother's birthweight and that of her offspring shows that there was a significant relationship between these two variables for the group as a whole ($p<0.001$, $r = 0.16$). However, when divided by gender it showed that this relationship was only significant for boys ($p = 0.002$, $r = 0.2$). Mother's birthweight was then examined in relation to the core fetal measurements and DXA readings, to ascertain if her own weight at birth had influence on the body composition of her offspring. The findings are tabulated in Table 5.22.

There were no associations between maternal birthweight and fetal scan measurements at 19 weeks (Table 5.22). However, increased maternal birthweight was associated with increased thigh volume at 34 weeks ($p = 0.05$, $r = 0.10$). This association weakened in boys after gender division ($p = 0.08$, $r = 0.13$), and was not apparent for girls alone ($p = 0.32$, $r = 0.07$). Additionally, at 34 weeks there was a significant association between maternal birthweight and fat / skin volume ($p = 0.04$, $r = 0.11$). Gender specific analysis showed as association in boys ($p = 0.03$, $r = 0.16$), but not in girls ($p = 0.47$, $r = 0.06$). There was no overall association between maternal birthweight and muscle or femur volumes in the fetus at 34 weeks. However, there was a significant association for femur volume in girls ($p = 0.02$, $r = 0.16$), which was not seen in boys.

With regard to bone parameters, there were only two significant associations between maternal birthweight and femur measurements at 34 weeks. Firstly, maternal birthweight and femur length ($p = 0.04$, $r = 0.10$); gender specific analyses showed that boys tended to have longer femur lengths if their mothers were heavier at birth ($p = 0.07$, $r = 0.12$), whereas girls showed no such association ($p = 0.35$, $r = 0.07$). Secondly, there was a positive association between maternal birthweight and proximal CSA in girls at 34 weeks ($p = 0.03$, $r = 0.16$). However, this association was not seen in boys ($p = 0.87$, $r = -0.01$). There were also no associations maternal birthweight and neonatal DXA readings.

	Maternal birthweight								
	both sexes			boys			girls		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm³)	–0.01	0.84	*379*	<0.001	0.99	*196*	–0.02	0.80	*183*
Fat / skin volume (cm³)	0.003	0.94	*375*	–0.002	0.98	*195*	0.01	0.85	*180*
Muscle volume (cm³)	–0.01	0.82	*375*	0.01	0.94	*195*	–0.03	0.74	*180*
Femur volume (cm³)	–0.008	0.88	*423*	–0.02	0.77	*218*	0.01	0.84	*205*
Femur length (cm)	–0.01	0.80	*441*	<0.001	1.00	*228*	–0.03	0.72	*213*
Proximal CSA (cm²)	0.04	0.44	*425*	–0.02	0.81	*221*	0.10	0.15	*204*
Mid-shaft CSA (cm²)	–0.03	0.58	*434*	–0.02	0.78	*223*	–0.04	0.61	*211*
Distal CSA (cm²)	–0.03	0.48	*418*	–0.11	0.10	*215*	0.05	0.45	*203*

	Maternal birthweight								
	both sexes			boys			girls		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm³)	**0.10**	**0.05**	*376*	0.13	0.08	*196*	0.07	0.32	*180*
Fat / skin volume (cm³)	**0.11**	**0.04**	*375*	**0.16**	**0.03**	*195*	0.06	0.47	*180*
Muscle volume (cm³)	0.08	0.15	*377*	0.09	0.22	*196*	0.07	0.36	*181*
Femur volume (cm³)	0.02	0.68	*421*	–0.10	0.15	*217*	**0.16**	**0.02**	*204*
Femur length (cm)	**0.10**	**0.04**	*428*	0.12	0.07	*220*	0.07	0.35	*208*
Proximal CSA (cm²)	0.06	0.22	*408*	–0.01	0.87	*210*	**0.16**	**0.03**	*198*
Mid-shaft CSA (cm²)	0.03	0.53	*426*	0.01	0.85	*219*	0.05	0.44	*207*
Distal CSA (cm²)	0.01	0.83	*407*	–0.05	0.48	*211*	0.09	0.24	*196*

	Maternal birthweight								
	both sexes			boys			girls		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	–0.03	0.67	*253*	–0.04	0.69	*130*	0.03	0.77	*123*
Bone mineral density	–0.06	0.31	*253*	–0.06	0.49	*130*	–0.05	0.58	*123*
Bone area	–0.01	0.84	*253*	–0.03	0.76	*130*	0.05	0.58	*123*

Table 5.22: Maternal birthweight and fetal core scan measurements; 19 and 34 weeks.

5.16.3 Maternal parity and fetal scan measurements

The SWS cohort was recruited from a population of women who included those who had never been pregnant (nulliparous) and those who had already had one child (primiparous) or children (multiparous). The effect of parity was then examined to see if this had any influence on the size, growth and skeletal development of the fetus.

	Maternal parity status					
Fetal measurement : 19 weeks		mean	95% CI	n	paired t- test	
					t	p
Thigh volume (cm^3)	null	6.358	6.194 – 6.521	*256*	–1.47	0.14
	multi	6.550	6.347 – 6.753	*175*		
Fat / skin volume (cm^3)	null	1.789	1.717 – 1.860	*254*	–0.88	0.38
	multi	1.840	1.748 – 1.932	*173*		
Muscle volume (cm^3)	null	4.078	3.396 – 4.200	*256*	–1.51	0.13
	multi	4.229	4.073 – 4.384	*173*		
Femur volume (cm^3)	null	0.470	0.459 – 0.481	*289*	–1.70	0.09
	multi	0.486	0.471 – 0.500	*195*		
Femur length (cm)	null	2.910	2.887 – 2.932	*302*	–0.81	0.42
	multi	2.924	2.897 – 2.951	*206*		
Proximal CSA (cm^2)	null	0.265	0.259 – 0.271	*292*	–0.85	0.39
	multi	0.270	0.261 – 0.278	*196*		
Mid-shaft CSA (cm^2)	null	0.130	0.128 – 0.133	*298*	0.25	0.80
	multi	0.130	0.126 – 0.134	*202*		
Distal CSA (cm^2)	null	0.226	0.220 – 0.232	*290*	–0.28	0.78
	multi	0.227	0.220 – 0.235	*189*		
	Maternal parity status					
Fetal measurement : 34 weeks		mean	95% CI	n	paired t-test	
					t	p
Thigh volume (cm^3)	null	91.760	89.899 – 93.621	*250*	**–2.11**	**0.04**
	multi	94.912	92.599 – 97.225	*178*		
Fat / skin volume (cm^3)	null	37.851	36.765 – 38.937	*249*	**–2.73**	**0.007**
	multi	40.208	38.889 – 41.527	*178*		
Muscle volume (cm^3)	null	51.208	50.172 – 52.244	*250*	–0.93	0.35
	multi	51.976	50.705 – 53.246	*179*		
Femur volume (cm^3)	null	2.769	2.707 – 2.832	*283*	–1.34	0.18
	multi	2.833	2.764 – 2.903	*200*		
Femur length (cm)	null	6.349	6.316 – 6.382	*290*	–1.21	0.23
	multi	6.380	6.342 – 6.417	*201*		
Proximal CSA (cm^2)	null	1.021	0.996 – 1.046	*277*	0.76	0.45
	multi	1.006	0.977 – 1.035	*193*		
Mid-shaft CSA (cm^2)	null	0.336	0.328 – 0.344	*288*	–0.95	0.34
	multi	0.342	0.332 – 0.352	*201*		
Distal CSA (cm^2)	null	0.786	0.763 – 0.810	*273*	0.33	0.74
	multi	0.781	0.755 – 0.806	*195*		

Table 5.23: Parity and core fetal measurements; 19 and 34 weeks; t-tests performed to highlight differences between nulliparous and multiparous mothers. (N.B. null = nulliparous, multi = multiparous).

It can be seen from Table 5.23 there were weak and non-significant associations between parity and the fetal measurements 19 weeks gestation. However, multiparous mothers are more likely to have male babies with larger femur volumes than nulliparous mothers ($p = 0.03$, $t = -2.13$). The full breakdown of sex differences for parity influences is included in Appendix VI. At 34 weeks it can be seen from Table 5.23 that there were two significant findings with regard to fetal volumes. Firstly, thigh volume in fetuses whose mothers were multiparous were significantly larger than those in fetuses of nulliparous mothers ($p = 0.04$, $t = -2.11$). Secondly, the fat / skin volume of these fetuses were greater if their mother was multiparous ($p = 0.007$, $t = -2.73$). On further inspection it was seen that this association was only apparent in boys ($p = 0.02$, $t = -2.28$), (shown in the Table in Appendix VI). Thus parity can be seen to influence specific fetal variables, but more particularly in boys.

There were no associations between maternal parity and neonatal DXA readings.

It is known from other studies that children born to women who have already carried children to full term that subsequent children are likely to be of increasing birthweight. By using regression analysis the effect of parity and birthweight was examined and is presented in Table 5.24 where it can be seen that for both sexes there is a mean increase of 182g from the first to the second baby ($p<0.001$, $t = 4.0$).

	Birthweight trend and parity								
	both sexes			**boys**			**girls**		
Weight increase	increase (g)	t	p	increase (g)	t	p	increase (g)	t	p
From 1st to 2nd baby	182	**4.0**	**<0.001**	186	**2.9**	**0.004**	168	**2.6**	**0.009**
From 1st to 3rd + baby	233	**3.8**	**<0.001**	263	**3.1**	**0.002**	182	**2.1**	**0.04**
Trend over 3 groups	133	**4.8**	**<0.001**	145	**3.7**	**<0.001**	112	**2.8**	**<0.001**

Table 5.24: The effect of parity on birthweight for the group.

5.16.4 Maternal smoking status and fetal scan measurements

The next maternal characteristic to be examined was the smoking status of the mother before pregnancy. Of the entire cohort at the time of the pre-pregnant interview, 380 reported that they were non-smokers and 137 had reported to be regular smokers. The smoking status of the individuals data collected during pregnancy was not available at the time of the analysis, but the data presented here does serve to show that effects in the growth and development of a fetus can be influenced if the mother has smoked prior to conception.

	Maternal smoking status					
Fetal measurement : 19 weeks		mean	95% CI	n	*t*- test	
					t	p
Thigh volume (cm^3)	non-smoker	6.391	6.245 – 6.536	*318*	–1.17	0.24
	smoker	6.562	6.302 – 6.823	*113*		
Fat / skin volume (cm^3)	non-smoker	1.786	1.725 – 1.848	*316*	–1.37	0.17
	smoker	1.876	1.747 – 2.005	*111*		
Muscle volume (cm^3)	non-smoker	4.125	4.013 – 4.236	*317*	–0.48	0.63
	smoker	4.178	3.985 – 4.370	*112*		
Femur volume (cm^3)	non-smoker	0.476	0.466 – 0.486	*358*	–0.10	0.92
	smoker	0.466	0.459 – 0.495	*126*		
Femur length (cm)	non-smoker	2.911	2.891 – 2.931	*372*	–0.90	0.37
	smoker	2.929	2.893 – 2.965	*136*		
Proximal CSA (cm^2)	non-smoker	0.267	0.261 – 0.273	*359*	<0.01	1.00
	smoker	0.267	0.257 – 0.277	*129*		
Mid-shaft CSA (cm^2)	non-smoker	0.130	0.128 – 0.133	*365*	0.26	0.79
	smoker	0.130	0.126 – 0.134	*135*		
Distal CSA (cm^2)	non-smoker	0.228	0.223 – 0.234	*354*	1.27	0.21
	smoker	0.222	0.214 – 0.230	*125*		
	Maternal smoking status					
Fetal measurement : 34 weeks		mean	95% CI	n	*t*-test	
					t	p
Thigh volume (cm^3)	non-smoker	93.149	91.510 – 94.789	*313*	0.18	0.86
	smoker	92.856	89.758 – 95.955	*115*		
Fat / skin volume (cm^3)	non-smoker	38.851	37.885 – 39.817	*312*	0.07	0.95
	smoker	38.787	37.052 – 40.522	*115*		
Muscle volume (cm^3)	non-smoker	51.608	50.709 – 52.508	*314*	0.32	0.75
	smoker	51.310	49.596 – 53.034	*115*		
Femur volume (cm^3)	non-smoker	2.805	2.751 – 2.859	*354*	0.66	0.51
	smoker	2.770	2.679 – 2.862	*129*		
Femur length (cm)	non-smoker	6.368	6.340 – 6.397	*361*	0.88	0.38
	smoker	6.343	6.293 – 6.393	*130*		
Proximal CSA (cm^2)	non-smoker	1.019	0.996 – 1.042	*344*	0.69	0.49
	smoker	1.000	0.970 – 1.038	*126*		
Mid-shaft CSA (cm^2)	non-smoker	0.339	0.332 – 0.346	*359*	0.28	0.78
	smoker	0.337	0.325 – 0.349	*130*		
Distal CSA (cm^2)	non-smoker	0.794	0.774 – 0.814	*344*	1.87	0.06
	smoker	0.757	0.725 – 0.789	*124*		

Table 5.25: Pre-pregnant smoking and core fetal measurements; 19 and 34 weeks.

T-tests were employed to see if the smoking status was associated with the 8 core measurements at 19 and 34 weeks and DXA readings. The results are shown in Table 5.25 where it can be seen that at 19 weeks there were small differences in the mean values for the core measurements and none of these differences were significant. It was the same picture at 34 weeks where there appeared to be no association between maternal pre-pregnant smoking status and the core measurements taken of her fetus at scan. The full tabulation of sex differences and smoking status are shown in Appendix VII.

There were, additionally, no associations between maternal pre-pregnancy smoking status and any of the readings for neonatal BMC, BMD or total bone area. Other studies have seen an association between smoking and bone density, but the data used was based on maternal smoking during the pregnancy which provided a clearer picture of maternal smoking habits.

5.17 Pregnancy influences on fetal size and growth

For a fetus to grow well its mother must provide adequate nutrition to aid musculo-skeletal development. As previously described in Chapter 1, vitamin D plays a crucial role as it is the essential precursor of 1,25-dihydroxyvitamin D_3, the steroid hormone required for calcium absorption, bone development and growth in children. For this reason maternal blood was taken so that vitamin D concentrations could be ascertained, so that correlation with fetal scan and DXA would be possible.

This aided the examination of possible associations between bone, muscle and fat / skin growth in the fetus and levels of vitamin D in its mother's blood. The concentrations were also correlated with the splaying index described previously, to examine possible associations between vitamin D levels and bone shape. The results are tabulated in Table 5.28. It was not within the SWS protocol to obtain blood samples from the mothers at 19 weeks and so bloods from the 34 week aliquots were used in the analysis.

5.17.1 Maternal vitamin D status and fetal scan measurements

At the time of the 34 week scan, a research nurse gained consent from the participant for blood to be taken. Three samples of blood were taken by venous puncture totalling in 27.5ml of whole blood and aliquots were frozen at –80°C in freezers at the MRC ERC. Out of the cohort of 517, some volunteers did not consent for a blood sample being taken for a variety of reasons and in some cases a sample could not be obtained. Therefore the number of blood samples obtained was 424 (82% of the study group). Concentrations of 1,25-dihydroxyvitamin D_3 of the 424 subjects were obtained using the frozen serum at a specialist laboratory at St. Thomas' Hospital, London. A summary of the measurements, expressed in nmol per litre (nmol/L) of blood serum, is shown on Table 5.16. A synopsis of the vitamin D immunoassay technique used to obtain the concentrations is included in the Appendix X.

The conventional marker of vitamin D status is plasma 25-hydroxyvitamin D, which is the gold standard for determining vitamin D status of an individual. This marker is employed because it reflects the main storage form of precursor substrate for vitamin D, 1,25-dihyroxyvitamin D, the formation of which is under homeostatic control, (Holick, 2003 and 2005).

The classification of normal and abnormal vitamin D status for non-pregnant individuals is currently under debate, but many centres involved in commercial assays for vitamin D adhere to the values listed below.

< 12.5 nmol/L	Deplete and associated with rickets or osteomalacia.
12.5 – 25 nmol/L	Deficient.
25 – 50 nmol/L	Insufficient.
50 – 70 nmol/L	Possibly insufficient.
> 80 nmol/L	Optimal for bone and muscle health.
> 200 nmol/L	Excessive or intoxicated.

Some centres in the United States quote values of 25 – 37.5 nmol/L to 137.5 – 162.5 nmol/L, as being the 'normal' range, but this is not truly reflective of whether a patient is vitamin D deficient or intoxicated. It is estimated that the body uses daily on average 3000 to 5000 IU of cholecalciferol and in the absence of sun exposure, 1000 IU is needed to maintain health at 78 nmol/L (30 ng/mL). Above 80 nmol/L (32 ng/mL) vitamin D has been shown to improve muscle strength and bone mineral density in adults. The current vitamin D requirements in the United States are based on protection against bone diseases, but these guidelines are being revised upward in light of new findings, especially for soft tissue health. Much debate has centred on

supplementing pregnant women, as their circulating vitamin D is in demand from the growing fetus. However, precisely how much is required and what is considered a 'normal' circulating level during pregnancy has not been established.

Publications from the Institute of Medicine in the United States suggest that women taking less than 3.8 μg (150 IU)/day had an average serum 25(OH)D concentration of 22.75 ± 3.75 nmol/L, while women taking more than 12.5 μg (500 IU)/day had a concentration of 27.75 ± 3.25 nmol/L. Although the authors noted no significant difference between these two values in the mothers, newborns of mothers who received the vitamin D supplement had a statistically higher serum calcium level on the fourth day of life than those from mothers with the lower vitamin D intake. Several studies have evaluated supplementation after the pregnant woman's first trimester with either 10 or 25 μg (400 or 1,000 IU)/day of vitamin D. It was concluded that vitamin D supplementation increased circulating concentrations of 25(OH)D in the mother and may improve neonatal handling of calcium. In the SWS cohort nutritional data suggested that very few women were taking supplementary vitamin D during pregnancy, about 3% of the entire cohort. Supplementation data for the 424 subgroup who gave blood for this study have not been examined.

The levels seen in pregnant women are currently under scrutiny and as presently there are no contemporary reference guidelines. Findings from the Institute of Health also reveal that during pregnancy, there is a gradual rise in a woman's serum $1,25(OH)_2D$ concentration that is paralleled by an increase in her blood concentration of vitamin D binding protein. During the last trimester, the woman's serum $1,25(OH)_2D$ level continues to rise without any change in the vitamin D binding protein level, causing an increase in the free concentration of $1,25(OH)_2D$. Evidence is strong that the placenta metabolizes 25(OH)D to $1,25(OH)_2D$ and therefore contributes to the maternal and possibly fetal blood levels of $1,25(OH)_2D$. These findings suggest a trend in the serum levels, but to date have not been quantified for a population.

Although there is ample evidence for placental transfer of 25(OH)D from the mother to the fetus the quantities are relatively small and do not appear to affect the overall vitamin D status of pregnant women. The recommendations published state that women, whether pregnant or not, who receive regular exposure to sunlight do not need vitamin D supplementation. However, at vitamin D intakes less than 150 IU/day, pregnant women during the winter months at high latitudes had a mean 25(OH)D concentration of 22.75 nmol/L at delivery. Thus, there is no additional need to increase the vitamin D oral intake during pregnancy above that required for non-pregnant women. However, an intake of 400 IU/day, which is supplied by prenatal vitamin supplements, would not be excessive.

Without recognised serum levels available for pregnant women, for the purposes of this study, vitamin D status was assessed using non-pregnant levels; below 25 nmol/L termed as 'deplete'; 25 to 50 nmol/L as 'insufficient'; between 50 and 70 nmol/L as 'possibly insufficient' and above 70 nmol/L as 'replete' or 'sufficient'. These levels have previously been adopted in UK studies prior to this work.

From the results obtained it was seen that the values of vitamin D concentrations ranged between 8 and 180 nmol/L for the 424 samples assayed, with a mean value of 65.6 nmol/L. The distribution for the cohort is shown in Figure 5.8, where it can be seen that 41.5% (176 women) have blood serum values above 70 nmol/L, in the *replete* or sufficient range. Another 98 women (23.1%) lie in the range between 50 and 70 nmol/L and can be considered 'possibly insufficient'. Those lying between 25 and 50 nmol/L numbered 125 women (29.5%) were considered vitamin D insufficient. The remaining 25 women (5.9%) showed values less than 25 nmol/L and were considered vitamin D deplete.

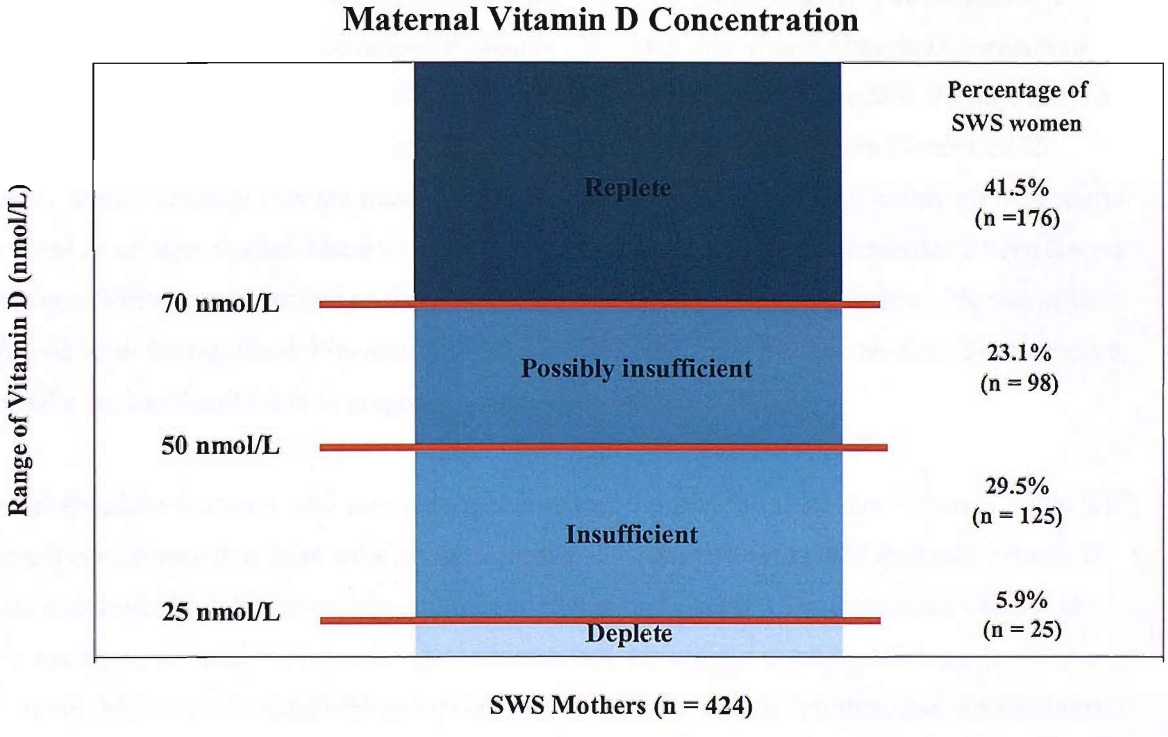


Figure 5.8: Categories of vitamin D sufficiency within the mothers of the study group.

The study cohort was examined with regard to any difference in the vitamin D concentrations depending on which sex child they were carrying. From Table 5.26 it can be seen that circulating vitamin D is slightly higher in concentration in the 209 women carrying girls (14 to 180 nmol/L, mean 66.25 nmol/L) than for the 215 women carrying boys (8 to 143 nmol/L, mean 65.02 nmol/L). However, a t-test showed that this difference was not statistically significant ($p = 0.64$, $r = -0.47$).

Maternal vitamin D (nmol/L)				
	n	min	max	mean
both	*424*	8	180	65.63
boys	*215*	8	143	65.02
girls	*209*	14	180	66.25

Table 5.26: Maternal vitamin D and fetal gender differences.

It has been seen in previous studies that skin tone has a significant effect on the amount of photochemistry needed to produce vitamin D within the blood and so the ethnicity and vitamin D concentrations of the women was examined. In the complete cohort of 517 women, 16 (3.09%) were non-white. However, mothers who gave a blood sample numbered 424 and of these women 2.46% were non-white in ethnic origin. When ranked by lowest concentration of vitamin D it was noticed that the lowest 4 values belonged to Asian women (3) and one black African woman. Their values ranged from 8 to 20 nmol/L of vitamin D. Of the remaining 6 women, 4 were within the lowest 30% of the values (1 black Afro-Caribbean, 1 Chinese and 2 Asians). The remaining 2 subjects were an Eastern Mediterranean woman of olive skin tone whose vitamin D concentration was 70 nmol/L, which slightly above the mean for the study cohort of 65 nmol/L. Additionally, a Chinese woman with some white Caucasian blood relatives showed a vitamin D value of 85 nmol/L, which was well over the mean for the group. The distribution found in this study supports the findings of other studies where vitamin D concentrations in pregnant women have been lowest in the non-Western mothers and as such are the women most at risk in producing offspring with impaired bone development. Previous data from the Princess Anne Study, also from Southampton, showed a similar distribution in pregnant women, (Javaid *et al.*, 2006).

The relationships between fetal core measurements and the concentrations are shown in Table 5.27, where it can be seen that there were no relationships between late pregnancy maternal vitamin D status and thigh, fat / skin or muscle volumes at 19 weeks. Lower levels of maternal vitamin D were weakly associated with greater fetal volumes, but this did not reach significance ($p = 0.09$, $r = -0.09$). Maternal vitamin D status was not related to femur length, but there was a weak inverse association with mid-shaft CSA ($p = 0.07$, $r = -0.09$). When examining the relationship between maternal vitamin D status and fetal femoral metaphyseal areas, it revealed that lower levels of maternal vitamin D were associated with greater proximal CSA ($p = 0.02$, $r = -0.12$). When divided by gender this finding was confined to the girls ($p = 0.02$, $r = -0.17$). There was also a weak inverse association between decreased levels of vitamin D and increased mid-shaft CSA ($p = 0.07$, $r = -0.09$). In addition, distal CSA was inversely associated with maternal vitamin D at 19 weeks ($p = 0.003$, $r = -0.15$). It could be seen that the association was similar in both sexes when divided

($p = 0.07$, $r = -0.13$ for girls and $p = 0.03$, $r = -0.15$ for boys); this is shown in Figure 5.27. At 34 weeks there were no significant associations between maternal vitamin D and fetal thigh tissue volumes. However there was a weak association between increased maternal vitamin D status and fetal fat / skin volume, but this did not reach significance ($p = 0.08$, $r = 0.09$). It can be seen from Table 5.27 there was a weak association between maternal vitamin D status and femur length in girls at 34 weeks ($p = 0.06$, $r = 0.13$). This relationship was not seen in the boys.

	Maternal vitamin D								
	both sexes			**boys**			**girls**		
Fetal measurement : 19 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	–0.06	0.23	*352*	–0.08	0.28	*178*	–0.02	0.78	*174*
Fat / skin volume (cm^3)	–0.05	0.40	*349*	–0.002	0.98	*177*	–0.08	0.30	*172*
Muscle volume (cm^3)	–0.04	0.46	*351*	–0.06	0.41	*179*	–0.003	0.97	*172*
Femur volume (cm^3)	–0.09	0.09	*395*	–0.08	0.24	*200*	–0.08	0.29	*195*
Femur length (cm)	0.03	0.61	*417*	–0.004	0.96	*211*	0.06	0.41	*206*
Proximal CSA (cm^2)	**–0.12**	**0.02**	*398*	–0.04	0.53	*202*	**–0.17**	**0.02**	*196*
Mid-shaft CSA (cm^2)	–0.09	0.07	*409*	–0.10	0.17	*205*	–0.08	0.28	*204*
Distal CSA (cm^2)	**–0.15**	**0.003**	*391*	**–0.15**	**0.03**	*196*	–0.13	0.07	*195*

	Maternal vitamin D								
	both sexes			**boys**			**girls**		
Fetal measurement : 34 weeks	r	p	n	r	p	n	r	p	n
Thigh volume (cm^3)	0.05	0.35	*360*	0.07	0.34	*188*	0.03	0.49	*172*
Fat / skin volume (cm^3)	0.09	0.08	*360*	0.10	0.17	*188*	0.09	0.25	*172*
Muscle volume (cm^3)	0.01	0.79	*362*	0.03	0.72	*189*	0.02	0.76	*173*
Femur volume (cm^3)	0.00	0.99	*406*	–0.03	0.68	*207*	0.04	0.56	*199*
Femur length (cm)	0.08	0.12	*414*	0.03	0.67	*210*	0.13	0.06	*204*
Proximal CSA (cm^2)	–0.06	0.20	*395*	–0.01	0.88	*202*	–0.12	0.10	*193*
Mid-shaft CSA (cm^2)	0.02	0.66	*412*	0.05	0.52	*209*	–0.005	0.94	*203*
Distal CSA (cm^2)	**–0.11**	**0.03**	*395*	–0.05	0.45	*203*	**–0.17**	**0.02**	*192*

	Maternal vitamin D								
	both sexes			**boys**			**girls**		
Neonatal DXA readings	r	p	n	r	p	n	r	p	n
Bone mineral content	0.09	0.19	*243*	0.04	0.64	*125*	0.13	0.15	*118*
Bone mineral density	–0.05	0.45	*243*	–0.12	0.18	*125*	0.03	0.78	*118*
Bone area	0.10	0.12	*243*	0.06	0.48	*125*	0.14	0.12	*118*

Table 5.27: Maternal vitamin D and core fetal scan measurements; vitamin D measured in nmol/L.

Additionally, maternal vitamin D status and proximal CSA were no longer associated by 34 weeks, nor were mid-shaft CSA. However the relationship seen at 19 weeks between maternal vitamin D status and distal CSA was significantly inversely related ($p = 0.03$, $r = -0.11$). It was interesting to observe that this inverse association was present in girls at 34 weeks ($p = 0.02$, $r = -0.17$), but not in boys ($p = 0.45$, $r = -0.05$).

To demonstrate the associations between maternal vitamin D and fetal distal metaphyseal enlargement, the vitamin D distribution was divided into quarters and plotted against distal CSA for 19 weeks (Figure 5.9). It can be seen that with decreased vitamin D, the values for the distal CSA increase.

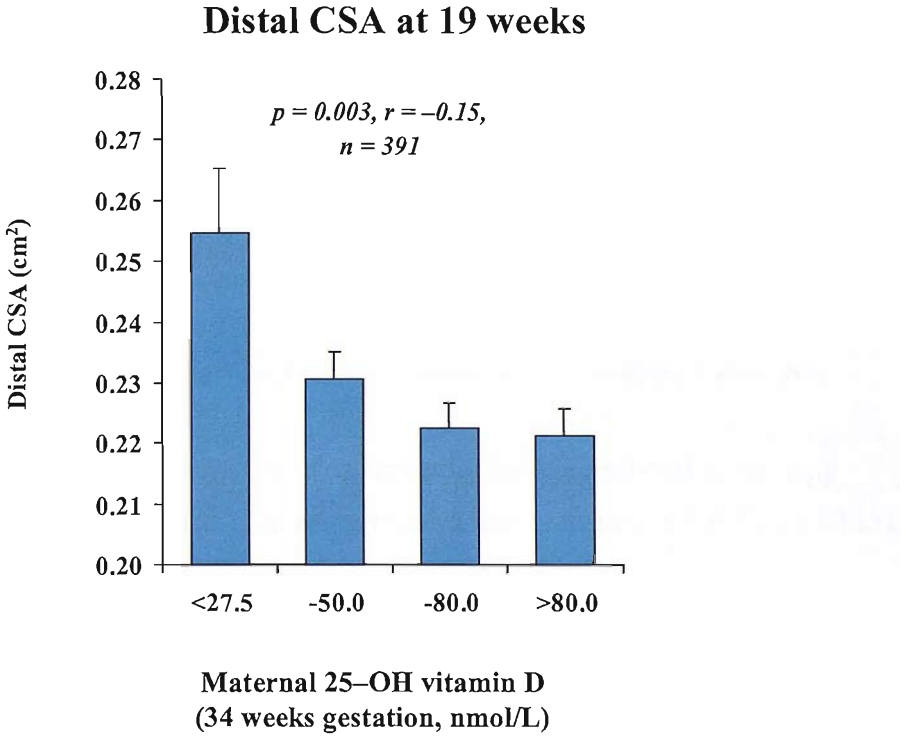


Figure 5.9: Maternal vitamin D and fetal distal CSA at 19 weeks. Values are means and 95% CI.

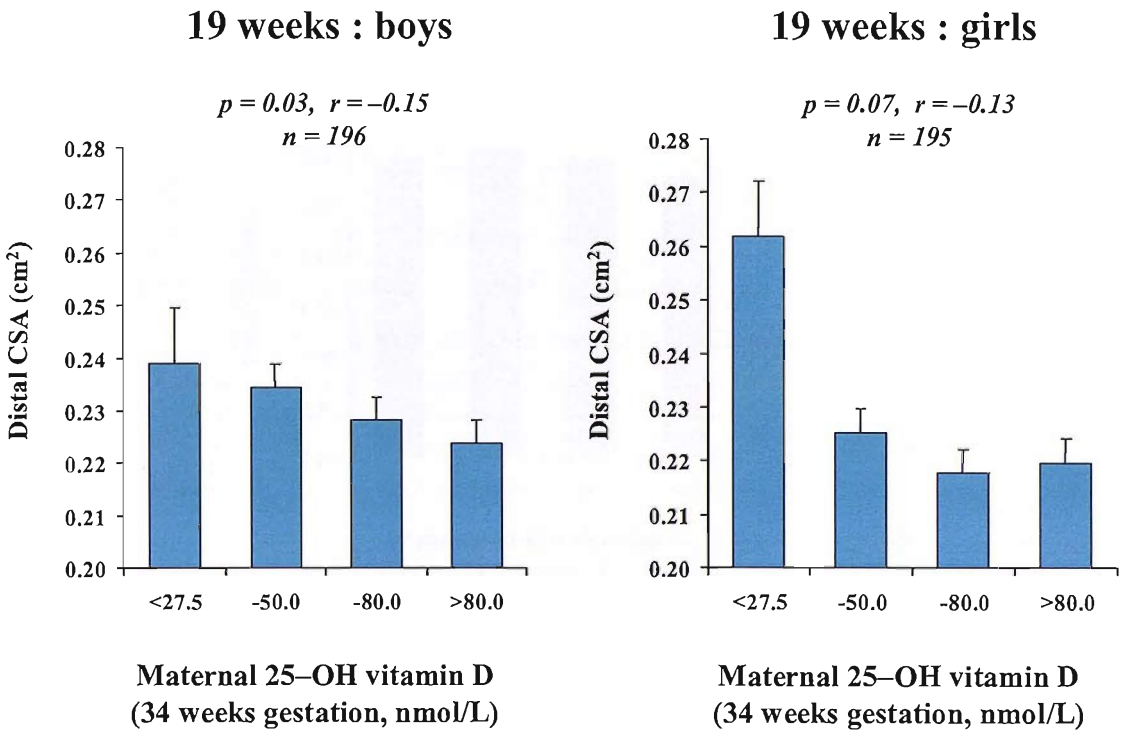


Figure 5.10: Maternal vitamin D and fetal distal CSA at 19 weeks. Values are means and 95% CI.

When divided by sex, as shown in Figure 5.10 demonstrates that the relationship was only significant in boys ($p = 0.03$, $r = -0.15$) and was greatly weakened in girls ($p = 0.07$, $r = -0.13$).

When the association between maternal vitamin D and fetal femoral distal CSA was examined at 34 weeks, it could be seen that the relationship remained inversely significant for the entire group ($p = 0.03$, $r = -0.11$). Figure 5.11 shows the distribution divided into quarters.

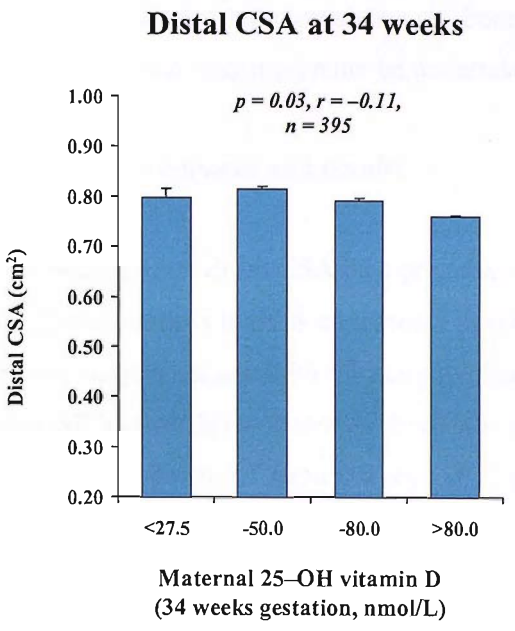


Figure 5.11: Maternal vitamin D and fetal distal CSA at 34 weeks. Values are means and 95% CI.

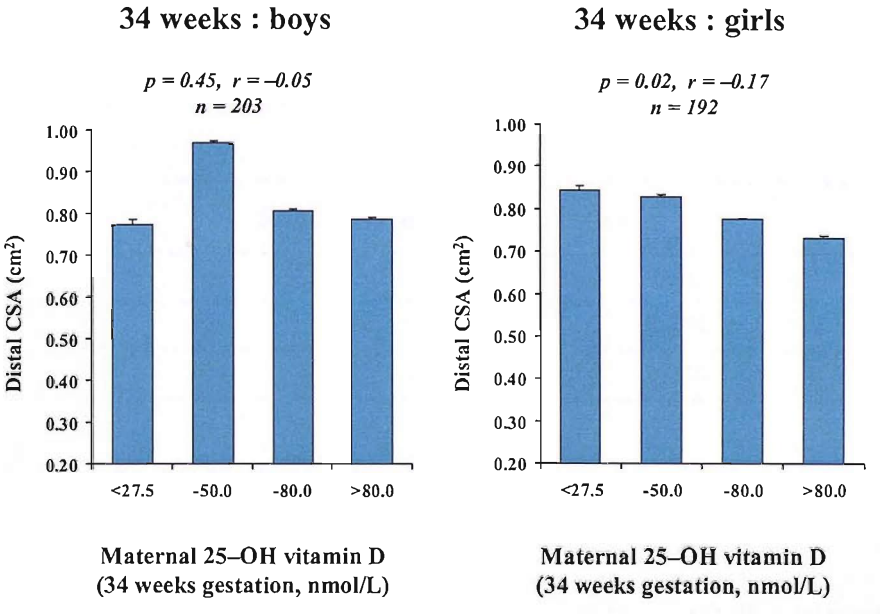


Figure 5.12: Maternal vitamin D and fetal distal CSA at 34 weeks (both sexes). Values: means and 95% CI.

On division for gender, the histograms in Figure 5.12 shows that the relationship only remained in girls ($p = 0.02$, $r = -0.17$), indicating that greater maternal blood vitamin D was associated with smaller distal CSA, particularly in girls at 34 weeks. These results indicate that lower levels of maternal vitamin D are having an effect on the geometry of the developing femur, which may be sex specific.

From the Table 5.27 it can be seen that circulating maternal vitamin D concentrations at 19 and 34 weeks were not associated with neonatal bone mineral content, bone mineral density or total bone area. This was surprising and further investigation must be undertaken must be done to answer this.

5.17.2 Splaying index development and results

It is possible that fetuses showing greater distal CSA than proximal CSA are displaying early signs of vitamin D insufficiency. This manifests itself as an increase in size at the growing ends of the metaphysis when vitamin D is not present to inhibit the activity of osteoblasts (bone-forming cells). This results in 'trumpet-shaped' bone ends most commonly seen in the long bones of children with vitamin D insufficiency (rickets) as shown in Chapter 4, page 91, Figure 4.3.

To quantify the degree of splaying, an index was devised which takes into account the length of the bone being studied and this index may aid the identification of fetuses showing this variation, perhaps associated with maternal vitamin D insufficiency. The index was derived by dividing the distal CSA by the femur length. Femur length was used as it is a measure which is strongly associated with gestational age, unless there is evidence of chromosomal abnormality in the fetus; distal CSA was used as it was considered that this structure was more likely to be influenced by altered concentrations of vitamin D. The summary results are shown in Table 5.28.

Summary of splaying index				
	n	min	max	mean
19 weeks	*391*	0.038	0.153	0.078
34 weeks	*395*	0.066	0.270	0.124

Table 5.28: Splaying index summary statistics for 19 and 34 weeks.

The splaying index showed there was a range of indices from 0.038 to 0.153 at 19 weeks (mean 0.078). The spread of values was similar at 34 weeks from a minimum of 0.066 to a maximum of 0.270 (mean 0.124). Lower index values indicated that the distal CSA showed less evidence of splaying in relation to the femur length, whereas higher indices denoted greater splaying of the distal femoral metaphysis in relation to the femur length.

To further investigate the influence of maternal vitamin D on the geometry of her offspring's femur, the derived splaying index was correlated against the logged vitamin D concentrations

(Table 5.29). The values were log transformed as the majority of the samples were similar, but there were outliers at the uppermost and lowest ends of the ranges. By logging the values regression analysis can be made possible.

	Maternal vitamin D and splaying index								
	both sexes			**boys**			**girls**		
	r	p	n	r	p	n	r	p	n
19 weeks	**–0.17**	**<0.001**	*391*	**–0.18**	**0.01**	*196*	**–0.16**	**0.03**	*195*
34 weeks	–0.09	0.07	*395*	–0.01	0.85	*203*	**–0.18**	**0.01**	*192*

Table 5.29: Maternal vitamin D and splaying index at 19 and 34 weeks.

The derived splaying index takes into account the degree of splaying in relation to the length of the bone. Lower late pregnancy maternal vitamin D concentration with greater metaphyseal splaying of the distal fetal femur impacts on the splaying index. This relationship is highly significant across both sexes ($p<0.001$, $r = -0.17$), Figure 5.13. This association was present for each sex when divided by gender ($p = 0.01$, $r = -0.18$ for boys and $p = 0.03$, $r = -0.16$ for girls).

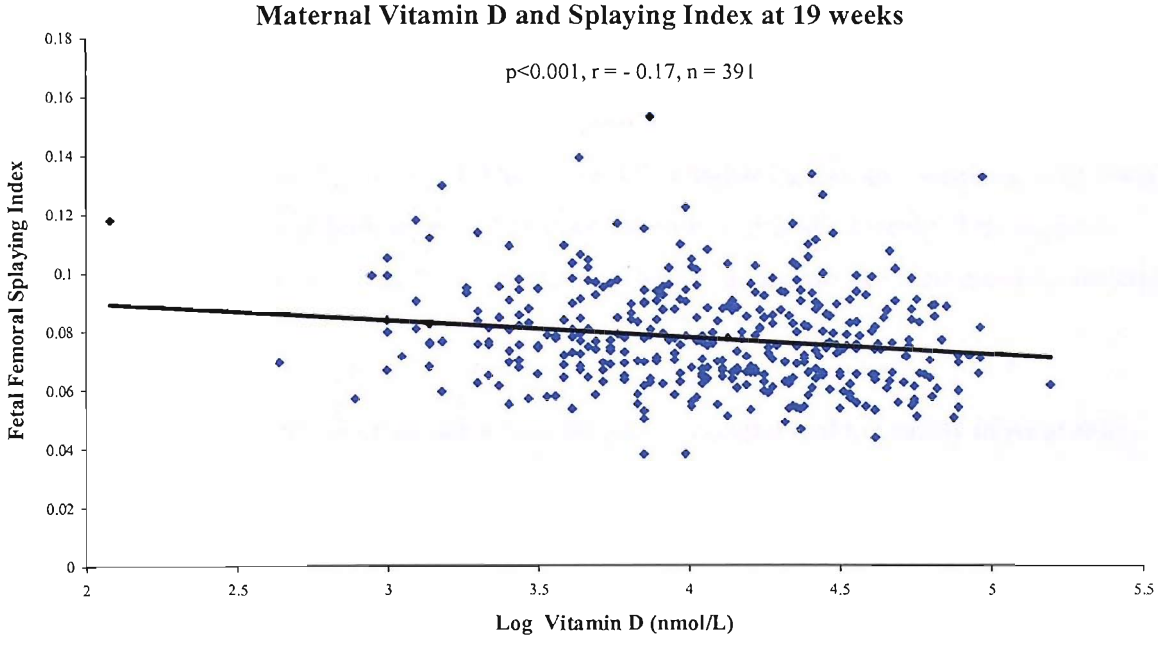


Figure 5.13: Maternal vitamin D and splaying index distribution at 19 weeks.

The scatter plot above demonstrates the relationship between maternal vitamin D concentrations and the splaying index at 19 weeks. The regression line clearly shows an inverse significant association, where decreased vitamin D concentration was associated with a decreased splaying index. The index was derived from distal CSA and femur length. This again illustrates the relationship between maternal vitamin D concentrations in the blood during pregnancy and the development of the fetal skeleton. The situation at 34 weeks (Figure 5.14) shows that this association was weaker ($p = 0.07$, $r = -0.09$).

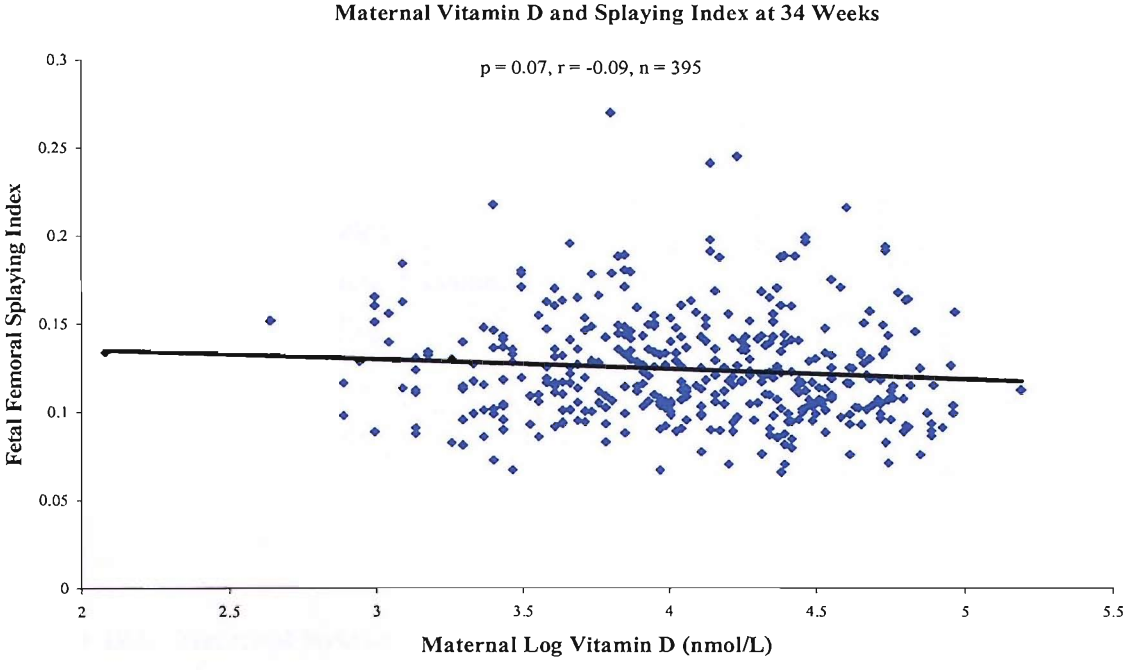


Figure 5.14: Maternal vitamin D and splaying index distribution at 34 weeks.

However, when divided by sex the association was not apparent for boys, but remained highly significant for girls ($p = 0.01$, $r = -0.18$). This denotes that higher indices are associated with lower vitamin D concentrations in both sexes at 19 weeks but only in girls at 34 weeks. This suggests they are at risk from vitamin D insufficiency that may lead to impairment of their musculo-skeletal development.

With further refinement the splaying index may be used as a useful tool to identify those at risk from vitamin D insufficiency and will be addressed in future work.

5.18 Fetal growth velocities and maternal influences

How well a fetus grows and develops can be influenced by the uterine environment. To be able to measure the effect of any maternal influence would be a useful tool in predicting fetal outcome. The difference in size of all of the core measurements between the 19 week and the 34 week scan was measured in percentage change, using measurements corrected to z scores at 19 and 34 weeks, so that regression analysis could be performed. The percentage change was then correlated with the maternal characteristics already outlined, to examine maternal influences on fetal growth. The maternal variables used were:

Maternal birthweight
Maternal age at delivery
Maternal height
Maternal body mass index
Maternal sum of skin-fold thicknesses
Maternal arm muscle area
Maternal parity status
Maternal smoking status
Maternal vitamin D status

5.18.1 Maternal birthweight and fetal growth velocity

From Table 5.30 it can be seen that higher maternal birthweight was associated with greater fetal thigh volume growth velocity ($p = 0.01$, $r = 0.14$); this association was of similar strength in boys ($p = 0.05$, $r = 0.15$) and girls ($p = 0.13$, $r = 0.12$). Higher maternal birthweight was also significantly associated with increased fat / skin volume growth velocity ($p = 0.05$, $r = 0.11$); this effect was more marked in boys ($p = 0.08$, $r = 0.14$) than in girls ($p = 0.37$, $r = 0.07$).

There was only a weak association between maternal birthweight and fetal muscle volume growth velocity, which did not reach significance ($p = 0.07$, $r = 0.10$). Although maternal birthweight had no overall association with femur volume growth velocity, Table 5.30 shows that there was a positive association in girls ($p = 0.04$, $r = 0.15$).

Table 5.30 also shows that higher maternal birthweight was strongly associated with greater fetal femur length growth velocity ($p = 0.001$, $r = 0.16$). This relationship was still significant in both sexes when the group was divided ($p = 0.01$, $r = 0.17$ for boys and $p = 0.05$, $r = 0.14$ for girls).

There were no associations between maternal birthweight and growth velocity of femur CSA.

	Maternal birthweight								
	both sexes			boys			girls		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	**0.14**	**0.01**	*323*	**0.15**	**0.05**	*168*	0.12	0.13	*155*
Fat / skin volume	**0.11**	**0.05**	*319*	0.14	0.08	*166*	0.07	0.37	*153*
Muscle volume	0.10	0.07	*321*	0.09	0.25	*167*	0.11	0.17	*154*
Femur volume	0.06	0.25	*296*	–0.03	0.73	*203*	**0.15**	**0.04**	*193*
Femur length	**0.16**	**0.001**	*419*	**0.17**	**0.01**	*215*	**0.14**	**0.05**	*204*
Proximal CSA	0.01	0.84	*387*	0.05	0.46	*200*	-0.04	0.57	*187*
Mid-shaft CSA	0.03	0.55	*410*	–0.05	0.45	*209*	0.10	0.14	*201*
Distal CSA	0.02	0.72	*378*	0.04	0.62	*194*	0.005	0.95	*184*

Table 5.30: Maternal birthweight and fetal growth velocity; core scan measurements form 19 to 34 weeks.

5.18.2 Maternal age at delivery and fetal growth velocity

Maternal age did not appear to have any significant association with growth velocity of bone or soft tissue volumes in the offspring. As can be seen from Table 5.31, there was a weak inverse association between age and growth velocity of the mid-shaft CSA in girls, but this did not reach significance ($p = 0.06$, $r = -0.12$).

	Maternal age								
	both sexes			boys			girls		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	–0.04	0.40	*368*	–0.007	0.93	*191*	–0.08	0.27	*177*
Fat / skin volume	–0.05	0.37	*364*	–0.04	0.59	*189*	–0.06	0.47	*175*
Muscle volume	–0.06	0.25	*366*	–0.03	0.67	*190*	–0.10	0.17	*176*
Femur volume	–0.03	0.58	*454*	–0.04	0.53	*231*	–0.001	0.99	*223*
Femur length	–0.06	0.26	*482*	–0.02	0.80	*245*	–0.09	0.18	*237*
Proximal CSA	–0.006	0.90	*446*	–0.09	0.20	*228*	0.08	0.22	*218*
Mid-shaft CSA	–0.04	0.35	*472*	0.04	0.59	*238*	–0.12	0.06	*234*
Distal CSA	0.005	0.92	*434*	0.009	0.89	*220*	0.004	0.95	*214*

Table 5.31: Maternal age at delivery and fetal growth velocity from 19 to 34 weeks.

5.18.3 Maternal height and fetal growth velocity

From Table 5.32 it can be seen that taller mothers had fetuses with greater thigh volume growth velocity ($p = 0.04$, $r = 0.11$), but this association was not maintained when the group was divided by sex. Maternal height was not associated with fat / skin or femur volume growth velocity, but was positively associated with muscle volume growth velocity ($p = 0.009$, $r = 0.14$).

	Maternal height								
	both sexes			**boys**			**girls**		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	**0.11**	**0.04**	*366*	0.12	0.11	*190*	0.10	0.20	*176*
Fat / skin volume	0.05	0.33	*362*	0.01	0.88	*188*	0.10	0.21	*174*
Muscle volume	**0.14**	**0.009**	*364*	**0.18**	**0.01**	*189*	0.08	0.30	*175*
Femur volume	0.06	0.23	*452*	0.009	0.89	*230*	0.11	0.10	*222*
Femur length	**0.12**	**0.009**	*480*	0.12	0.06	*244*	0.12	0.08	*236*
Proximal CSA	0.05	0.28	*444*	0.06	0.38	*227*	0.05	0.51	*217*
Mid-shaft CSA	–0.02	0.71	*470*	–0.08	0.20	*237*	0.05	0.44	*233*
Distal CSA	0.004	0.94	*432*	0.04	0.53	*219*	–0.04	0.56	*213*

Table 5.32: Maternal height and fetal growth velocity from 19 to 34 weeks.

When divided by sex this association was apparent in boys ($p = 0.01$, $r = 0.18$), but not in the girls ($p = 0.30$, $r = 0.8$). Maternal height was weakly associated with femur length growth velocity for both sexes ($p = 0.06$, $r = 0.12$ for boys and $p = 0.08$, $r = 0.12$ for girls), but became significant in the group as a whole ($p = 0.009$, $r = 0.12$). However, maternal height was not associated with CSA growth velocity.

To illustrate the associations between maternal height and the growth velocity of her offspring's bone there follows two histograms showing the relationship of firstly, maternal height and femur volume growth velocity (Figure 5.15) and secondly, maternal height and femur length growth velocity, (Figure 5.16). These histograms serve to demonstrate that maternal height has influence on the growth velocity of the length of her baby's bones, but not necessarily the volume. It can also be seen that increased femur length growth velocity is more likely to occur if the mother is above 164 cm in height.

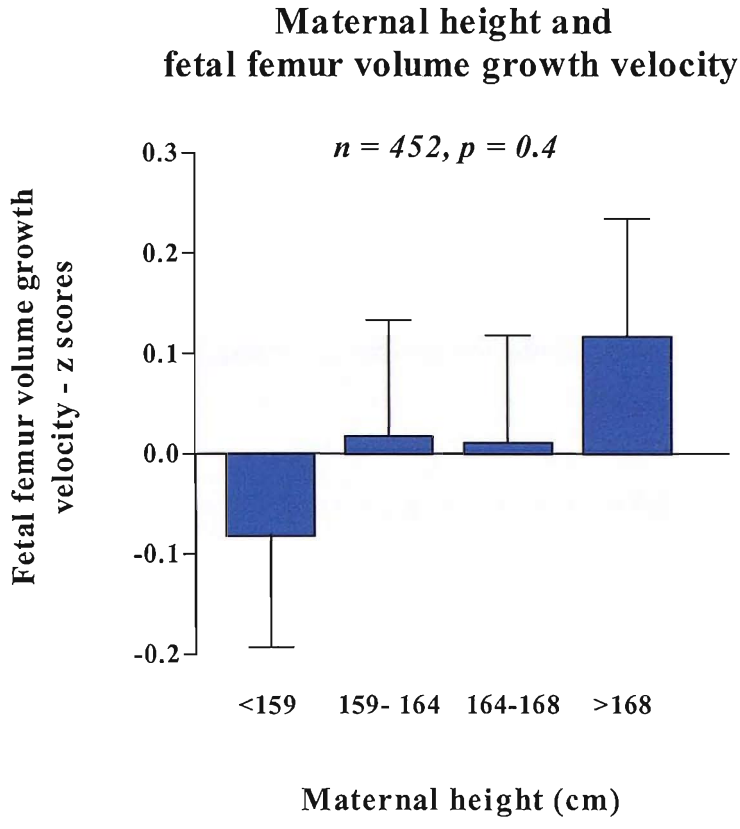


Figure 5.15: Maternal height and fetal femur volume growth velocity. Values are means and SEM.

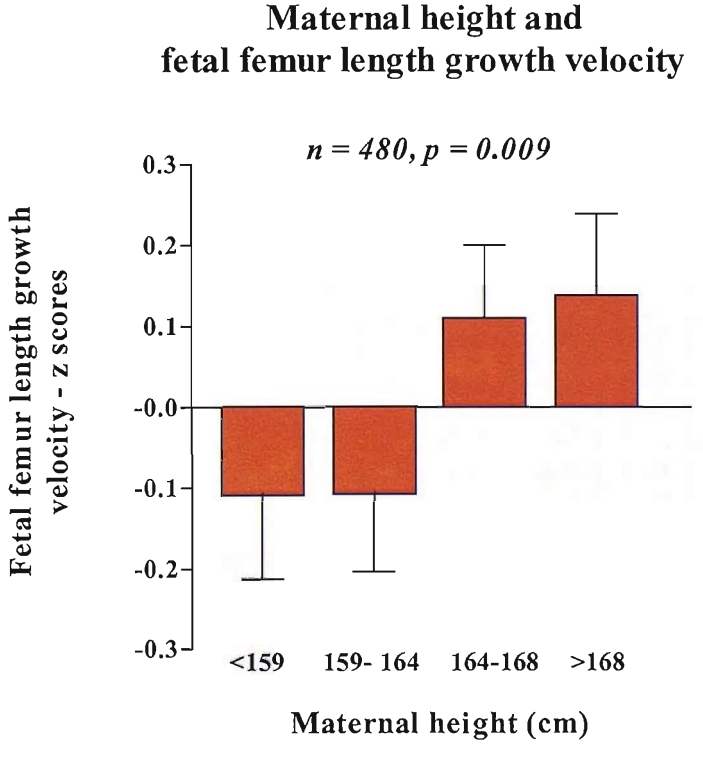


Figure 5.16: Maternal height and fetal femur length growth velocity. Values are means and SEM.

5.18.4 Maternal body mass index and fetal growth velocity

From Table 5.33 it can be seen that maternal BMI was significantly associated with fetal thigh volume, fat / skin volume and muscle volume growth. Women with the greatest BMI had fetuses with the greatest fetal thigh volume growth velocity ($p = 0.008$, $r = 0.14$); this association was similar in boys ($p = 0.04$, $r = 0.15$) and in girls ($p = 0.09$, $r = 0.13$). This pattern was also seen for increased BMI values being associated with increased growth velocity of the fat / skin volumes in the group, ($p = 0.006$, $r = 0.14$); again this association was similar in boys ($p = 0.03$, $r = 0.16$) and in girls ($p = 0.08$, $r = 0.13$).

The influence of higher BMI was also seen in the growth velocity of fetal muscle volume ($p = 0.03$, $r = 0.12$); this effect was somewhat stronger in boys ($p = 0.05$, $r = 0.15$) than in girls ($p = 0.08$, $r = 0.32$).

Maternal BMI was not associated with fetal bone growth velocity, except for an isolated inverse association for the growth velocity of the mid-shaft CSA in girls ($p = 0.009$, $r = -0.17$); suggesting this part of the femur grew less well in girls whose mothers had a higher BMI.

	Maternal BMI								
	both sexes			**boys**			**girls**		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	**0.14**	**0.008**	*366*	**0.15**	**0.04**	*190*	0.13	0.09	*176*
Fat / skin volume	**0.14**	**0.006**	*362*	**0.16**	**0.03**	*188*	0.13	0.08	*174*
Muscle volume	**0.12**	**0.03**	*364*	**0.15**	**0.05**	*189*	0.08	0.32	*175*
Femur volume	–0.05	0.26	*452*	–0.07	0.32	*189*	–0.04	0.52	*222*
Femur length	0.03	0.54	*480*	0.06	0.37	*244*	–0.009	0.89	*236*
Proximal CSA	0.02	0.69	*444*	–0.03	0.70	*227*	0.08	0.27	*217*
Mid-shaft CSA	–0.07	0.15	*470*	0.03	0.67	*237*	**–0.17**	**0.009**	*233*
Distal CSA	–0.05	0.35	*432*	–0.09	0.19	*219*	0.004	0.96	*213*

Table 5.33: Maternal BMI and fetal growth velocity from 19 to 34 weeks.

5.18.5 Maternal sum of skin-fold thicknesses and fetal growth velocity

From Table 5.34 it can be seen that higher maternal SSF was associated with greater growth velocity in fetal thigh volume, fat / skin volume and muscle volume. The association between maternal SSF and fetal thigh volume growth velocity was highly significant ($p<0.001$, $r = 0.22$) for the entire group and this association was very strong in both sexes ($p = 0.003$, $r = 0.22$ for boys and $p = 0.002$, $r = 0.23$ for girls).

Increased maternal SSF influenced the growth velocity of fetal fat / skin volumes in both sexes ($p<0.001$, $r = 0.22$), which remained true for each sex separately ($p = 0.007$, $r = 0.20$ for boys and $p<0.001$, $r = 0.26$ for girls).

Greater maternal SSF was also associated with increased fetal muscle volume growth velocity in the study group ($p = 0.004$, $r = 0.15$), but this association was only present in boys when divided by sex ($p = 0.01$, $r = 0.18$).

There were no significant associations between maternal SSF and fetal femur volume, length or CSA, although there was a weak negative relation between maternal SSF and mid-shaft CSA in girls ($p = 0.07$, $r = -0.12$).

	Maternal sum of skin-fold thicknesses								
	both sexes			**boys**			**girls**		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	**0.22**	**<0.001**	*364*	**0.22**	**0.003**	*188*	**0.23**	**0.002**	*176*
Fat / skin volume	**0.22**	**<0.001**	*360*	**0.20**	**0.007**	*186*	**0.26**	**<0.001**	*174*
Muscle volume	**0.15**	**0.004**	*362*	**0.18**	**0.01**	*187*	0.10	0.19	*175*
Femur volume	–0.02	9.71	*449*	<0.001	1.00	*228*	–0.04	0.58	*221*
Femur length	0.07	0.12	*477*	0.04	0.50	*242*	0.10	0.12	*235*
Proximal CSA	–0.004	0.94	*441*	–0.07	0.30	*225*	0.07	0.31	*216*
Mid-shaft CSA	–0.04	0.36	*467*	0.03	0.63	*235*	–0.12	0.07	*232*
Distal CSA	0.003	0.96	*429*	–0.03	0.64	*217*	0.04	0.55	*212*

Table 5.34: Maternal SSF and fetal growth velocities from 19 to 34 weeks.

5.18.6 Maternal arm muscle area and fetal growth velocity

From Table 5.35 it can be seen that greater maternal AMA was associated with higher growth velocity of fetal thigh volume ($p = 0.02$, $r = 0.12$) and muscle volumes ($p = 0.002$, $r = 0.17$), but no other fetal variables. Much of the fetal thigh comprises muscle tissue and so it is perhaps not surprising that maternal AMA was associated with both thigh and muscle volume growth velocities. The association of increased maternal AMA with thigh muscle growth velocity was significant for both sexes ($p = 0.01$, $r = 0.18$ for boys and $p = 0.04$, $r = 0.16$ for girls).

	Maternal arm muscle area								
	both sexes			**boys**			**girls**		
Growth velocity : 19 to 34 weeks (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	**0.12**	**0.02**	*366*	**0.15**	**0.04**	*190*	0.11	0.16	*176*
Fat / skin volume	0.05	0.37	*362*	0.08	0.26	*188*	0.009	0.90	*174*
Muscle volume	**0.17**	**0.002**	*364*	**0.18**	**0.01**	*189*	**0.16**	**0.04**	*175*
Femur volume	–0.04	0.47	*450*	–0.07	0.28	*229*	0.01	0.85	*221*
Femur length	0.02	0.63	*478*	0.02	0.73	*243*	0.02	0.77	*235*
Proximal CSA	–0.007	0.89	*443*	0.006	0.92	*227*	–0.008	0.90	*216*
Mid-shaft CSA	0.02	0.76	*468*	0.06	0.35	*236*	–0.02	0.75	*232*
Distal CSA	–0.05	0.33	*431*	–0.06	0.35	*219*	–0.02	0.81	*212*

Table 5.35: Maternal AMA and fetal growth velocity from 19 to 34 weeks.

5.18.7 Maternal parity status and fetal growth velocity

From Table 5.36 it can be seen that there were no significant associations between the parity of the mother and growth velocity of the core fetal scan measurements from 19 to 34 weeks. However, when the group was divided by sex there was an isolated significant finding in boys. It showed that the proximal CSA growth velocity was greater in male fetuses born to mothers who were multiparous when compared with those who were primiparous ($p = 0.01$, $t = 2.49$).

The breakdown for each sex is tabulated in Appendix VIII. So from these results it appears that parity status has little influence on the 19 to 34 week growth velocity of the fetal tissues of the thigh.

Fetal growth velocity (z scores)	Maternal parity status					
		mean	95% CI	n	*t*- test	
					t	p
Thigh volume (cm^3)	null	0.01	–0.131 – 0.149	*215*	–0.48	0.63
	multi	0.06	–0.102 – 0.224	*153*		
Fat / skin volume (cm^3)	null	–0.04	–0.210 – 0.125	*213*	–1.13	0.26
	multi	0.11	–0.097 – 0.316	*151*		
Muscle volume (cm^3)	null	0.03	–0.110 – 0.170	*214*	0.39	0.70
	multi	–0.01	–0.171 – 0.147	*152*		
Femur volume (cm^3)	null	0.04	–0.106 – 0.176	*268*	0.31	0.76
	multi	0.001	–0.176 – 0.179	*186*		
Femur length (cm)	null	–0.01	–0.135 – 0.114	*285*	–0.42	0.68
	multi	0.03	–0.124 – 0.188	*197*		
Proximal CSA (cm^2)	null	0.71	–0.077 – 0.219	*266*	1.61	0.11
	multi	–0.12	–0.316 – 0.067	*180*		
Mid-shaft CSA (cm^2)	null	–0.05	–0.203 – 0.099	*279*	–0.81	0.42
	multi	0.05	–0.147 – 0.243	*193*		
Distal CSA (cm^2)	null	0.07	–0.087 – 0.222	*259*	0.54	0.59
	multi	0.001	–0.187 – 0.188	*175*		

Table 5.36: Maternal parity and fetal growth velocity from 19 to 34 weeks. (N.B. null = nulliparous, multi = multiparous)

5.18.8 Maternal smoking status and fetal growth velocity

From Table 5.37 it can be seen that fetal thigh volume growth velocity tended to be slower in fetuses whose mothers smoke ($p = 0.06$, $t = 1.88$) although this relationship did not quite reach significance. This was also true of femur length, where mothers who smoked tended to have fetuses whose femur length growth velocity was slower, although this did not reach significance.

When the group was divided by sex it was seen that for mothers who smoked, the proximal CSA growth velocity was slower in boys ($p = 0.05$, $t = 1.95$); similarly smoking mothers produced offspring with the slower distal CSA growth velocity ($p = 0.08$, $t = 1.76$). This did not reach significance and was not seen in girls. The breakdown for sex is tabulated in Appendix IX.

	Maternal smoking status					
Fetal growth velocity (z scores)		mean	95% CI	n	*t*- test	
					t	p
Thigh volume (cm^3)	non-smoker	0.091	–0.025 – 0.208	*270*	1.88	0.06
	smoker	–0.137	–0.371 – 0.097	*98*		
Fat / skin volume (cm^3)	non-smoker	0.069	–0.071 – 0.209	*268*	1.22	0.22
	smoker	–0.114	–0.416 – 0.189	*96*		
Muscle volume (cm^3)	non-smoker	0.060	–0.057 – 0.177	*269*	1.48	0.14
	smoker	–0.119	–0.345 – 0.107	*97*		
Femur volume (cm^3)	non-smoker	0.050	–0.078 – 0.177	*333*	0.79	0.43
	smoker	–0.052	–0.278 – 0.174	*121*		
Femur length (cm)	non-smoker	0.062	–0.048 – 0.171	*353*	1.84	0.07
	smoker	–0.143	–0.347 – 0.060	*129*		
Proximal CSA (cm^2)	non-smoker	0.025	–0.115 – 0.164	*325*	0.89	0.37
	smoker	–0.095	–0.314 – 0.123	*121*		
Mid-shaft CSA (cm^2)	non-smoker	–0.017	–0.161 – 0.127	*344*	–0.16	0.87
	smoker	0.005	–0.208 – 0.217	*128*		
Distal CSA (cm^2)	non-smoker	0.075	–0.068 – 0.218	*318*	0.93	0.35
	smoker	–0.053	–0.267 – 0.160	*116*		

Table 5.37: Maternal smoking and with fetal growth velocity from 19 to 34 weeks.

5.18.9 Maternal vitamin D status and fetal growth velocity

Growth velocity of the 8 core fetal measurements was correlated with maternal vitamin D concentrations and the results are tabulated in Table 5.38. It can be seen there was a weak association between maternal vitamin D status and fetal fat / skin volume growth velocity which did not quite reach significance ($p = 0.06$, $r = 0.11$). However, division by sex showed this relationship was significant in girls ($p = 0.04$, $r = 0.17$), but not in boys ($p = 0.59$, $r = 0.04$).

There were no other relationships between maternal vitamin D status at 34 weeks and fetal growth velocity. As the blood samples were only analysed from one point in the pregnancy (34 weeks) this may have influenced the analysis of maternal vitamin D and its relationship with fetal growth velocity.

	Maternal vitamin D								
	both sexes			**boys**			**girls**		
Fetal growth velocity (z scores)	r	p	n	r	p	n	r	p	n
Thigh volume	0.08	0.17	*309*	0.06	0.42	*161*	0.09	0.27	*148*
Fat / skin volume	0.11	0.06	*307*	0.04	0.59	*160*	**0.17**	**0.04**	*147*
Muscle volume	–0.01	0.85	*309*	0.006	0.94	*161*	–0.03	0.72	*148*
Femur volume	0.07	0.17	*380*	0.05	0.53	*193*	0.10	0.19	*187*
Femur length	0.06	0.20	*407*	0.03	0.71	*206*	0.11	0.12	*201*
Proximal CSA	0.05	0.36	*374*	0.03	0.72	*191*	0.07	0.37	*183*
Mid-shaft CSA	0.07	0.16	*397*	0.08	0.27	*199*	0.05	0.50	*198*
Distal CSA	0.03	0.60	*366*	0.06	0.38	*185*	–0.03	0.73	*181*

Table 5.38: Maternal vitamin D and fetal growth velocity from 19 to 34 weeks.

The data produced from the analysis covers correlations between fetal and maternal characteristics, and DXA readings. The significant findings have been tabulated and appear in the Appendices X, XI and XII. The salient points and significance of the results described here will be discussed in the next chapter.

Chapter 6 – Discussion

Introduction

The research presented here has accomplished a number of purposes; namely the development of a novel technique for imaging fetal bone *in utero* and the application of this technique to measure fetal musculo-skeletal development. Another purpose was to validate the techniques employed in the acquisition of volume data sets for reproducibility obtained using 3D ultrasound. This validation had not been present in literature published prior to this research.

Additionally the use of 3D in fetal imaging has led to analysis of maternal influences on fetal body composition, bone shape, fetal size and growth. An unexpected finding was the relationship between circulating vitamin D levels and changes in fetal femur geometry, which may prove a useful tool in the diagnosis of vitamin D insufficiency in the unborn child. The successes and limitations of the research discussed here evolved from the basic premise of devising a reproducible method for imaging bone in *utero* and access to a population based study, the Southampton Women's Survey.

The Southampton Women's Survey cohort comprised a set of recruits attending the SWS Ultrasound Unit for their pregnancy scans. This use of convenience sampling may have biased the profile of the group as these women were already committed to a research programme. It is possible that those who declined to take part in the survey may be in the group whose offspring could exhibit musculo-skeletal anomalies or a predisposition for an adult disease, which may be associated with a group of less motivated or nutritionally aware women. There have been studies in the SWS showing that individuals who have lower educational attainment are likely to eat an unbalanced "imprudent" diet, (Robinson *et al.,* 2004). Despite this the data has shown that women with a wide variety of maternal, fetal and biochemical characteristics have been studied. Standard European measurement charts were used for measurements at each scan and in particular femur charts were used to compare the range of sizes with the normal ranges devised by Chitty and Altman in 2002. Gestational age was plotted against femur length and in both the SWS cohort and the distribution was matched with the Chitty charts. These suggest that the SWS cohort is representative of the general reference population, a conclusion also made in a publication describing the cohort, (Inskip *et al.*, 2005).

The concept that the prevalence of adult disease may be 'programmed' *in utero* by nutrient restriction to the fetus has been explored by researchers over the last few decades. This hypothesis of developmental origins of health and disease underpins the purpose of the SWS. Research has

shown that adaptations made by the fetus can increase the risk of ill-health in adult life and has been the subject of prominent publications, (Aihie-Sayer *et al.,* 2004; Barker, 1994, 1995, 1998 and 2004; Cooper *et al.,* 2002; Cooper, 2002; Couzin, 2002; Gale *et al.,* 2001; Gluckman and Hanson, 2005; Harvey and Cooper, 2004; Javaid and Cooper, 2002a; Javaid *et al.,* 2002b; O'Brien *et al.,* 1999; Sydall *et al.,* 2005). Fetal adaptations permanently change the body's structure and function culminating in later disease. The aforementioned researchers suggest that muscle and bone development, size, density and strength, or predisposition for disease, could all be influenced or limited by the nutritional state of the mother, in addition to their predetermined genetic script. Thus the SWS has proven an invaluable source of data to support these hypotheses.

The collection of prenatal data has, until recently, been restricted to biochemistry, radiographs and interrogation of the mother. Ultrasound for use as a research tool has grown from a crude technique reserved for the unusual, (Goldstein *et al.,* 1979; Robinson, 1973; Robinson and Fleming, 1975), through its refinements to a useful diagnostic tool, not only in obstetrics, but in abdominal, small parts and vascular imaging, (Kuo *et al.,* 1992; Lees *et al.,* 1994; Lessoway *et al.,* 1990). Obstetric vascular studies also followed, which strengthened the research connection in fetal medicine, (Kiserud *et al.,* 2003). The next refinement which has made collection of obstetric data feasible was the innovation of volume scanning in the form of 3D and 4D ultrasound pioneered in the 1990s, (Hata *et al.,* 1998; Merz, 1995; Riccabona *et al.,* 1995 and 1996). Further adaptation of this technique has made it possible for data to be collected from the fetus, not only of size and shape, but also growth, organ volume and body composition, (Chang *et al.,* 2002 (a, b), 2003 (a, b, c), 2005 and 2006; Cohen *et al.,* 2001; Huang *et al.,* 2005; Kalache *et al.,* 2006; Kurjak *et al.,* 2005; Lee *et al.,* 2004; Pang *et al.,* 2006). Obtaining information safely about the growing fetus at such an early stage in pregnancy has been possible due to the advances in ultrasound technology outlined here.

To commence the study the appropriate equipment was chosen and how this was done will be discussed with the evaluation of the system for the purpose of imaging musculo-skeletal development in the fetus. The lengthy process of developing a suitable technique for volume acquisition and measurement on stored data will also be reviewed, and the application of the finalised techniques to gather data regarding bone and soft tissues of the fetal thigh.

Arising from this scan data collection has been the desire to combine it with maternal variables gathered by anthropometry and interview within the SWS, to answer questions of maternal influence on fetal musculo-skeletal development. The discussion culminates in the implications the research has highlighted for the risk of musculo-skeletal health in the offspring.

6.1 Choice of system and machine evaluation

Many human developmental studies have been handicapped by lack of knowledge of the prenatal phase of life and to address this problem new imaging techniques must be formulated, to allow investigation of musculo-skeletal growth. The imaging modality most often used for exploring fetal anomalies is medical ultrasound and with the advent of 3D and 4D modes the field of prenatal research has been revolutionised. The criteria identified in order to successfully accomplish the study were, the ability to visualise small objects with optimum resolution, acquire the volumes quickly before fetal movement became a problem, store the data without the quality degrading and be able to measure and manipulate the data at any stage in the future. The KretzGE Voluson® 730 met these criteria as it comprised a mechanical sweep transducer, the timing of which could be manipulated by the operator, so that fast repeatable volume acquisition could take place without losing resolution. In addition the system was supplied with specialist software (Sonoview® 2000) which allowed post-processing of the images at a remote computer with a high definition screen.

Soft tissue and tissue boundaries could be identified in even the smallest fetus, but bone, even in the fetus, is difficult to image by ultrasound. As the skeleton matures, imaging of bone becomes more problematic as the bone becomes denser. With the Voluson® 730 it was possible to visualise fetal bone and make measurements upon it and in addition it was possible to demonstrate anatomical features not seen before. These included the medullary canal in long bones and sheaths surrounding thigh muscle groups. Some of these features made the images confusing until familiarity with the images and anatomy was gained. In order to assess if the structures seen were 'real' and not artefact, a scan acquisition technique for acquiring volumes was devised and novel measurements taken on previously unseen planes was explored.

It was also apparent that some of the measurements were difficult to obtain due to increased bone density and shadowing at later gestations. Maternal body composition, ultrasound system peculiarities and movement also hindered creation of exact images and as a consequence after reviewing stored data some measurements were not attempted on volumes with poor resolution.

In general the quality of the images produced by the Voluson® 730 system was excellent for 3D scanning and volume acquisition, but after performing experiments with the test-tank, reconstruction errors and measurement discrepancies in the far-field became apparent. The distance between the transducer and femur also affected the resolution in the C plane. At depth, there was loss of information due to an increase in voxel size furthermost from the transducer. The volume of data acquired was not an exact cuboid matrix but comprised voxels of smaller near-field elements and larger far-field data that appear cuboid in shape. This made images in the far-field appear

blurred to the eye, which worsened the further away from the transducer the region of interest was placed. Boundaries then become difficult to assess subjectively by the operator and consequently difficult to measure.

The system's manufacturers, KretzGE, recognise their Voluson® 730 had limitations in its imaging capabilities, which have been addressed since the SWS acquired their system in 2002. They agreed resolution in the reconstructed plane C was considerably lower than that of the primary scan plane A. The system's resolution was particularly poor for low-contrast targets in reconstructed planes and these images also contained significant system artefacts parallel to the face of the transducer, (Voluson® 730 Operation Manual). Discarding measurements taken on the C plane, which is 90° to the longitudinal aspect of the femur and thigh, meant excluding useful measurements made in the coronal aspect. This plane cannot be reproducibly achieved by free-hand scanning.

The image quality produced by the Voluson® 730 was excellent for 2D, 3D and 4D soft tissue and earlier gestation bone images, with the exception of the C plane. However the equipment does not possess a measurement package of the same standard. The ability to perform measurements on surface-rendered images would have been useful as the 'skeletal' or 'maximum' mode enabled precise edge detection of bone, but due to scaling problems this facility has not been developed, and is now being addressed by competing manufacturers. Overall the KretzGE Voluson® 730 system performed well, but limitations in resolution, measurement error and lack of definition when imaging dense bone were noted.

6.2 Technique development

When performing an ultrasound examination optimal equipment settings and standardisation of operation should enable a repeatable, reliable technique, which is important even for the most experienced technician. Sceptics of 3D ultrasound believe that acquiring a volume for manipulation later is removing operator skill, but this is untrue. High-quality 2D technique is needed for 3D ultrasound or the resultant volumes are of questionable value. During the technique development stage of this study it was necessary to identify the best method of scan acquisition and equipment settings. A set of core measurements was devised, which were revised or discarded as their usefulness, complexity or reproducibility was evaluated. It was also important to develop a repeatable method of obtaining measurements on stored volume data, assess any variability and refine the technique so that reproducibility could be achieved. In this way any measurements found to be consistently variable were excluded.

The 15 core measurements decided on at the commencement of the study were revised to 8 after trial and evaluation. It took many weeks to hone the techniques for volume data-set acquisition so that the volume acquired was repeatable and usable. In addition it also took many weeks to acquire the skills to accurately and reproducibly produce linear, trace and volume measurements on these core variables. In the analysis the mean of a triplet of measurements was always used for accuracy and this added to the processing times required for each subject.

The study was limited by the scan protocol for the SWS ladies as ethics approval allowed scans to be performed at 11, 19 and 34 weeks only. Ideally serial scans to assess longitudinal growth velocity and anatomical shape evolution could have provided more data. It would also have been possible to assess at which gestation volume acquisition could have been commenced, as the 11 week scan was too early to obtain more than a femur length for each subject. Despite these limitations, valuable data has been collected and the volumes themselves have been stored for interrogation in future studies.

6.2.1 Scan acquisition

The method for volume acquisition was suggested by the manufacturers of the KretzGE ultrasound system and involves placing the long axis of the transducer longitudinally on the maternal abdomen, with the ROI over the uppermost fetal thigh. Within the ROI, the femur must be perpendicular to the direction of the wave of ultrasound being emitted from the transducer. For this study, the volume was only then acquired if the fetal thigh was directly over the opposing limb, so that suitable landmarks, such as the pelvis and fetal bladder could be used to ensure that the femur was not rotated by any amount. This ensured that anterior, lateral, posterior and medial borders of the femur could be identified. In a publication by Chang and co-workers (Chang *et al.,* 2003), when they examined the humerus, they did not mention the orientation of the bone when acquiring volumes, so the accuracy of their acquisition may have been questionable. From scanning many femora it is quite clear that posterior shadowing causes visualisation problems for the operator and the degree of shadowing varies with the orientation of the bone. By standardising the bone orientation the degree of variability can be minimised and may therefore only be consistent in one plane. By using it on the femur, the technique needed to be adapted to take into account the dense nature of bone at 34 weeks and the irregularities of the growing bone ends, something not so apparent in the humerus.

Scan acquisition time was kept to a minimum if the fetus was restless by decreasing the size of the ROI and depth of the ROI box, so that the volume was only taken from as small an area as necessary. Some of the earliest scans had not included enough of the soft tissue for thigh, muscle

and fat / skin volumes, as the ROI or angle of sweep had not been optimised, but the useable data of the bone was still included in the analyses. Care was thereafter taken to ensure all of the thigh tissues were included on volume acquisitions; the angle and sweep time adjusted to compensate.

Maternal or fetal movement artefacts appear as wavy lines on the 3D display and occur mainly when fetal movement, maternal respiration, coughs, hiccups, maternal blood vessel pulsation or operator hand movement was faster than scan acquisition time. To compensate for this a decrease in scan acquisition had to be made, but again this degraded image quality, so finding the balance was important in developing the acquisition technique. Poor fetal positioning or lack of fluid (oligohydramnios) was found to create soft-tissue distortion and if the fetus was too flexed or had its lower limbs pressed together, it was difficult to judge which boundaries represented the skin surface. By relieving pressure on the transducer, it was sometimes possible to reduce this distortion. Bone compresses less than soft tissue, so it was only possible to measure the bone component of the fetal thigh in some cases, due to poor position or fluid deficit. Where there were structures overlying the fetal thigh, such as an upper limb or the spine, shadowing affected how much of the thigh and femur could be seen. Occasional manipulation of the fetus by pressing the maternal abdomen or waiting for the fetus to move, usually improved images.

Obese women with excess adipose tissue also made imaging difficult as it affected the degree of speckle caused by multiple structures within the adipose tissue. Adjusting the power, gain or use of harmonic imaging settings had to be employed. Some resultant volumes were too poor to be used when they were reviewed. Usually at the time of examination several volumes were acquired using different equipment settings so a selection of images could be chosen from.

6.2.2 Measurement technique

Conventional measurements of the femur were reproducible, but innovative CSA and volume calculations were more challenging. To obtain these measurements from the stored volume data, the information was transferred onto compact disk and loaded onto a computer equipped with Sonoview® 2000 software which was used to enhance the image, expand or reduce magnification, allowed measurements to be made on the image, but more importantly the software allowed interrogation of the 3D volume from any angle or plane, by slicing into the volume from any direction. Any changes made at this stage cannot affect the stored data-set which can be interrogated as many times as necessary.

Linear and trace measurements were traced in the conventional way on-screen with electronic callipers. The distances and areas created were displayed on-screen and noted down on paper pro-

formas ready for entry onto a database. The technique employed for the volumes involved the 3D multi-plane facility incorporated into the Sonoview software. In this study the fetal thigh was called up onto the computer screen in 3 orthogonal planes (A, B and C) with the longitudinal axis of the femur displayed horizontally on-screen in plane A. This orientation was manipulated electronically using the active software and computer mouse.

In Figure 6.1, plane A shows the femur as a horizontal white structure with a small yellow dot on the left-sided metaphyseal end of the shaft. This was placed by the operator at the furthermost point of the bone and it is from this position that all volume traces were commenced in this study; for thigh volume, muscle volume or bone volume. The increment between each slice was adjusted by the operator, as small as 1 mm apart, until the furthermost point of the bone was reached. In the literature, little indication of start and finish points have been mentioned and orientation of the limb or bone ignored. Standardised position and incremental consistency appeared arbitrary in other studies and so for this research it was decided that at 19 weeks, the increments would be 1 mm apart on the irregular bone ends, as this is the smallest increment achievable by the equipment and 3 mm apart for the more uniform shape of the shaft. At 34 weeks 1 mm increments were again used at each metaphyseal end and 5 mm intervals for the shaft. A larger number of slices gave a better result, although this increased processing time, as observed by others, (Farrell *et al.*, 2001; Nelson *et al.*, 2002). This was a painstaking technique as each run for thigh, fat / skin / muscle boundary and femur volumes were performed 3 times per volume. However reproducible results were generated. Some investigators state that it is not necessary to create the largest number of image planes possible to obtain an accurate volume (Pang *et al.*, 2006), but this research was performed using commercial phantoms containing tissue-mimicking structures far larger than the bone and tissue volumes used in this study.

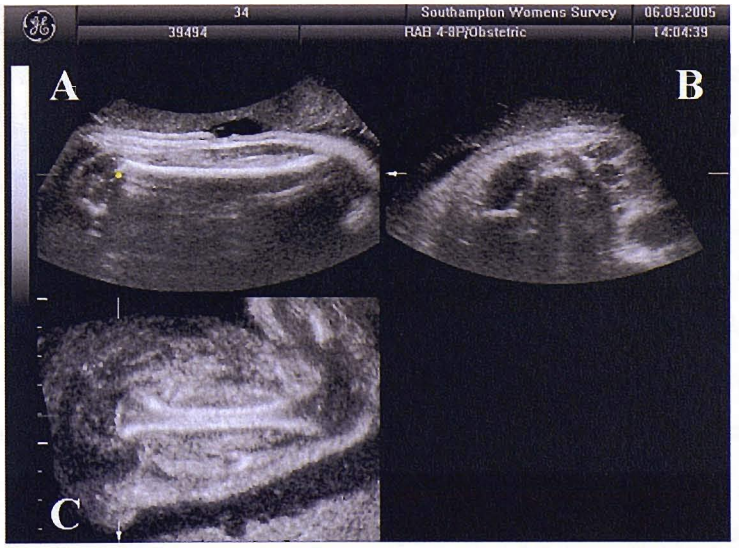


Figure 6.1: Three orthogonal planes of a 34 week thigh volume before measurement.

6.2.3 *In vitro* tests and reproducibility

Previous researchers overlooked potential measurement discrepancy in their studies, assuming their results to be valid. In this study measurements have been authenticated in a scientific and logical way with the aid of the test-tank. For the purpose of this study, a commercially available phantom would not have been adequate, as they do not contain materials that accurately mimic bone. To assist in identifying difficulties with bone imaging, for this study, a basic test-tank was constructed, into which organic and non-organic materials were placed, submerged in water. Rods and lamb bones placed in the test-tank and they were scanned through a port cut into the side. Volumes of the objects were acquired and stored for later measurement. As the articles had been physically measured with callipers before testing, finite measurements of diameter and length could be compared with those taken on the stored volumes.

These *in vitro* tests were used to assess depth and measurement accuracy of linear measurements. There were limitations in this period of testing as bone volumes could not be attempted using the test tank. The discrepancy between bone and water experienced during these tests gave rise to shadowing and flaring, which made it impossible to visualise the entire length of the bones being used. This was especially apparent in the larger bones which contained more mineral content and by the time these lambs were 6 months old, the metaphyseal ends had expanded greatly, forming the articulations of the hip and knee. At this great size it was also impossible to include the entire bone in the ROI of the scan field.

Tests performed to evaluate axial and lateral measurements on non-reconstructed planes, showed minimal discrepancy. In the far field (10–15 cm from the transducer), both lateral and axial measurements were slightly larger (1–2%). This indicated that the ultrasound system measurement registration was accurate in 2D, especially in the axial direction, parallel to the direction of beam propagation. However, in reconstructed planes the further away from the transducer the target was placed, the greater the degree of distortion, leading to greater measurement size. In obstetrics, the best images are taken in the near field, with the transducer approximately 5 to 10 cm from the fetus. Even at 5 cm with this test, there was a 22% increase in the on-screen measurement compared with known size. At 12 cm, a 78% increase in measurement was produced, which was unacceptable. In addition, measurements were rendered more difficult to perform as the system had an in-built reliance on 'smart processing'. This was where cursor placement by the operator was not always accepted by the equipment, if the digital on-screen cursor straddled a boundary between 2 pixels.

The test-tank produced was basic in design, but it exposed inaccuracies when volume data sets were reconstructed. The tests proved that a repeatable technique had been achieved, but

measurement discrepancies arose from inherent failings within the reconstruction process. The major cause of poor resolution concerned the distance between the transducer and target, predominantly in the reconstructed C plane. This impacted on calliper placement, influencing variability and inaccuracies within the measurements obtained and as a result non-reproducible measurements such as FPDP and FDAP taken on the reconstructed C plane, were excluded from further analysis. FMSTS and FMAP were also discarded as these variables suffered from shadowing and flaring even though they were taken on the A plane. Ultrasound imaging is subject to this phenomenon, especially where a linear structure which is perpendicular to the ultrasound wave-front, is of a dense medium such as bone. Once machine and operator capabilities had been established the next step was to repeat the measurements devised on living subjects in the SWS and evaluate trace and volume measurements not possible the *in vitro* setting.

6.2.4 *In vivo* tests and technique confirmation

The previous tests performed *in vitro*, assessed inter-operator variation and evaluated accuracy within the technique, but to see how precise the technique was in highlighting differences between individuals, the measurements taken on volumes from living fetuses *in vivo* were scrutinised from the triplets of core measurements to ascertain biological differences between fetal subjects. Practical issues, such as movement elimination whilst taking a volume acquisition, were assessed, together with placement of the transducer over the area of interest for the best image quality. Angle of sweep, time of sweep, resting state of the fetus, depth of focus, image quality settings, maternal position and breathing and the taking of multiple sweeps on a variety of different settings were experimented with to gain experience and hone technique for volume acquisition. Additionally, the first 29 fetuses had additional measurements of both femora at 19 weeks. Paired t-tests showed there was no significant difference between the length, CSA or volume dimensions of either left or right femur ($p<0.001$, $r = 0.79$). Thereafter, only the most easily accessible femur, lying perpendicular to the long axis of the ultrasound beam was used.

While no participant was excluded from the study because of increased adiposity, the resultant images were considered carefully for quality. Some of the volume data sets were excluded or measured again at this stage for reproducibility and any measurements which showed greater than 5% discrepancy within a triplet were excluded. The figure of 5% is commonly used as a quantification of variability in medical studies above which results are considered too variable to be safely used. Thus this figure was adopted in this study as a cut-off marker.

Intra-operator variability was tested in this study, but inter-operator variability was deferred. To accomplish the scan acquisition and measurement techniques developed for this study took many months of trial and error and it was not feasible to transfer a new technique until it had been fully explored. In other studies where femur length alone has been tested, the intra-operator CV for this linear measurement for 10–16 week fetuses was 4.6% and 2% in laboratory conditions, (Rosati *et al.*, 2004). The within-subject CV for each triplet of linear measurements in this study was 0.57% at 19 weeks and 0.37% at 34 weeks, which reflects the simplicity of a linear measurement. Whereas for trace measurements the within-subject CV was 5.6% at 19 weeks and 3.8% at 34 weeks, indicating the greater difficulty in achieving the hand-traced measurement. With regard to between-subject differences, linear, trace and volume measurements at both 19 and 34 weeks all showed greater CV. This suggests low measurement error relative to biological variability between fetuses. In laboratory studies using commercial phantoms the CV of intra-operator variation has been shown to vary from 10% to 20% for volume measurements, (Riccabona *et al.*, 1996). However, in this study the intra-operator CV of soft tissue volumes was 0.94% at 19 weeks and 0.54% at 34 weeks. With the difficulty in imaging smaller bones at 19 weeks and denser, more reflective bones at 34 weeks, it was not surprising that the intra-operator CV was higher; 3.4% at 19 weeks and 1.73% at 34 weeks. Achieving consistency between operators may need intense training and practice, another phase of testing would be necessary and this will be discussed in Chapter 7 – Future Work.

Visual size on-screen was found to be the cause of difficulties in measuring some of the core measurements. Mid-shaft CSA and MSST measurements, because of their small dimensions, were difficult to perform. By expanding the B plane on which they were taken, to the full size of the screen, calliper placement became easier and so more of these measurements were included in the later part of the study.

In the second trimester of pregnancy, fat / skin layers of the fetal thigh are not distinguishable as separate layers by scan, so this structure was assessed as one layer; fat / skin layer. Fat begins to be accrued in subcutaneous layers after 28 weeks of gestation as fetuses develop, so it is after this time that these layers can be distinguished separately. Although these layers could be visualised and differentiated at 34 weeks, for the purpose of this study the fat / skin layers were measured together, as they were at the 19 weeks. MSST was dropped from the core measurements, even after technique refinement as it was found that fat / skin volumes were more useful and descriptive than a simple linear measurement. To support this, in a previous study (Bernstein, 2004) where a 2D linear measurement of thigh fat / skin layers was taken on a CSA of the thigh, it was found that this measurement was not as valuable as taking a CSA at that level and suffered from 27% inter-operator measurement discrepancy. In addition MTMCSA and MTCSA were also discarded.

Whilst they were easily acquired, again, they did not have the descriptive potential as the volume measurements. Thus the remaining 8 core measurements of thigh volume, fat / skin volume, muscle volume, femur length, proximal CSA, mid-shaft CSA and distal CSA continued to be collected from the stored volume data-sets.

6.3 Application of 3D technique

Once 3D acquisition and measurement techniques had been developed, the information obtained was examined to see if 3D ultrasound could be applied to the study of musculo-skeletal development. Fetal body composition, fetal bone imaging and fetal gender differentiation were then explored using this modality.

6.3.1 Body composition evaluation

The amount of fat accrued by a neonate correlates directly with energy stores and birthweight comprises 12–14% of fat in the normal birthweight range. However, fat content in the entire range can vary by 46% in the neonate, when taking into account growth-retarded fetuses, diabetic mothers and smoking mothers, (Catalano *et al.,* 1992; Bernstein *et al.,* 2000). Being able to estimate fat accrual accurately in the fetus is important if these conditions are to be addressed and with such a wide range of tissue variation, ultrasonic techniques have been instrumental in helping to quantify body composition.

Previous researchers had attempted to address the evaluation of fetal body composition before the advent of 3D ultrasound for the purpose of assessing the growth retarded or compiling values of subcutaneous fat stores in normal and macrosomic fetuses, (Larciprete *et al.,* 2003 and 2005). These studies predominantly involved 2D linear measurements of fetal fat tissues using the abdomen at the level of the umbilical vein and CSA of fetal thigh to assess lean mass. In addition a linear measurement of the subscapular fat / skin layer was taken at the lower scapular border on coronal section of the fetal trunk. These measurements were then used to quantify fat and muscle within the fetus. Whilst this methodology takes into account different areas of the body, it only provides a 'snapshot' of fetal body composition. Using volume calculations of the entire thigh, which includes all tissues, plus the separate volumes of fat / skin, muscle and bone, a more complete and descriptive picture of tissue proportion can be visualised.

In this study, using 3D technology, the measurements made on soft tissues of the fetal thigh did not prove as difficult as those made on bone. This was assisted by the amniotic fluid / skin boundary being well defined, as was the fat / skin to muscle boundary, which made it possible to perform

evaluations of fetal tissue volumes and demonstrate the different proportions of tissue within the thigh at different gestations, as seen below. These volumes were then correlated with maternal body composition markers such as arm muscle area, height and sum of skin-fold thicknesses.

Proportions of fetal thigh tissues				
19 weeks	**range (cm^3)**	**mean (cm^3)**	***n***	**% of thigh volume**
Thigh volume	2.83 – 11.91	6.44	*431*	100
Fat / skin volume	0.35 – 4.11	1.81	*427*	27
Muscle volume	1.37 – 7.23	4.14	*429*	64
Femur volume	0.24 – 0.81	0.48	*484*	7
34 weeks	**range (cm^3)**	**mean (cm^3)**	***n***	**% of thigh volume**
Thigh volume	51.36 – 145.98	93.07	*428*	100
Fat / skin volume	20.07 – 67.52	38.83	*427*	41
Muscle volume	27.39 – 77.91	51.53	*429*	55
Femur volume	1.66 – 4.19	2.80	*483*	3

Table 6.1: The proportions of thigh volume tissues; 19 and 34 weeks, for both sexes.

Tissue volumes were most valuable measurements for fetal body composition assessment. Of the fetal thigh volume, 64% comprised muscle tissue at 19 weeks and 7% bone; the remaining fat / skin layers made up 27% of the total thigh volume. By 34 weeks the proportions differed where just over half of the thigh volume was now muscle (55%), bone only 3%, but the fat / skin layers had increased to 41% of the total thigh volume. Boys in general were larger at 19 weeks and remained larger at 34 weeks, but there was no statistical difference between genders in the distribution of tissues in the thigh. The increased fat / skin layer was a direct result of fat accrual by the fetus after 28 week's gestation, the exact timing of which cannot be assessed by this study where there have only been 2 points in time where fetal body composition has been assessed, but the growth velocity of these tissues between 19 and 34 weeks showed an increase in fat / skin layers, which was greatest in boys. These results show that 3DUS of the fetal thigh can be applied successfully to demonstrating fetal body composition and gender differences within the tissues. This may prove a useful technique for future studies in body composition, fetal growth or future obesity.

6.3.2 Bone imaging

Bone is difficult to image because of the discrepancy between surrounding soft tissues and the mineralised bone itself. Propagation of ultrasound through bone is affected by this severe mismatch of acoustic properties causing scattering of the ultrasound in all directions at the boundary. This results in loss of detail as the echoes do not return to the transducer. Imaging bone by ultrasound also produces an intense upper echo causing shadowing which blackens out the posterior bone boundary. In this study major image adjustment by use of the TGC was necessary to increase smaller echoes returning from within the bone and decreasing brighter images in the near field which would otherwise obscure information. This effect had to be compensated for mainly at the 34 week scan where the fetal skeleton has a greater mineral content. At the 19 week scan the femur appeared homogeneous in echogenicity and it was easier to assess where the bone edges lay. Artefact at the end of the femoral diaphysis at both 19 and 34 weeks could be mistaken for bone, but after many weeks of becoming familiar with the new images, it was recognised that these structures related to dense capsular material at the joint and so were excluded from measurements.

From repeated examination of femora it became apparent that in approximately 10-15% of the bone images, there was splaying at the metaphyseal ends. This may have been anatomical variation, but the appearances were reminiscent of the bones affected by vitamin D deficiency (rickets). It was therefore hypothesised that these fetuses may be the offspring of women low in circulating vitamin D during their pregnancy. Further investigation of their bone density, bone mineral content, bone area and their mother's vitamin D levels was then vital in forging a link between the bone shape and impaired bone development. This will be discussed later in this chapter.

6.3.3 Gender differences

Bone, muscle and fat / skin volumes were seen to vary in proportion within the fetal thigh at different gestations and the means of tissue volumes showed differences between the sexes. Total thigh volume was significantly different between boys and girls at 19 weeks, with boys having a greater mean volume than girls. Additionally at this gestation, the fat / skin, muscle and bone volumes within boys were significantly greater than girls. By 34 weeks the mean thigh volume in boys was significantly higher in boys than girls. When broken down into the component tissues, bone content tended to be slightly higher in boys, which was unsurprising. This difference was not significantly greater than in girls, which was unexpected. There was no difference in fat / skin volumes between boys and girls, which was surprising as it was anticipated that girls may have had

more fat / skin layers than boys. Boys were seen to have significantly greater muscle volume, which was perhaps an anticipated result.

At 19 weeks femur length, proximal and mid-shaft CSA showed no difference between the sexes, however, boys tended to have greater CSA dimensions than girls, although not reaching significance. By 34 weeks there were no significant differences between the sexes for these characteristics or growth velocity differences for any scan measurement.

With regard to neonatal DXA results, it could be seen that boys had significantly larger bone area and greater bone mineral content than girls, although there was no difference in the bone mineral density readings.

6.4 Assessment of maternal influences on fetal body composition, size and growth

Epidemiological studies have shown that prevalence of adult health and disease may have developmental origins. To assess influences occurring during development the newly collected scan and DXA data were correlated with maternal variables and in this way, associations of fetal body composition, fetal size and growth velocity with maternal factors were examined.

Maternal birthweight was scrutinised for association between the mother's own size at birth and that of her offspring. The birthweight mean of the mothers was 204g smaller than the mean birthweight of their offspring. As there was a mean of 31 years difference between the birth of the fetuses and their mothers this was not surprising. The trend for birthweight to increase over generations under conditions where there is improved nutrition and healthcare is a known phenomenon. The smallest and largest of both groups also showed an increase in approximately 200g in the fetus as compared with the group of mothers. The cohort of babies contained some infants who were premature and some post-mature and a small group who were induced before term for medical reasons, but despite this it was seen that a mother's birthweight and that of her offspring were significantly related, if she delivered a male fetus. There are numerous contemporary studies which address factors affecting birthweight (Wilkin and Murphy, 2006; Olafsdottir *et al.*, 2006; Sekiya *et al.*, 2006; Griffiths *et al.*, 2006), but none of these publications separate the sexes, so it is not know if the effect seen in this study has been replicated elsewhere, or if this is an isolated finding. Animal studies such as those performed on sheep, occasionally reveal gender specific growth patterns, but these are the result of nutrient restriction and twin studies which cannot be performed in human fetuses, (Cleal *et al.*, 2006). The true reason for the association between maternal birthweight and the birthweight of male fetuses cannot be explained by this study.

6.4.1 Bone determinants

Examination of correlations between maternal and fetal characteristics has allowed insight into which elements are important for the development of bone, fat / skin and muscle components of the fetal thigh.

Maternal birthweight was not associated with bone size at 19 weeks, but by 34 weeks female fetuses showed increased femur volume, greater proximal CSA and increased femur volume growth velocity. Increased maternal birthweight appeared to influence bone length, volume and but the 3 CSA dimensions remained unaffected.

Maternal age and arm muscle area played no part in the size and speed of growth of her offspring's skeleton, or the bone mineral accrual in the neonate.

Maternal height influenced femur volume at 34 weeks in girls and femur length and growth velocity by 34 weeks, indicating that taller mothers tend to produce fetuses with accelerated linear bone growth. There were no significant associations between maternal height and the CSA measurements taken of the femur at 19 or 34 weeks, which supports the idea that growth is occurring in length rather than CSA. It was interesting that maternal height played no significant part in the bone mineral content, bone mineral density or total bone area of her offspring, but this is perhaps under the control of paternal characteristics, or placental function.

Maternal BMI was associated with fetal bone geometry at 19 weeks, seen as an inverse association between proximal CSA. This effect persisted to 34 weeks in girls and for mid-shaft CSA, suggesting that increased maternal BMI was associated with smaller proximal CSA in girls. This indicates that this area of mid-shaft of the femur in addition to the proximal CSA grew less well in girls if their mother had a high BMI. There was also a negative association with between BMI and femur volume. Unexpectedly, maternal BMI was strongly associated with neonatal BMC and bone area in boys, but there was a negative trend with all three DXA parameters in girls.

Maternal SSF was not found to be associated with bone volume or length at 19 weeks. However, there was a negative association between SSF and proximal CSA and mid-shaft CSA at 34 weeks in girls. There was also a positive association between SSF and BMC and bone area in boys. The increased maternal SSF perhaps reflects the amount of stored nutrient supply that would be available to her fetus and perhaps in the case of boys, this extra supply has a positive effect on skeletal development.

Maternal parity influences the size of core measurements, with greater ranges and mean values seen in fetuses at 19 and 34 weeks in multiparous women, although none of these associations reached significance, with the exception of a relationship with femur volumes in boys alone at 34 weeks. This suggests greater femur volumes in male offspring born to multiparous mothers and reflects the data which demonstrates that multiparous women produce increasingly heavier babies with each subsequent pregnancy. Parity was not associated with DXA results.

Maternal smoking was not associated with bone size or growth at 19 weeks. However, this analysis was using the smoking status of the mother prior to pregnancy, not her smoking status throughout the pregnancy, as this data was not available at the time of the analysis. It would be interesting to re-run this analysis with pregnancy data as other studies have noticed a developmental delay of the ossification centres in babies in smoking women (Namgung *et al.*, 2003) and reports from other studies support Namgung's findings of height and weight reduction in offspring of smoking mothers. These babies were also found to have lower lumbar and femoral neck bone mineral density, although this was not an observation possible in this study.

Maternal vitamin D status was inversely associated with proximal and distal CSA at 19 weeks, which supported the hypothesis that a reduction in the amount of circulating maternal vitamin D would cause the femoral metaphyseal dimensions to increase ($p = 0.02$, $r = -0.12$ for proximal CSA and $p = 0.003$, $r = -0.15$ for distal CSA). When divided by sex distal CSA remained significantly associated for boys, but this was not true for girls. Maternal vitamin D levels were inversely associated with distal CSA at 34 weeks ($p = 0.03$, $r = -0.11$).

There was no relationship between maternal vitamin D and bone growth velocity, but this may be because the blood samples were taken at 34 weeks and may not reflect changes occurring over the preceding 15 weeks. As part of the SWS protocol, blood samples were taken from the recruits before pregnancy and at the 11 week scan appointment and it may be possible in the future to assay these bloods for vitamin D concentrations, as they may further demonstrate the relationship between vitamin D and the variables under influence. These results indicate that lower levels of maternal vitamin D are having an effect on the geometry of the developing femur, which may be sex specific. These are important findings as this has confirmed the hypothesis that lower maternal vitamin D levels during pregnancy have an impact on the growth, shape and development of the fetal femur, even as early as 19 weeks gestation.

It was surprising to see that there was no correlation between vitamin D and neonatal DXA results in light of a study previously carried out in Southampton. This publication reported that very low

levels of vitamin D (below 15 nmol/L) were significantly associated with decreased BMC (Javaid *et al.,* 2006), but this effect was not seen in this group.

6.4.2 Fat / skin determinants

Previous studies have used upper arm measurements of the fetus to study fat content, suggesting that using this area was more sensitive than using the thigh, (Catalano *et al.,* 1992). However, these studies pre-dated 3D ultrasound which has aided with imaging of these tissues. Here thigh volume and fat / skin volume has been used to examine the maternal influences affecting their accrual. As much of fetal body composition comprises fat / skin and muscle components rather than bone, it is reasonable to suggest that maternal birthweight, and thus soft tissue influence, is a real predictor of infant birthweight and fat / skin components.

Maternal birthweight was not associated with any of the fetal scan measurements at 19 weeks, confirming previous research which has shown that the fetus does not lay down fat until after 28 weeks gestation. By 34 weeks there was a significant association between increased maternal birthweight and increased thigh and fat / skin volumes and their growth velocities. These later gestation results remained stronger for boys than girls when the group was divided by sex. This suggests that maternal birthweight predominantly influences the fat / skin accrual of male infants.

Maternal height was significantly associated with fat / skin volume and its growth velocity by 34 weeks. Taller mothers produced offspring with greater fat / skin volumes at 34 weeks.

Maternal BMI, if greater, was associated with increased thigh volume at 19 weeks in boys, but at 34 weeks there was a significant correlation for the entire group. This suggests that increased maternal BMI influences fetal body mass as reflected in fetal thigh volume. However, this affect was most prevalent in boys and may have been due to muscle mass accrual being greater in boys in addition to the fat / skin layers.

Maternal SSF was used as comparator to fat / skin and thigh volumes of her fetus. From the data it could be deduced that at 19 weeks there were no associations between maternal SSF and any of the tissue volumes of her fetus. This may be in part due to the gestation at which this was tested in the fetus. Subcutaneous fat stores do not accumulate in the fetus until 28 weeks and so this layer at 19 weeks was small in comparison with the other thigh tissues. However, by 34 weeks greater maternal SSF prior to pregnancy was associated with greater fetal thigh and fat / skin volumes and their growth velocities.

Maternal AMA was used as a comparator to fetal muscle mass and at 19 and 34 weeks it was strongly associated with thigh volume and its growth velocity, but after gender division the effect was most apparent in boys. Increased AMA was also associated with increased fat / skin volume at 34 weeks.

Maternal parity status was associated thigh volume. In fetuses whose mothers were multiparous the thigh volumes were significantly greater than those in fetuses of nulliparous mothers. Additionally the fat / skin volumes of these fetuses were greater if their mother was multiparous, but this association was most apparent in boys. These findings support evidence that increased parity increases the birthweight of subsequent fetuses. The trend over the nulliparous, to primiparous and multiparous groups shows that there was a mean increase of 133g with each subsequent pregnancy which was a significant increase. The trend was slightly greater for boys over the 3 groups, 144.9g as opposed to 111.8g for girls and supports previously seen trends in obstetrics publications.

Maternal smoking prior to pregnancy did not appear to influence thigh or fat / skin volumes at 19 weeks, but at 34 weeks the growth velocity of fetal thigh volume tended to be slower in fetuses whose mothers smoked before pregnancy, although this relationship does not quite reach significance. This data is flawed in that the smoking status of the mother during this pregnancy could not be added to the model and the analysis was performed using reported smoking at the time of the pre-pregnant interview. The analysis may reveal other outcomes if the data for smoking during pregnancy becomes available and will be discussed in Chapter 7 – Future Work.

Maternal vitamin D status did not appear to influence fetal measurements at 19 or 34 weeks, except for a significant association with fat / skin volume growth velocity in girls. This suggests that higher levels of vitamin D in maternal blood increases the fat / skin volume growth velocity in female fetuses, but this data is incomplete as the blood samples were only analysed from one point in the pregnancy (34 weeks) and this may have rendered the analysis ineffective for relationships between growth velocity and maternal vitamin D status. Further analysis of blood taken earlier in the pregnancy may be useful to assess the relationship.

6.4.3 Muscle determinants

A study of neonates (Rohl *et al.*, 2001) reported that tissues other than fat account for 85% of birthweight and so accurately measuring fat free mass is important in evaluating fetal body composition. What determines the amount of muscle accrued by the fetus appears to be lead by maternal muscle mass. In this study the data showed that mother's own birthweight did not

influence the muscle mass or muscle volume growth velocity of her child, nor did her age at delivery, parity, smoking or circulating vitamin D.

Maternal height was associated with muscle volume 34 weeks and its growth velocity, suggesting that taller mothers produce offspring with greater muscle mass. When divided by sex this association remained in boys, but not for girls. Taller individuals have longer bones with greater muscles attached to them so this association was not so surprising.

Maternal BMI was associated with muscle volume at 34 weeks with larger mothers producing offspring with greater muscle volume and growth velocity.

Maternal SSF was found to be strongly associated with muscle volume at 34 weeks, indicating that increased subcutaneous maternal fat was associated with greater muscle volume and greater growth velocity in the fetus. This again suggests that increased fat stores within the mother before pregnancy provides adequate nutrition for muscle development in her fetus.

Maternal AMA was significantly associated with greater thigh volume at 19 and 34 weeks, with increased thigh volume growth velocity for boys. Greater maternal AMA was also associated with greater muscle volume at 34 and its volume growth velocity. The effect was more apparent in boys and these results indicate that increased thigh volume, driven by the increase in muscle tissue laid down, was especially significant in boys.

By way of a summary the three tissue components of the fetal thigh have been examined in relation to maternal tissues and the following histogram in Figure 6.2, briefly outlines the comparisons for tissue volumes. Fetal femur volume was plotted against maternal height, AMA and SSF demonstrating that maternal height was the premium determinant of fetal volume of the variables examined. All maternal variables were significantly associated with increased muscle content of the fetuses, but AMA had the greatest association. For fetal fat / skin volume, all maternal variables played a part in determining the fat / skin content of the fetal thigh, but maternal SSF appeared to be the most significant determinant.

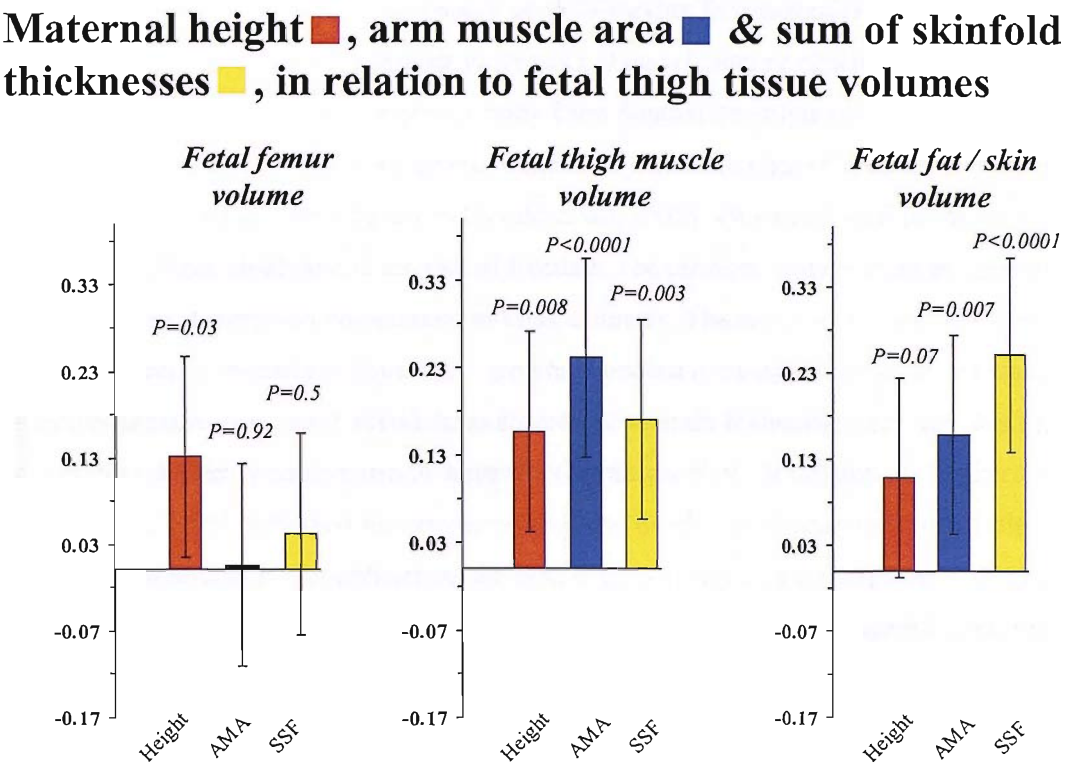


Figure 6.2: Maternal body composition and fetal tissue volumes at 34 weeks.

6.5 Application of 3D as a useful diagnostic tool for vitamin D insufficiency

The identification of a set of fetuses whose metaphyseal ends were larger than their peers began the development of the hypothesis that these children may in some way have been different from the rest of the group. The appearance was reminiscent of the appearances seen on radiographs of the wrists and knees of children suffering vitamin D deficiency, the severity of the bone deformity is variable and some researchers have composed a 10 point grading system to assess severity in children, (Thacher *et al.*, 2000), such is the concern about treatment and aetiology. This condition can be very serious as it has the potential for morbidity and mortality as the child can develop hypercalcaemia leading to seizures, failure to thrive and an increased susceptibility to infection. However, it is easily treatable once diagnosed and only the extreme cases or those diagnosed late into the condition are at risk form chronic disorders and skeletal deformities. Due to the higher incidence of immigrant populations arriving from more temperate climates, the incidence of childhood vitamin D insufficiency is increasing, as darker skinned people are less able to synthesise vitamin D in their skin. As an estimated 90% of an individual's daily requirement for vitamin D is derived from sunlight, complexion and covering the body for cultural or religious reasons in a sunlight-poor region will be impaired. This is extremely important for pregnant

women, as the developing fetus is dependent upon its mother for vitamin D and calcium for skeletal development. Should the mother be vitamin D insufficient or deplete, the skeletal health of the fetus would be compromised. A recent study from Southampton has shown a relationship with impaired bone mineral accrual in 9 year old children as a consequence of their mothers being vitamin D insufficient during pregnancy, (Javaid *et al.,* 2006). This nutritional insult impacts on bone health for these children and the risk of fracture. The problem is increasing as more and more people leave their homelands and migrate to other climates. The rising incidence of vitamin D insufficiency and its related conditions is a growing concern in many countries where immigrant populations become those most at risk from nutritional vitamin D insufficiency and this includes their offspring. Recently researchers in Australia (Robinson *et al.,* 2006) and the Netherlands (van der Meer *et al.,* 2006) published figures on the growing incidence of vitamin D insufficiency in immigrant populations. Both publications advocated the screening of all pregnant women at risk, so that supplementation could be commenced and in this way avoiding late diagnosis and treatment for the child. The Netherlands study gained their information from vitamin D assays taken from the woman's booking blood test at 12 weeks of pregnancy. At this stage in the United Kingdom this is not routine as for many years, rickets and other vitamin D related bone conditions have been declining. Now with rising incidence of these conditions, sometimes the diagnosis of vitamin D insufficiency is overlooked as it is not expected. Early diagnosis of vitamin D insufficiency could prevent development of chronic musculo-skeletal impairment and this is why this chance finding of metaphyseal splaying so early on in the pregnancy is so exciting. Not only could this technique be used as a diagnostic tool but it could also provide a means of monitoring the condition once supplementation has commenced.

6.5.1 Recognition of metaphyseal splaying

It was apparent that some femoral shapes varied from the norm. Most fetuses showed parallel-sided femora, as in Figure 6.3, but some showed metaphyseal splaying as in Figure 6.4. This appearance was present in approximately 10% of fetuses at 19 weeks, but was more difficult to assess by image at 34 weeks because of the flare and shadowing. Thus it was necessary to devise a method to accurately measure this structure. Once volume acquisition technique had been achieved, careful tracing of the metaphyseal ends was developed. To obtain the correct landmarks, the furthermost point of the bone was viewed in cross-section and the image was advanced along the femoral shaft until the widest area of metaphyseal cross-section was within the ROI. At this point the area was traced around and this area was perpendicular to the axis of the femoral shaft. It was performed at both ends of the metaphysis (proximal and distal CSA) and it was noted from the raw data as it was collected that in most cases the proximal CSA was larger than the distal CSA, whether it was taken at 19 or 34 weeks.

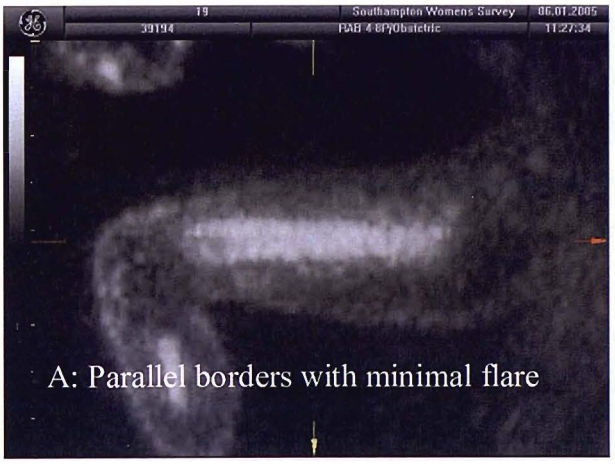


Figure 6.3: Parallel-sided metaphyseal borders.

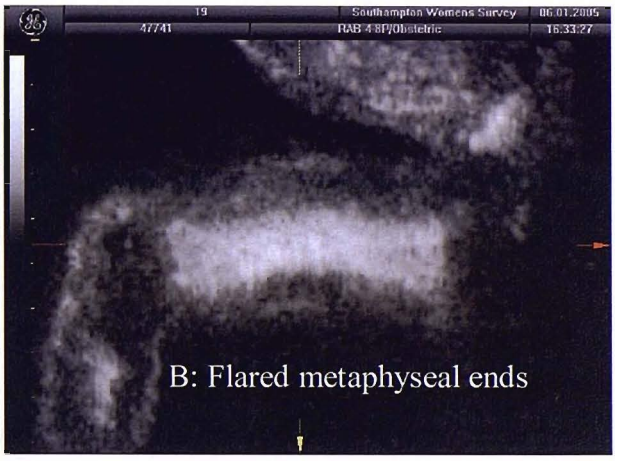


Figure 6.4: Flared metaphyseal ends.

Those with distal CSA measurements greater than proximal may be individuals showing an anatomical variation, or as hypothesised here, may be fetuses of mothers, whose circulating vitamin D was decreased, causing an imbalance in homeostasis. Thus bone production was greater than bone reabsorption, leading to increased metaphyseal CSA, a feature common to childhood vitamin D insufficiency.

It was necessary to establish if this anomaly was an anatomical variation or if the hypothesis that these fetuses were being carried by mothers who were vitamin D insufficient or deplete. Two lines of investigation were pursued. Firstly, an index was formulated to try to quantify the degree of splaying for each individual. In this way 'normal' and 'abnormal' splaying could be categorised. The index took into account the length of the femur so that the proportion of splaying to bone size was calculated. It is known from biometric studies that bone length is proportional to gestational age and so femur length rather than femur volume was used in the equation to identify increased splaying in bones whose length was proportional to the gestational age of the fetus. Secondly, maternal blood samples taken at 34 weeks were assayed for vitamin D concentrations to examine whether fetuses with splayed metaphyseal bone ends had vitamin D insufficient mothers.

6.5.2 The role of maternal vitamin D in splaying index validation

The results showed that even at 19 weeks there was an association between circulating maternal vitamin D and geometry of the fetal femur. This was interesting at the blood samples had been taken later in the pregnancy, at 34 weeks. Maternal vitamin D concentrations were found to be significantly inversely associated with greater fetal proximal and distal CSA dimensions. This association confirmed the hypothesis that lower levels of vitamin D did indeed coincide with

metaphyseal splaying. By 34 weeks the association remained with lower levels of vitamin D being associated with greater fetal distal CSA. However, the CSA measurements were an indication of finite size and did not show if the splaying was increased in proportion to bone length. It was tested by using an index which took into account the bone length. This index was derived by dividing the CSA of the distal metaphysis by the femur length. The vitamin D results had been log transformed so that they could be regressed and so the two variables were correlated for each subject.

At 19 weeks lower vitamin D levels and increased splaying index were associated, suggesting the index was useful as a tool for detecting fetuses likely to be at risk from vitamin D insufficiency. However, at 34 weeks as the association became weaker. As previously described in Chapter 5, the lowest vitamin D concentrations belonged to Asian women (3) and one black African woman, which is a finding supported by other studies who have seen an increase in rickets and osteomalacia in children from non-Caucasian, mainly immigrant, populations who have moved to more temperate climates. When the maternal vitamin D levels were checked it was found that these women were also vitamin D insufficient or deplete, (De Lucia and Nelson, 2002; Holick 2003; Nozza and Rodda, 2004).

6.6 Influences on musculo-skeletal development and the risk of adult disease.

Maternal body composition has been seen to play an important role in the body composition and growth velocity of her fetus. Should the mother have a large BMI or greater subcutaneous fat, then this predicts a fetus with greater fat / skin volumes. Some women experience rapid weight gain during pregnancy, especially if they are diabetic or develop gestational diabetes, which again influences fetal adiposity *in utero,* (Rohl *et al.,* 2001).

Weight gain accrued as a result of the pregnancy itself can have an impact on future offspring. Mothers who do not lose weight sufficiently in the first year or so after their first child (less than 25% of the weight gained throughout the pregnancy), create for themselves a larger BMI that has the knock-on effect on the next pregnancy, (Gunderson *et al.,* 2001 and 2004). As seen in this study parity increased fat / skin volumes in the offspring, so for each subsequent pregnancy if the mother's BMI was not maintained to her pre-pregnant state there was the potential for producing offspring with higher fat / skin volumes, that may go on to be fatter children and adults. Thus multiparous women with a high BMI and particularly those who become pregnant fairly quickly following the birth of a child, are most at risk of producing heavier offspring.

Should a fetus deliver prematurely or have a small birthweight for gestational age, then muscle bulk (lean mass) is affected, not fat mass, (Hediger *et al.,* 1998). We have seen from this study that

there was a significant association between mother's own birthweight and that of her baby, even if the delivery was premature or post-mature. However, the premature fetus was likely to have less muscle as well as fat, as muscle development would not have been completed. Post-mature babies have time to accrue more fat, but their musculo-skeletal development has considerably slowed by 40 weeks, so that the birthweight is affected by fat stores and not increased bone size. Once delivered, rapid weight gain affects the length of long bones of the fetus (Neufeld *et al.,* 2004), but does not increase the maturity of the skeleton and therefore children with increased weight are more susceptible to skeletal problems as well as obesity and heart conditions, which can as easily result from low birthweight, (Cameron *et al.,* 2003).

One of the maternal factors influencing musculo-skeletal development in the fetus was the mother's smoking status. Although the data in this study was limited to smoking status prior to pregnancy, many studies depict the environmental influence of smoking in pregnancy on bone and muscle development, as affecting skeletal dimensions. Smoking has also been associated with heart and kidney size in the offspring (Lampl *et al.,* 2005) and it needs further scrutiny to determine whether smoking in pregnancy affects the tissue volumes or CSA dimensions in the same way. One study has already focussed on the abdominal circumferences in babies as an indication of reduced lean mass in offspring born to smoking mothers and a sign of reduced fat deposition in the fetal thigh, (Bernstein *et al.,* 2000). However, this was not performed with 3D ultrasound and relied on linear measurements across the fat / skin layers. The head circumference and femur length of the babies were not influenced by smoking and they concluded that smoking shows selective reduction in the mass of certain compartments, which may also be a reason for not seeing much affect in this study, where only the thigh was used in isolation. Bernstein also suggested that the smaller abdominal circumferences taken after 33 weeks gestation may be caused by liver growth restriction in the smoking mothers. In previous Southampton studies smoking has been seen to be a precursor to lower bone mineral content in the offspring and this in turn leads to impaired bone strength and increased fracture risk in the individual, whether as a child or later as an adult, (Godfrey *et al.,* 2000; Cooper, 2002). Whilst some effects of smoking can appear pre-natally, peri-natally or in early infancy, the legacy of the mother's habit can increase the risk of diseases in the childhood, such as asthma (Moshammer *et al.,* 2006) or in adulthood, when osteoporosis risk is significantly increased, (Harvey and Cooper, 2004).

Studies performed in Southampton, prior to and including participants of the SWS, showed that 31% mothers were insufficient in vitamin D in their diet and 18% were deficient in late pregnancy in the Southampton area, (Javaid *et al.*, 2006). This had a 'knock-on' effect to the children by the age of 9, whose bone density, examined by DXA, was still depleted if there had been poor circulating vitamin D in late pregnancy. Thus another maternal influence has been shown to affect

the musculo-skeletal development of her fetus, which as far as this study has shown, begins early in the pregnancy. Many studies have intimated that adequate circulating vitamin D in the maternal bloodstream is particularly beneficial to fetal skeletal health in the third trimester of pregnancy. From the data in this study it could be seen that by 19 weeks there were already differences in growth between the sexes, in babies whose mothers had a high BMI, babies with taller or fatter mothers, those with more muscle and those who had been pregnant before. Although this study was limited to one blood sample at 34 weeks, it showed that the relationship between bone size, shape and growth velocity and maternal vitamin D was not limited to the third trimester.

Early recognition of bone changes is important to the diagnosis of vitamin D insufficiency and in the living fetus this had not been possible until this study. Some research in Bejing where there has been a reported 25.4% incidence of nutritional rickets in children (Liu, 1991), has used post-mortem specimens of a random selection of cadavers to examine fetal long bones for changes in bone architecture. Within the specimens 35.5% were assigned the diagnosis of *vitamin D insufficient* after histological examination, suggesting many more infants could be susceptible to early bone changes that were not overt. The study also concluded that microscopically there was evidence of impaired bone development as early as 30 weeks of gestation. So it was important here to devise a technique that could recognise early bone changes associated with vitamin D insufficiency to allow intervention which may prevent long-term skeletal alteration.

Studies have suggested supplementation with vitamin D of pregnant women during the winter months to prevent insufficiency which could potentially reduce the risk of osteoporotic fracture in later life for the offspring, (Javaid *et al.,* 2006; van der Meer *et al.,* 2006). It is known that supplementation in childhood and adolescence can have a positive effect on bone accrual, as seen in longitudinal studies of calcium and dairy product supplementation (Matkovic *et al.,* 2005), but there is not enough evidence to suggest that dietary manipulation of maternal calcium and vitamin D intake can have an effect on the skeletal maturation of the offspring. The idea of supplementation has not been universally accepted and further testing of its efficacy is anticipated. Some centres also advocate screening programmes to identify those at risk from vitamin D insufficiency, by taking maternal blood samples in early pregnancy, (van der Meer *et al.,* 2006; Robinson *et al.,* 2006). It has been shown here that bony changes could be distinguished in pregnancy in those at risk suggesting that 3D ultrasound could provide a diagnostic tool in the detection of pre-natal vitamin D insufficiency.

Prevention of vitamin D insufficiency at this early stage could have a vast impact on bone health in future generations. Childhood fracture and ill health could be reduced and those individuals once at risk could continue into life with less risk of osteopenia, osteoporosis and fracture. Multiple

sclerosis, cancer, insulin-dependent diabetes mellitus and schizophrenia have also been linked to low prenatal and peri-natal vitamin D concentrations, as this affects functional characteristics of the body tissues, in addition to bone. It is therefore essential to establish musculo-skeletal health at the very beginning of life.

In summary, the results obtained comprised anticipated and unexpected relationships and it must be acknowledged that factors affecting the results could include sample size and over-reporting of gender-specific findings. Caution must be taken when reporting subgroup specific results, such as sex differences, because of the danger of over-interpretation of apparent (but not real) differences between the findings within the subgroups. Studies should be designed with enough power to make self-contained inferences specific to each subgroup. Gender is a variable that is well-known to influence many disease processes and growth patterns. By presenting data from subgroups, (i.e. each gender) implies that the subgroup specific results are more accurate than overall results. This may or may not be true as the significance of results can be influenced by two components, bias and variance. Bias is the inaccuracy caused by ignoring the variability of the true relationship; for example, measured maternal vitamin D levels and distal metaphyseal splaying in different sex fetuses. Variance is the inaccuracy caused by sampling error; in this case having a cohort that is too small to give a precise relationship results when divided by sex of the fetus.

In this research lower levels of maternal vitamin D were associated with increased splaying of the femoral distal metaphysis; at 19 weeks there was a strong inverse correlation in both sexes combined ($p = 0.003$, $r = -0.15$), but this relationship was not significant in girls when the group was divided by sex, ($p = 0.03$, $r = -0.15$ for boys and $p = 0.07$, $r = -0.13$ for girls). This apparent gender difference is most likely due to inadequate power and statistical tests of interaction were non-significant. To test the bias and variance of this finding for sex specificity, a repeat of the same study model on a matched cohort could be attempted and additionally a repeat trial using a greater sample. These methods may confirm the accuracy of the sex-specific relationships, or conversely highlight the possibility that the result was a chance finding. Where possible, further work using a larger cohort may be necessary to corroborate the results and allow formal statistical testing for gender interactions.

Chapter 7 – Future Work

Introduction

The Southampton Women's Survey is an important resource of data for epidemiological studies. The information obtained from the participants and their families, together with the scan volumes acquired in this study has the potential for being a crucially important resource for future studies in musculo-skeletal development. The directions which this research could take arise from some of the limitations and questions posed by the work previously detailed.

7.1 Inter-operator studies for technique reproducibility

The introduction of 3D ultrasound, particularly in the UK, has been accepted and used by very few centres, as until recently it was viewed as a means of taking 'baby pictures' for public consumption and not a serious research tool. It has therefore been largely overlooked by many clinicians and sonographers and as such remains in a niche field. The research here has shown that 3D ultrasound is a suitable method for examining growth and body composition in addition to its frequent use as a fetal anomaly diagnostic tool. If these techniques are to be transferred, inter-operator studies should be performed so that the same level of expertise can be employed elsewhere.

The scan acquisition and measurements were all performed by the author and these techniques were developed over a period from March 2002 to December 2005. The process was time-consuming but necessary to ensure that methodology was appropriate, reproducible and resulted in an acceptable intra-operator coefficient of variation for volume measurements of 3.4% at 19 weeks and 1.73% at 34 weeks. To achieve such good results between operators may need intense training and practice, which may be addressed in another study.

7.2 Further development of splaying index

The splaying index devised here, was a simple method used to assess the degree of metaphyseal splaying within a long bone, which can give an indication of developmental impairment of bone in the fetus. With its association with lower circulating levels of maternal vitamin D it can used to detect fetuses at risk from vitamin D insufficiency, during pregnancy. Whilst the splaying index may be simplistic, the development of other indices based on scan findings may be possible and this may lead to the realisation of a useful clinical tool for the sonographer and clinician in the identification of fetuses at risk from vitamin D insufficiency.

7.3 Vitamin D trials in pregnancy

Worldwide various agencies have expressed a wish to undertake large vitamin D interventional studies in pregnant women. Some researchers believe that it will be beneficial to supplement women at risk of vitamin D insufficiency as it may reduce the risk of rickets and other bone mineral conditions, but these women have to be identified first, (Prentice, 2003). Should vitamin D trials come to fruition it would be possible to use the techniques developed here to identify those at risk, monitor those taking part in the study and monitor the progress of any intervention that may be necessary. By identifying small bone size changes in the developing fetus it may also be possible to pinpoint crucial periods of bone growth or response to supplementation. The splaying index or distal CSA measurement could play an important part in this type of trial.

The study here was limited to 19 and 34 weeks for volume acquisition, but in a planned trial of monitoring it would be advantageous to commence volume acquisition at earlier gestations, perhaps 15 weeks, and at more frequent intervals, possibly every 2 weeks until term. This proposes a great deal of work, but it could provide an enormous resource of data that could be used for other studies. For example, fat accrual or muscle development studies, with the volumes being saved for future investigation.

7.4 Analysis of 11 week femur length and maternal 11 week blood samples

At the onset of this work, it was considered using data collected at the 11 week scan that each participant had, but it proved impossible to obtain thigh volumes at this early gestation with the equipment used. However, at this scan, performed between 10 weeks and 6 days and 13 weeks, other measurements were obtained. These were crown-rump length, bi-parietal diameter, head circumference, abdominal circumference and femur length. Using the femur data a longitudinal study of femur growth could be performed. Adding strength to the study would be the analysis of the blood samples for vitamin D concentrations taken from the mothers at this first trimester scan appointment. In this way a fuller picture of vitamin D status could be acquired and would be of great interest with regard to growth velocity of fetal bone and subsequent metaphyseal splaying. These bloods have been stored since being collected at the 11 week scan appointment and are awaiting analysis.

7.5 Analysis of other fetal biometry and maternal data

At all of the scans fetal biometry was performed as described in the section above and in addition at 19 week and 34 week scans thoracic circumference was also obtained. The relationships between

these measurements and those already analysed in this study could be very interesting. As previously mentioned, this study utilised the fetal thigh in isolation. From studies of body composition there is the suggestion that growth, fat and muscle accrual and bone size may vary with the section of the body being studied at different gestations. By including the other measurements it may be possible to see a wider picture of growth patterns and fetal development.

Another element which was not considered in this study was the categorisation of medical conditions with may have influenced the musculo-skeletal development of each fetus. Retrospective analysis of pregnancies complicated by diabetes, osteoporosis, nutritional impairment, hypertension or toxins, or fetuses who were premature or post-mature, or any condition that may have had an impact on the musculo-skeletal outcome of the offspring, could be undertaken using the data already obtained.

7.6 Smoking, alcohol and nutrition in pregnancy

As previously seen the data concerning maternal smoking was limited to the pre-pregnant status. At the time of analysis the pregnancy smoking data was not ready for analysis, but once this has been completed the analysis could be re-run using the scan and DXA data, to ascertain any relationships between smoking and musculo-skeletal development of the fetus. Studies of women who smoke during pregnancy have shown that this activity can have a detrimental effect on their unborn child. The known complications which can arise from smoking include pre-term labour, reduced birthweight (Steyn *et al.,* 2006), pre-eclampsia, increased risk of placental abruption and neonatal asphyxia, (Hammoud *et al.,* 2005). Impairment of skeletal formation can also result from maternal smoking as osteocalcin levels in cord blood is lower in smoking mothers than non-smokers, but such additional analyses will only be possible with more thorough pregnancy data. Smoking and high alcohol consumption not only affect the bone mineral content of the fetal skeleton, but also the development of the placenta, (Colak *et al.,* 2002). Alcohol, smoking and diet have all been recorded for the participants before and during pregnancy and it would be interesting to see if they influence the offsprings' musculo-skeletal development, as predicted by other studies.

7.7 Sunlight exposure during pregnancy and effects of seasonality

The importance of sunlight in the production of vitamin D in the skin has been explained previously. The sunlight hours experienced in the Southampton area is affected by seasonal variation and this factor has been reported as an influence in the vitamin D levels found in pregnant women. This in turn affects the woman's own bone density and the bone mineral accrual of her developing child. In a study of seasonal variation and bone density in Korea, it was found that there

was an increase in fetal bone mineral content in babies born in the summer compared with winter ($p<0.005$). In real terms this was calculated as a reduction of 8% in bone mineral content. In the USA where supplementation does not take place routinely for pregnant mothers, the reduction can be as much as 12%, (Namgung and Tsang, 2003). Sunlight variation affects birthweight, although the mechanisms are not fully understood and in a multiple centre Australian study (Mc Grath *et al.,* 2005), peak birthweight was recorded in the spring, with a smaller peak appearing in the autumn. This was unaffected by the latitude at which the babies were born. To assess if seasonality and hours of sunlight affect the musculo-skeletal development of the SWS babies, sunlight hours recorded by the University of Southampton Oceanographic Centre, will be correlated with data collected here. Scan and DXA data could be used in conjunction with maternal 34-week blood analysis already performed, in addition to the 11-week samples, once assayed.

7.8 Maternal heel ultrasound and exercise in pregnancy

Many studies have highlighted the advantage of physical exercise to maintain bone strength in the adult skeleton, (Carter *et al.,* 1996; Borer, 2005; Jamsa *et al.,* 2005; Engelke, 2006). However it has been shown in studies of pregnant mothers, that optimum bone density and strength is maintained during pregnancy to the detriment of the fetal skeleton if the activity is too strenuous. The effect can be seen by measuring maternal bone strength using quantitative ultrasound on the calcaneous of the mother. Obviously DXA using radiation is contraindicated on a pregnant subject, so ultrasound can be used instead. In previous SWS studies excessive exercise manifested as a decrease in calcaneal bone density taken on the pregnant mother at the 11 and 34 week scan appointment, (Godfrey *et al.,* 2001; Javaid and Cooper, 2002a). Seasonality can also lead to variable results in the quantitative heel ultrasound (Javaid *et al.,* 2005a) probably due to the amounts of ultraviolet light varying from summer to winter and the effect this has on the maternal synthesis of vitamin D. By using the heel ultrasound data collected in the SWS, scan measurements and growth velocity patterns could be assessed in the fetuses.

7.9 Grip-strength

Increased hand grip-strength has been shown to be an indication of muscle strength and health in the adult and if the individual grows less well in early life then there will be less muscle strength as an adult. Grip-strength is most strongly associated with birthweight in men and women after adjusting for height and weight, (Aihie-Sayer *et al.,* 2004). As part of the SWS interviews both the mother and father had their grip-strengths tested, by research nurses using a hand-held grip-strength machine. This was performed on the father at any time, but the women were tested at the 19 week scan appointment for consistency. At late gestations grip can be impaired by swelling of the

extremities commonly associated with pregnancy. The measurements recorded for this SWS component could be analysed in conjunction with the muscle volume data of the fetus, to examine any parental influences on the muscle development of their child.

7.10 Paternal data

Whilst this study has predominantly studied maternal influences on fetal musculo-skeletal development, paternal variables may exert similar or very different influences on the growth and development of the fetus. The SWS has been very thorough in the collection of data from both the mother and father of each child and there is a wealth of paternal data which could be analysed in conjunction with the scan and DXA data of their offspring. Variables collected include birthweight, height, weight, grip-strength, saliva samples, blood samples and DXA. All of these will be useful in examining the picture of parental influence on the growth, size and development of their fetuses.

7.11 Children's follow-up studies

The SWS has been instrumental in collecting important data from 12,500 women in the Southampton area before and during pregnancy. Ongoing is a scheme to follow-up the children where research nurses visit each family at 6 months, 12 months, 2, 3 and 4 years after the birth of the SWS baby. At these visits information is recorded concerning the child's diet, exercise, allergies, behaviour, growth, development and ill-health. By studying the outcomes of these children in relation to the measurements taken on them as fetuses, sense can be made of the size, growth and shape of the bone, muscle, and fat / skin layers in relation to any illnesses or conditions they may develop as they grow. The first delivery of the 517 cohort used in this study was in February 2003 and so already follow-up data is available for many of the subjects, which may confirm some of the outcomes predicted by this study.

Conclusion

From the beginning of this thesis four key questions were posed as the aims of the research. At this point re-evaluation of those aims will reveal whether the study has accomplished the task it set out to do. The first question was pertinent to the development of the new technique of 3D ultrasound;

Can we measure bone and tissue volumes in the unborn fetus?

This key question arose as a response to the lack of technical validation of the use of 3D ultrasound imaging for tissue volumes of fetal bone and body composition. With the new technology of 3D ultrasound available it was hypothesised whether the acquisition of bone and soft tissue volumes of the developing fetus was possible. The work entailed methods development of a novel 3D ultrasound technique to measure the geometry and volumes of fetal thigh bone, muscle and fat /skin layers. This was accomplished by trials of many subjects and evaluating the equipment using a test-tank. The humeral volume technique described by Chang and co-workers proved a useful bench mark and with some adjustments for the irregular shape of the femur, an accurate and reproducible technique evolved.

The reproducibility of the volumes and, more importantly, the measurement accuracy, were key to the development of this technique for research in large epidemiological studies where collection of precise data is essential. The technique development stage proved that data sets and measurements could be repeated with a small degree of variability in all measurements excepting those using the reconstructed planes. Knowledge of the equipment's limitations and lengthy acquisition times were necessary to produce the best images, from which the most precise measurements were obtained. In this respect the accuracy of using 3D volumes for measurement acquisition has been explored and its reproducibility tested. This proved successful and furnished the possibility of using the data collected for further studies on fetal musculo-skeletal development.

Between-measurement accuracy for the operator is an important factor in technique, but what is crucial to medical studies of populations is the sensitivity achieved by a technique in demonstrating differences between subjects, however small. With the accuracy of the methodology ascertained, the next question proposed to test the sensitivity of the measurements obtained in showing small changes;

Can we highlight sex differences and growth velocities in these pregnancies?

The next key aim was to use the measurements collected using 3D ultrasound to try to demonstrate gender, size and growth velocity differences in the fetuses of the population under study. It was shown that using the data collected it was possible to show size and growth variability between sexes. Whilst some of the results showed a sex specific bias, such as muscle and fat / skin accrual in boys of multiparous women, some showed unexpected findings in both sexes. The negative association between mothers with greater BMI and their offspring having smaller bone CSA was one of these curiosities. Three-dimensional ultrasound has been shown to be sensitive enough to tease out sex differences in size and growth velocities between the sexes and indeed, between subjects of the same sex. This work provided a comprehensive database of measurements for the fetal thigh, which could then be correlated with maternal variables in order to identify the effect of maternal influences on musculo-skeletal development in the fetus.

The next key question involved the use of the scan measurements obtained from the fetus and the data acquired from the mother prior and during pregnancy.

Can we demonstrate how maternal influences affect the body composition of the fetus?

It was hypothesised that by using the pre-pregnant maternal data gathered from the Southampton Women's Survey, influences on fetal size and growth could be shown. Studies have already shown developmental origins of health and disease manifesting as childhood or adult conditions, based on observations of newborn weight, body composition and bone mineral accrual. This study aimed to show a picture of body composition prior to birth with this new modality of 3D ultrasound and relate it to maternal characteristics. By scanning the fetus with ultrasound, then correlating the results with known measurements and variables from the mother, a picture of maternal influences has been drawn. Strong predictors of fetal size, such as maternal birthweight, BMI, height, arm muscle area and sum of skin-fold thickness have been shown in these fetuses, whilst other characteristics seem to have little influence. These included maternal age at delivery and pre-pregnant smoking status. Some maternal influences were particular to a tissue type within her fetus, for example her height influenced the length and volume of her baby's femur and how well it grew. This also applied to muscle volume and growth. However, maternal height played little part in the accumulation of fat / skin layers in her offspring. Precise timing of increased bone, muscle and fat / skin volumes within the developing fetus have not been identified by this study, which was limited to scanning at two time-points in the pregnancy. Further investigation at different gestations may be necessary to unravel critical moments of growth for each tissue type. Despite this, 3D ultrasound has proven a useful tool for epidemiological studies concerning size, body composition and growth of fetuses in the second and third trimester and how these tissues relate to the influences imposed by maternal size and body composition.

After analysing the correlated data the next proposed question involved applying this data to the developmental origins hypothesis, to see if 3D fetal ultrasound would be a useful tool in helping predict the subsequent risk of adult disease.

Can we speculate about the implications for the programming of adult disease into the offspring?

Three-dimensional ultrasound has facilitated study of the fetus previously impossible and this offers the possibility of using the technology to predict programming of health and disease in the individual. Since the inception of the developmental origins hypothesis numerous studies have been performed to prove the relationship of developmental influences the fetus is exposed to in the uterine environment and health in later life. Three-dimensional ultrasound could make some of these connections more profound as it has the ability to visualise structure and change *in utero*.

It has been shown that 3D ultrasound technology in obstetric research can now be used to quantify developmental plasticity. Fetal accretion of fat and lean body mass is now visible and quantifiable by ultrasound and could be used to explore nutritional insults to the fetus, (Bernstein *et al.,* 2002; Chang *et al.,* 2002 and 2003; Song *et al.,* 2000). These individuals have an increased risk of coronary heart disease, hypertension, non-insulin-dependent diabetes and hypercholesterolaemia in adulthood (Javaid and Cooper, 2002) and ultrasound assessment will prove helpful in determining the mechanisms by which these conditions and growth trajectories are programmed *in utero*. Body composition can be assessed by 3D ultrasound, invaluable in developmental studies concerning the origins of obesity, diabetes and coronary heart disease. Thigh volume has also proven a good predictor of birthweight (Chang *et al.*, 2002; Liang *et al,* 1997), confirmed by data in this study.

Now the problems of imaging bone have been recognised and partially overcome, 3D bone ultrasound can provide a valuable role in quantifying bone mass and shape. This information could be used to predict the risk of osteopenia, rickets or fractures in infancy and childhood. If calcium and bone status was a feature that could be monitored *in utero*, then long-term skeletal health could be assisted, (Koo and Warren, 2003; De Lucia and Carpenter, 2002); with 3D ultrasound in mind this may be possible. Identifying those at risk at such an early stage could facilitate intervention in the form of maternal vitamin D and calcium supplementation which in turn would alleviate the risk of future bone conditions in the individual. Children with lighter skeletons have a higher risk for childhood fractures and an increased risk of osteoporosis and fracture in later life, (Cooper *et al.*, 2000; Cooper *et al.,* 2002; Javaid and Cooper, 2002; Dennison *et al.*, 2001; Kalkwarf *et al.*, 2002 and 2003). By identification of those at risk, earmarking at which point in pregnancy supplementation would be most effective and monitoring any intervention with ultrasound, the future for bone health in some individuals may be assured. Only large scale trials may be able to

assess the efficacy of this approach, but hopefully there will be a place to utilise the successful 3D ultrasound techniques developed here.

Principal Finding

To conclude this thesis, the important discovery regarding vitamin D and its relationship with bone change seen by ultrasound, must be highlighted. This result is most valuable and emanated from the recognition of variable bone shape during the scans. The vitamin D concentrations in maternal blood revealed a very strong inverse correlation between femur distal CSA at 19 weeks and maternal vitamin D status. This suggested that mothers with lower levels of circulating vitamin D produced offspring with greater femur distal CSA commonly seen in fetuses displaying vitamin D insufficiency. This inverse correlation remained significant at 34 weeks and was a finding mirrored by the splaying index results. This discovery requires further exploration. The reason why girls maintain the significant relationship in the third trimester and not boys is unclear, but as the growth pattern of proximal and distal femoral metaphyseal ends appears to be different in boys and girls, gender specific programming of the hip and knee joint should be considered. Also, the relationship of mothers with high BMI being associated with smaller distal, mid-shaft and proximal CSA if they are carrying girls should also be investigated in relation to bone size programming.

It was of interest that the very lowest maternal vitamin D concentrations were from women whose ethnicity was of non-white European origin. This supports others studies (Robinson *et al.*, 2006; van der Meer *et al.*, 2006), where ethnic groups within a population appeared to be at risk from vitamin D insufficiency, especially after migration to a more temperate climate. They also recommended that all measures to identify those at risk, such as screening women in pregnancy for vitamin D insufficiency, should be routinely performed. Other studies support the idea of vitamin D and calcium supplementation for pregnant women, especially in winter months when there is less sunshine, to prevent osteopenia and impaired bone development in the fetus. The study here found that pregnant women with less circulating vitamin D were more likely to be non-white with greater fetal distal measurements and higher splaying index. These babies are therefore at risk of impaired bone formation, rickets and conditions later in life such as osteoporosis.

The ability of 3D ultrasound to assist in the detection of these individuals is a significant bonus to the main premise of the work. Developing the techniques of volume acquisition and measurement upon those volumes has lead to a greater understanding of fetal body composition in relation to maternal influences and paves the way for further research in vitamin D insufficiency in pregnancy and paternal influences on fetal musculo-skeletal development.

APPENDIX

Appendix I

18/7/01

Southampton Women's Survey

Southampton and S.W. Hants Joint Research Ethics Committee Approvals

Full applications

LREC no. 276/97 – Survey of diet, body composition and hormone levels in young women in Southampton (Professor DJP Barker; pre-pregnancy phase of the SWS). Approval granted 28/11/97.

LREC no. 307/97 – The effects of maternal nutrition, body composition and cardiovascular status on the fetal development and metabolic programming (Professor DJP Barker; pregnancy phase of the SWS). Approval granted 28/11/97.

LREC no. 339/97 – Nuclear family studies of obesity by association and linkage. (Professor I Day; genetic studies of obesity in women, their sisters and parents). Study not conducted within the SWS – approval letters etc. with Ian Day.

LREC no. 340/97 – Southampton Women's Survey; Study of genes interacting with fetal programming (Professor I Day; studies of maternal genetics and programming). Approval granted 19/12/97.

LREC no. 125/98 – An investigation into the relationship between neonatal lung function, early life wheezing disorders and asthma in later life (Dr J Clough; neonatal lung function).

LREC no. 018/99 – Genetic influences on fetal growth and adult disease (Professor I Day; fetal, parental and (maternal) grandparental genetic studies). Approval granted 9/3/099.

LREC no. 089/99 – Follow-up of infants born to women in the Southampton Women's Survey (Professor DJP Barker; follow-up at 6, 12, 18 and 24 months, with skin prick tests at 12 and 24 months). Approval granted 30/4/99.

LREC no. 153/99 – Fetal growth and neonatal bone mass (Professor C Cooper; maternal heel ultrasound and neonatal DXA). Approval granted 29/6/99.

LREC no. 257/99 – Cardiovascular function in women before and during pregnancy and its relationship to fetal growth (Mr T Wheeler; cardiac output, arterial compliance and vascular reactivity prior to pregnancy and at 19 and 34 weeks of pregnancy). Conditional approval granted 31/8/99.

LREC no. 324/99 – The influence of maternal diet and body composition on protein metabolism in pregnancy and its relation to fetal growth (Dr KM Godfrey; protein synthesis and urea kinetics in early and late pregnancy). Approval granted 3/11/99.

LREC no.335/99 – Factors associated with depression among young women in the Southampton Women's Survey (Professor A Kendrick; GHQ12 in the non-pregnant population, EPDS 6 months post-delivery). Approval granted 18/11/99.

LREC no. 329/00 – A study into the relationship between lifestyle factors and premenstrual symptoms in a group of women taking part in the Southampton Women's Survey. (Dr C Sadler). Approval granted 15/11/00.

LREC application submitted – Paternal lifestyle, body build and skeletal status as determinants of neonatal bone mass (Professor C Cooper; paternal DXA scans).

Protocol amendments

276/97 – Repeatability study – to re-visit up to 150 women to repeat most of the initial interview to assess changes that occur over a two year period. Approval granted 31/10/00.

276/97 – Amendments to the recruitment procedure agreed in letters from the Ethics Committee dated 2/2/00, 5/4/01 and 15/6/01.

089/99 – Revision of protocol to assess children at 36 months instead of at 18 months as requested initially, to ask mothers to take part in an infant diet validation study and to obtain access to the children's Guthrie cards. Approval granted 4/4/01.

307/97, 089/99 and 153/99 – Professor DJP Barker, Professor C Cooper and Dr KM Godfrey; 36 week fetal liver and neonatal ultrasound scans. Approval granted 4/4/01.

Appendix II

DOHaD Bone and Joint Division

Risk Assessment Form

Name of procedure/chemical/equipment to be assessed (please attach method sheets for procedures)					
Name (of assessor)	Pamela Mahon Superintendent Sonographer	**Location**	**SWS Ultrasound Unit** Room 113 Level F Princess Anne Hospital Southampton SO16 5YA	**Date** (implemented)	**1/10/2003**
Please note here when this Risk Assessment has been reviewed		**Previous assessment carried out on 22/11/2002**			

Required reading List any other risk assessments or procedures that must be read in conjunction with this assessment
Incident forms

List Hazards These are things which can potentially cause harm		**List existing control measures** (usually "Good Laboratory Practice" and/or in-built safety controls on equipment).	**Assess Risk** This is the product of *probability* of harm occurring and *severity* of harm. Define as LOW, MEDIUM or HIGH
NAME (equipment/chemical etc – eg centrifuge)	**HAZARD** (type of harm – eg flammable, toxic)		
1. Medical ultrasound system. (Kretz GE Voluson 730, manufactured and commissioned March 2002).	Electric shock.	Only used by qualified staff, regular manufacturers service and upgrades, unplugged when not in use, machine power output and operating guidelines adhered to.	Low

2. Motorised examination couch. (Huntleigh Akron, supplied new in March 2002).	Electric shock and mechanical injury.	Verbal warnings when in use, unplugged when not in use.	Low.
3. Ultrasound coupling gel. (Diagnostic Sonar, regularly supplied in sterile containers).	Skin reaction in some patients. Becomes warm when placed in specialised gel warmer.	Allergy tested gel. Thermostatically controlled gel warmers in use.	Low
4. Gel warmer. (Diagnostic Sonar, supplied new March 2002).	Electronically heated metal block.	Thermostatically controlled, still under guarantee. Kept away from patients and visitors.	Low
5. Ultrasound transducer cleaning fluid. (Virkon, supplied regularly).	Toxic if ingested. Skin irritant.	Kept in powder form until needed, when it is mixed with water. Discarded after ultrasound transducers have been soaked or wiped with it. This process in only performed when there are no patients or visitors in the department and takes place away from the clinical areas. Gloves are worn when handling this substance and all surfaces cleaned after it has been used.	Low
6. DXA bone density scan equipment. (Lunar DPX, installed 1998).	X-radiation and mechanical injury from moving parts.	Only used by appropriately trained staff. The equipment has safety cut-off features installed. Staff are monitored for radiation safety, supervised by the Radiation Protection Officer.	Low

Appendix III

Current guidelines for the safety of medical ultrasound

High system power output does not necessarily give optimum diagnostic images and so it is the responsibility of the 'user' to limit the power generated. A visual indicator, which is now present on the most modern machines, is the thermal index (TI) and mechanical index (MI). This can be seen throughout an examination and allows the user to make decisions about appropriate examination exposures. The TI should always be less than 0.5 (displayed values) and MI should always be less than 0.3 (Barnett, 2003) and recommended by the International Society for Ultrasound in Obstetrics and Gynecology, (ISUOG, 2003).

Stanley Barnett and his colleagues have pooled together research taken over many years on bioeffects manifested in lower animals in experiments conducted *in vivo* and *in vitro*, (Barnett *et al.*, 2000). These laboratory-based experiments, show some biological effects after exposure, such as a reduction in immune response, change in sister chromatid exchange frequencies, cell death, change in cell membrane functions, degradation of macromolecules, free radical formation and reduced cell reproduction in mice, (Child *et al.*, 1990; Raeman *et al.,* 1996) and 10 day old pigs, (Dalecki *et al.,* 1997). These exposures were far higher than can be achieved by medical US equipment designed for use on humans and their offspring. To cause such harm a probe frequency of 2.3 MHz with a pulse repetition frequency (PRF) of 100 Hz for 16–20 minutes was used (Dalecki *et al.,* 1997) and much of the damage seen was following insonation of gas-filled cavities such as the lung or intestine. This is called 'transient cavitation' and occurs when gas bubbles expand rapidly, especially at water–air interfaces, then collapse as a reaction to one or more ultrasound pulse beams. This results in high local temperatures and pressures. If the bubbles oscillate repeatedly this can have effects on neighbouring cell membranes and this is known as 'stable cavitation.' This may result if the probe is placed continually over one area being scanned, which is to be avoided in obstetric scanning. A recent study using mice showed that by lengthy exposure to ultrasound of a high frequency, micro-bubbles could be produced in the tissues which cause irreparable damage to the blood vessels and muscles. Increase in neovascularisation of the tissues was so great that it was hypothesised that this technique could be used as an alternative therapy for certain musculo-skeletal conditions, where the patient is unsuitable for surgery, (Chappell *et al.,* 2005). Such is the effect that ultrasound can produce at high frequencies.

Thermal damage can be caused if the ultrasound system power output is unnecessarily high, or the same tissue location is scanned for an unnecessarily long period. This 'dwell-time' is the length of time that the ultrasound beam is fixed on a specific tissue target and is an important component of

thermal dosage. The duration of exposure to ultrasound is directly related to operator skill and examination difficulty, (Barnett *et al.,* 2000). Frequent exposure of the same subject should be avoided and there are published guidelines for the safe management of volunteers and patients for practical training in ultrasound scanning, (Barnett, 2003). This extends to the research setting where this study in on-going.

Temperature elevation of less than 1.5°C above normal physiological levels (37°C) is considered to present no hazard to human or animal tissue, including a human embryo or fetus, even if maintained indefinitely, (Barnett *et al.*, 2000). However, temperature elevations in excess of 1.5°C may cause harm and a temperature elevation of 4°C, maintained in excess of 5 minutes, is considered to be potentially hazardous to a fetus or embryo. These figures have been taken from guidelines published by British Medical Ultrasound Society (BMUS) Safety Group in August 2000, after their independent tests. Some centres still feel that greater caution should be taken than recommended guidelines as heating effects *in vivo* have still not been adequately explored, (Lubbers *et al.*, 2003).

In practice, power levels and exposure times are kept as low as reasonably achievable, especially in the earliest gestations of a pregnancy when exposure of embryonic tissues is critical because they are rapidly proliferating, and because of the potential developmental changes which may be caused. Collagen and bone have the highest acoustic absorption coefficients, so the soft tissue closest to bone will display the highest temperature effects, (Barnett *et al.*, 1997).

Diagnostic ultrasound has an enviable safety record in comparison with radiographic examinations, which have known genetic and somatic risks associated with ionising radiation. Magnetic resonance imaging (MRI) has been the cause of several hundred superficial burns, trauma from flying objects attracted by the strong magnets used to operate the system and death from interference with the patient's pacemaker, (Duck, 2002). Therefore ultrasound is the method of choice.

Appendix IV

Simple and multiple linear regression

Simple linear regression and multiple linear regression are related statistical methods for modelling the relationship between two or more random variables using a linear equation. Simple linear regression refers to a regression on two variables while multiple regression refers to a regression on more than two variables. Linear regression assumes the best estimate of the response is a linear function of some parameters (though not necessarily linear on the predictors). This statistical method was employed to equalise the variation between each subject for gestational age. Not all scans were performed at exactly the same preferred time point (19 weeks and 3 days for the 19 week scan and 34 weeks and 4 days for the 34 week scan) and so adjustment had to be made to make the measurements comparable. Explained simplistically, a line of 'best fit' was drawn through the scatter plot of the gestational dates and the points were slid along in a parallel direction to the β coefficient slope of the line to bring all of the measurements to the same virtual time point for comparison.

Nonlinear regression models

If the relationship between the variables being analysed is not linear in parameters, a number of non–linear regression techniques may be used to obtain a more accurate regression.

Coefficient of variation

Coefficient of variation (CV) is a measure of dispersion of a probability distribution. It is defined as the ratio of the standard deviation σ to the mean : μ

$$c_v = \frac{\sigma}{\mu}$$

The coefficient of variation is a dimensionless number that allows comparison of the variation of populations that have significantly different mean values. It is often reported as a percentage (%) by multiplying the above calculation by 100.

The coefficient of variation is often used when discussing the normal distribution for positive mean values with the standard deviation significantly less than the mean. This application may be reasonable for many models, but breaks down theoretically unless the distribution is known to be positive valued, since there is a non–zero probability that the distribution will assume a negative value. When the mean value is near zero, the coefficient of variation is sensitive to change in the standard deviation, limiting its usefulness. Within-subject CV was also used to study measurement error when reproducibility of measurements was being analysed.

Appendix V

Protocol For Anthropometric Measurements: a protocol for fieldworkers at the MRC Environmental Epidemiology Unit, University of Southampton.

Protocols prepared by Dr. Sarah Duggleby and Professor Caroline Fall, MRC ERC 1998.

1. **Height:** Height must be measured with a stadiometer to the nearest 0.1 cm. Place the base-plate on the floor, selecting as firm and level a surface as possible, and preferably near a perpendicular, such as a door architrave, which helps the eye to ensure that the tape is vertical. Ask the subject to remove her shoes and stand on the base-plate with her back to the tape. She should be told to stand as tall and straight as possible with feet together and arms held loosely at the side and shoulders relaxed (to avoid lordosis). She should stand far enough forward on the base-plate such that the tape is not distorted when pulled to vertical. Check that the tape is inserted correctly into the base plate. Raise the tape vertically and place the head plate on the top of the subject's head, using the spirit level to check that the plate is horizontal. The head should be placed in the Frankfurt Plane, such that an imaginary line joining the upper margin of the external auditory meatus and the lower border of the orbit of the eye is horizontal.

If a short person is measuring a very tall person, considerable error can be introduced when reading off the height scale: their eyes will read the scale at an angle (parallax effect). Measurers should be aware of this and aim to read the scale from as level a position as possible. If there is a lot of height disparity the measurer should try to get level with the scale, by standing on, for example, telephone directories. Read the height to the nearest 0.1cm and beware of digit preference. Make one measurement of height.

2. **Sum of skin-fold thickness:** The measurement of a skin-fold is a direct measure of a double thickness of skin and subcutaneous fat and the purpose of measuring skin-folds is twofold. Firstly, they can be used to assess how much fat someone has and this reflects nutritional status. Low amounts of body fat show that the individual is undernourished. In this country, too much fat is more common. The problems associated with this are well known. We use a series of equations that convert skin-fold thicknesses into an amount of fat or fat mass. Secondly, it is now realised that *where* the fat is stored on the body is important. Different patterns of fat distribution are known to predict the risk of developing coronary heart disease and diabetes. Central fat (on the trunk) appears to be more unfavourable than peripheral fat (on the limbs). We think that the metabolic differences in fat stores at different places in the body may also influence fetal growth. In order to calculate sum of skin-fold thicknesses, 4 standard skin-fold sites are used: triceps, biceps, subscapular and upper suprailiac. These

sites were selected from 93 sites originally assessed. They are easily located in relation to bony landmarks, the skin-fold can be raised from the underlying tissue and measurements are reproducible.

Apparatus: There are three types of skin-fold callipers are in common use in this country: the one we use is the Harpenden 'John Bull' model with external springs. They come with a plastic carrying case and should always be kept and transported in their case. They are delicate precision instruments. The blades of the callipers are 90 mm^2 and open to 50 mm. The large dial reads up to 20 mm, and a smaller scale on the dial registers whether you have already gone once or twice round the scale. They exert a constant pressure of 10 g/mm^3. Divisions on the dial are every 0.2 mm, but it is usually possible to read to the nearest 0.1 mm. There is a screw adjuster on the side of the dial. Loosening this screw allows you to move the dial face relative to the needle, so that you can adjust the instrument to 0 with the blades closed. However, you should only let the appointed person for your study adjust your callipers. Callipers can be tested and calibrated using machined metal blocks to check the readings about once a month. They can be sent back to the manufacturers to be calibrated, but this is costly and should not be necessary if they are treated carefully.

Technique: There is no international consensus as to which side of the body should be used for skin-fold measurement. Some use the non-dominant side, others always the right or always the left. There is no statistically significant difference between measurements made on different sides of the body, even when there are considerable differences in muscularity, as in a tennis player. All measurements were taken on the non dominant side. To acquire the correct amount of tissue for a skin-fold, the best technique is to sweep the index or middle finger and thumb together over the surface of the skin from about 6 to 8 cm apart and to collect the subcutaneous tissue pushed away from the underlying muscle fascia by this action. The measurement of skin-folds should not cause undue pain to the subject and by this sweeping method the correct quantity of subcutaneous tissue can be obtained. It is easier to use both hands initially, to massage up a tube of skin with the thumb and fingers of both hands. One hand then remains holding the skin-fold throughout the measurement of the skin-fold, and the measurer picks up and uses the callipers with the other hand. The positioning of the blades of the calliper on the skin-fold will vary with the size of the fold of skin, but in general should be at least one blade-breadth in from the apex of the skin-fold. Be careful not to twist the callipers while striving to read the dial. There are different techniques for timing the readings. Reading of the meter can take place 2, 4 or 5 seconds after closing the blades or to wait until the needle on the dial has stopped moving. At SWS waiting until 5 seconds was used as standard. This measures compressed fat thickness, which may be important as people vary in the compressibility of their fat. Female fat is more compressible than male fat. The callipers should be released fully before beginning to count to 5. This is particularly important if a measurer has small hands because it is possible that some pressure will be maintained on the lever of the callipers not allowing them to exert full pressure. The dial should be read at 5 seconds even if it

is still moving. Do not drag the callipers off the fold at the end of the measurement, as this is uncomfortable and may damage the callipers. Consciously open the jaws to remove them. At least 3 measurements are taken at each site, releasing the skin-fold and picking it up again each time. In the analyses the average of three measurements was used.

Triceps skin-fold technique: The subject stands with their back to the measurer, arms hanging by their sides. The tip of the acromion (the point of the shoulder) is palpated and marked. With the subject's arm flexed at 90°, the olecranon (tip of the elbow) is palpated. Put the tape measure on the mark on the acromion and drop it down to the elbow, by the side of the arm. Read the exact distance as if you had drawn an imaginary horizontal line from the bottom most point of the elbow to your tape measure. Mark a point on the arm halfway between the acromion and elbow. This marks the vertical level at which the circumference will be measured. It is important that this measurement is made with the arm flexed, otherwise the tape takes an oblique course across the upper arm, and the mid-point is too high up. The subject is then asked to relax, with the arm hanging by their side. This is important as a very different reading may be obtained if the arm is not fully relaxed. The tape is placed around the upper arm with the upper border of the tape at the level of the mark, as if to measure mid-upper arm circumference. With the tape in position a horizontal line is drawn on the skin posteriorly and anteriorly at the level of the first mark. The posterior line is used for the triceps fold and the anterior line for the biceps fold. To determine the side-to-side position at which the skin-fold is measured, you must "eyeball" the mid-point and the most dorsal (i.e., the part which sticks out furthest posteriorly) part of the upper arm at the level of your horizontal mark. Make a vertical mark to form a cross. The skin-fold is picked up in a vertical "tube", with two hands, at least 1cm. above and below the cross. The skin-fold callipers are applied at the level of the cross, with the cross on the apex of the fold. It has been shown that the precise site is important, and that very different readings can be obtained, especially by displacement laterally and especially in obese subjects.

Biceps skin-fold: The subject faces the measurer with their arms hanging down and the (non dominant side) palm facing forward. An anterior horizontal line already marks the level at which the skin-fold will be measured. As with the triceps skin-fold, you need to "eyeball" the point along this line where the arm bulges forward the most–the mid point of the belly of the biceps muscle. Mark a vertical line here to form a cross. There is sometimes a prominent blood vessel visible here, but you can ignore it: it will not be damaged by the callipers. The skin-fold is picked up vertically and the callipers are applied at the level of the cross, with the cross on the apex of the fold.

Subscapular skin-fold: The subject stands with the shoulders and arms relaxed. The lowermost tip of the scapula is identified. This is easy in a slim subject but may be difficult in the obese. It may help to follow the medial border of the scapula downwards until the inferior angle is felt. Alternatively, you

can make the scapula stand out by asking the subject to put their arm behind their back, in a half-nelson. Once it is located, however, the subject must relax their arm again before you mark the skin with a cross, immediately below the lowermost tip of the scapula. The skin-fold is picked up obliquely, in the natural cleavage of the skin and the callipers are applied at the level of the cross, with the cross on the apex of the fold.

Upper suprailiac skin-fold: Stand behind the subject. They should stand straight and relaxed with their arms folded in front of them. Locate the iliac crest, the large curving pelvic bone, just below the waist. In obese subjects, you need to palpate firmly, and in all subjects, it helps to feel both sides together. Draw a horizontal line just above the crest at the side. Next find the mid axillary line: ask the subject to lift up their arm. The apex of the axilla is at the lowest point of the axillary "hollow", just behind the thick fold made by the pectoral muscle. Drop an imaginary vertical line down from the apex of the axilla. This is the mid axillary line. Draw a line where this imaginary vertical line meets the horizontal line. Pick up the fold in the natural creases of the skin and apply the callipers at the level of the cross, with the cross on the apex of the fold. It may help to ask the subject to tilt towards you to ease the tension on the skin while picking up the skin-fold.

3. **Arm muscle area:** This measurement is calculated using triceps skin-fold thickness (cm) and mid-arm circumference (cm) measurements. In practice this measurement overestimates arm muscle area by 20 to 25% as it includes mid-arm bone area, when compared with CT evaluation of the same tissues. To correct for this an equation developed by Heymsfield and co-workers (1982), was applied which subtracted the bone component of the upper arm from the figure obtained. Corrected AMA equations for men and women were respectively: [(MAC - pi x TSF) 2/4 pi] - 10, and [MAC - pi x TSF) 2/4 pip] - 6.5. It was this method used in AMA assessment in SWS subjects and subsequently used for the maternal variables in this study.

Appendix VI

19 weeks		Maternal parity and fetal measurements	mean	95% CI	n	paired t- test	
						t	p
Thigh volume (cm³)	boys	null	6.564	6.308 – 6.819	*124*	–0.90	0.37
		multi	6.771	6.494 – 7.048	*96*		
	girls	null	6.202	5.960 – 6.367	*132*	–0.91	0.36
		multi	6.362	6.028 – 6.622	*79*		
Fat / skin volume (cm³)	boys	null	1.865	1.756 – 1.974	*124*	–0.05	0.96
		multi	1.869	1.735 – 2.004	*95*		
	girls	null	1.716	1.623 – 1.809	*130*	–1.14	0.26
		multi	1.804	1.681 – 1.928	*78*		
Muscle volume (cm³)	boys	null	4.204	4.018 – 4.389	*126*	–1.12	0.27
		multi	4.364	4.147 – 4.582	*95*		
	girls	null	3.955	3.793 – 4.117	*130*	–0.79	0.43
		multi	4.063	3.843 – 4.283	*78*		
Femur volume (cm³)	boys	null	0.475	0.459 – 0.492	*143*	**–2.13**	**0.03**
		multi	0.504	0.483 – 0.525	*105*		
	girls	null	0.465	0.450 – 0.480	*146*	0.02	0.98
		multi	0.465	0.444 – 0.485	*90*		
Femur length (cm)	boys	null	2.917	2.89 – 2.95	*150*	–0.52	0.60
		multi	2.930	2.89 – 2.97	*111*		
	girls	null	2.902	2.87 – 2.94	*152*	–0.58	0.57
		multi	2.918	2.88 – 2.96	*95*		
Proximal CSA (cm²)	boys	null	0.263	0.255 – 0.272	*146*	**–2.28**	**0.02**
		multi	0.279	0.268 – 0.290	*106*		
	girls	null	0.267	0.257 – 0.277	*146*	1.10	0.27
		multi	0.258	0.246 – 0.270	*90*		
Mid-shaft CSA (cm²)	boys	null	0.131	0.126 – 0.135	*147*	–0.33	0.75
		multi	0.132	0.127 – 0.137	*108*		
	girls	null	0.13	0.126 – 0.134	*151*	0.74	0.46
		multi	0.128	0.122 – 0.133	*94*		
Distal CSA (cm²)	boys	null	0.226	0.218 – 0.233	*142*	–1.91	0.06
		multi	0.238	0.227 – 0.248	*102*		
	girls	null	0.227	0.218 – 0.236	*148*	1.56	0.12
		multi	0.216	0.205 – 0.226	*104*		

Tabulation of maternal parity status and core fetal measurements in boys and girls at 19 weeks.

		Maternal parity and fetal measurements					
34 weeks			mean	95% CI	n	paired t- test	
						t	p
Thigh volume (cm^3)	boys	null	92.949	90.282 – 95.617	*126*	–1.70	0.09
		multi	96.47	93.321 – 99.612	*97*		
	girls	null	90.551	87.936 – 93.165	*124*	–1.16	0.25
		multi	93.051	89.610 – 96.491	*81*		
Fat / skin volume (cm^3)	boys	null	37.968	36.553 – 39.382	*125*	**–2.28**	**0.02**
		multi	40.619	38.745 – 42.492	*97*		
	girls	null	37.734	36.063 – 39.404	*124*	–1.54	0.13
		multi	39.717	37.844 – 41.589	*81*		
Muscle volume (cm^3)	boys	null	52.352	50.746 – 53.958	*126*	–0.53	0.60
		multi	52.972	51.352 – 54.593	*97*		
	girls	null	50.046	48.752 – 51.340	*124*	–0.65	0.52
		multi	50.796	48.785 – 52.808	*82*		
Femur volume (cm^3)	boys	null	2.790	2.701 – 2.880	*140*	–1.66	0.10
		multi	2.902	2.807 – 2.996	*107*		
	girls	null	2.749	2.662 – 2.836	*143*	–0.10	0.92
		multi	2.756	2.654 – 2.857	*93*		
Femur length (cm)	boys	null	6.329	6.283 – 6.376	*143*	–1.40	0.16
		multi	6.381	6.323 – 6.437	*107*		
	girls	null	6.368	6.322 – 6.329	*147*	–0.30	0.77
		multi	6.379	6.329 – 6.428	*94*		
Proximal CSA (cm^2)	boys	null	1.041	1.003 – 1.080	*135*	0.91	0.36
		multi	1.016	0.978 – 1.054	*105*		
	girls	null	1.002	0.969 – 1.035	*142*	0.27	0.79
		multi	0.994	0.949 – 1.040	*88*		
Mid-shaft CSA (cm^2)	boys	null	0.334	0.324 – 0.345	*142*	–0.75	0.46
		multi	0.341	0.326 – 0.356	*107*		
	girls	null	0.338	0.326 – 0.349	*146*	–0.62	0.54
		multi	0.343	0.330 – 0.356	*94*		
Distal CSA (cm^2)	boys	null	0.800	0.765 – 0.836	*137*	0.53	0.60
		multi	0.787	0.753 – 0.821	*104*		
	girls	null	0.772	0.742 – 0.803	*136*	–0.04	0.97
		multi	0.773	0.734 – 0.813	*91*		

Tabulation of maternal parity status and core fetal measurements in boys and girls at 34 weeks.

Appendix VII

		Maternal smoking status and fetal measurements					
19 weeks			mean	95% CI	n	paired t- test	
						t	p
Thigh volume (cm^3)	boys	non-smoker	6.588	6.368 – 6.808	*153*	–0.81	0.42
		smoker	6.754	6.395 – 7.113	*67*		
	girls	non-smoker	6.208	6.017 – 6.398	*165*	–0.36	0.72
		smoker	6.283	5.912 – 6.654	*46*		
Fat / skin volume (cm^3)	boys	non-smoker	1.845	1.755 – 1.935	*152*	–0.75	0.45
		smoker	1.915	1.726 – 2.104	*67*		
	girls	non-smoker	1.731	1.647 – 1.816	*164*	–0.92	0.36
		smoker	1.816	1.656 – 1.976	*44*		
Muscle volume (cm^3)	boys	non-smoker	4.252	4.081 – 4.423	*153*	–0.43	0.67
		smoker	4.318	4.067 – 4.570	*68*		
	girls	non-smoker	4.005	3.860 – 4.150	*164*	0.28	0.78
		smoker	3.960	3.662 – 4.259	*44*		
Femur volume (cm^3)	boys	non-smoker	0.489	0.474 – 0.504	*175*	0.30	0.76
		smoker	0.484	0.458 – 0.511	*73*		
	girls	non-smoker	0.464	0.450 – 0.478	*183*	–0.21	0.83
		smoker	0.467	0.443 – 0.492	*53*		
Femur length (cm)	boys	non-smoker	2.919	2.892 – 2.946	*183*	–0.49	0.62
		smoker	2.932	2.883 – 2.981	*78*		
	girls	non-smoker	2.903	2.873 – 2.932	*189*	–0.70	0.49
		smoker	2.924	2.871 – 2.978	*58*		
Proximal CSA (cm^2)	boys	non-smoker	0.269	0.262 – 0.277	*177*	–0.18	0.86
		smoker	0.271	0.257 – 0.285	*75*		
	girls	non-smoker	0.264	0.255 – 0.273	*182*	0.33	0.74
		smoker	0.261	0.247 – 0.276	*54*		
Mid-shaft CSA (cm^2)	boys	non-smoker	0.132	0.128 – 0.136	*177*	0.83	0.41
		smoker	0.129	0.124 – 0.134	*78*		
	girls	non-smoker	0.129	0.125 – 0.133	*188*	–0.42	0.67
		smoker	0.131	0.124 – 0.138	*57*		
Distal CSA (cm^2)	boys	non-smoker	0.232	0.224 – 0.239	*171*	0.66	0.51
		smoker	0.227	0.216 – 0.239	*73*		
	girls	non-smoker	0.225	0.217 – 0.233	*183*	1.37	0.17
		smoker	0.214	0.202 – 0.226	*52*		

Tabulation of maternal smoking status and core fetal measurements in boys and girls at 19 weeks.

		Maternal smoking status and fetal measurements					
34 weeks			mean	95% CI	n	paired t- test	
						t	p
Thigh volume (cm³)	boys	non-smoker	94.597	92.350 – 96.843	*152*	0.17	0.87
		smoker	94.227	89.937 – 98.518	*71*		
	girls	non-smoker	91.783	89.400 – 94.166	*161*	0.44	0.66
		smoker	90.645	86.314 – 94.975	*44*		
Fat / skin volume (cm³)	boys	non-smoker	38.921	37.631 – 40.211	*161*	–0.51	0.61
		smoker	39.563	37.209 – 41.917	*71*		
	girls	non-smoker	38.785	37.344 – 40.227	*161*	0.81	0.42
		smoker	37.535	34.988 – 40.082	*44*		
Muscle volume (cm³)	boys	non-smoker	52.951	51.672 – 54.229	*152*	0.83	0.41
		smoker	51.918	49.562 – 54.274	*71*		
	girls	non-smoker	50.349	49.101 – 51.597	*162*	0.02	0.99
		smoker	50.33	47.821 – 52.837	*44*		
Femur volume (cm³)	boys	non-smoker	2.860	2.784 – 2.937	*170*	0.99	0.32
		smoker	2.790	2.663 – 2.916	*77*		
	girls	non-smoker	2.754	2.678 – 2.830	*184*	0.15	0.88
		smoker	2.742	2.609 – 2.875	*52*		
Femur length (cm)	boys	non-smoker	6.351	6.310 – 6.392	*173*	0.02	0.99
		smoker	6.351	6.277 – 6.424	*77*		
	girls	non-smoker	6.384	6.344 – 6.424	*188*	1.24	0.22
		smoker	6.332	6.270 – 6.394	*53*		
Proximal CSA (cm²)	boys	non-smoker	1.043	1.009 – 1.078	*165*	1.39	0.17
		smoker	1.002	0.960 – 1.044	*75*		
	girls	non-smoker	0.100	0.966 – 1.027	*179*	–0.307	0.76
		smoker	1.007	0.950 – 1.064	*51*		
Mid-shaft CSA (cm²)	boys	non-smoker	0.339	0.329 – 0.350	*172*	0.64	0.52
		smoker	0.333	0.318 – 0.348	*77*		
	girls	non-smoker	0.339	0.329 – 0.348	*187*	–0.39	0.70
		smoker	0.343	0.323 – 0.363	*53*		
Distal CSA (cm²)	boys	non-smoker	0.814	0.783 – 0.845	*166*	**2.35**	**0.02**
		smoker	0.751	0.712 – 0.791	*75*		
	girls	non-smoker	0.775	0.748 – 0.801	*178*	0.31	0.75
		smoker	0.766	0.709 – 0.822	*49*		

Tabulation of maternal smoking status and core fetal measurements in boys and girls at 34 weeks.

Appendix VIII

BOYS	**Maternal parity status**					
Fetal growth velocity (z scores)		mean	95% CI	n	paired t- test	
					t	p
Thigh volume (cm³)	null	–0.055	–0.266 – 0.157	*106*	–0.68	0.50
	multi	0.052	–0.173 – 0.278	*85*		
Fat / skin volume (cm³)	null	–0.163	–0.391 – 0.064	*105*	–1.62	0.11
	multi	0.137	–0.162 – 0.436	*84*		
Muscle volume (cm³)	null	0.062	–0.158 – 0.282	*106*	0.53	0.60
	multi	–0.023	–0.252 – 0.206	*84*		
Femur volume (cm³)	null	0.037	–0.173 – 0.247	*131*	0.58	0.57
	multi	–0.059	–0.322 – 0.203	*100*		
Femur length (cm)	null	–0.099	–0.282 – 0.083	*140*	–0.96	0.34
	multi	0.035	–0.174 – 0.244	*105*		
Proximal CSA (cm²)	null	0.209	–0.008 – 0.426	*130*	**2.49**	**0.01**
	multi	–0.217	–0.479 – 0.046	*98*		
Mid-shaft CSA (cm²)	null	–0.079	–0.285 – 0.128	*136*	–0.27	0.079
	multi	–0.032	–0.317 – 0.252	*102*		
Distal CSA (cm²)	null	0.146	–0.083 – 0.374	*127*	1.61	0.11
	multi	–0.140	–0.407 – 0.128	*93*		
GIRLS	**Maternal parity status**					
Thigh volume (cm³)	null	0.070	–0.116 - 0.257	*109*	–0.01	0.99
	multi	0.723	–0.170 – 0.315	*68*		
Fat / skin volume (cm³)	null	0.076	–0.171 – 0.323	*108*	0.01	0.99
	multi	0.074	–0.209 – 0.358	*67*		
Muscle volume (cm³)	null	–0.002	–0.179 – 0.176	*108*	–0.02	0.98
	multi	0.002	–0.219 – 0.222	*68*		
Femur volume (cm³)	null	0.038	–0.160 – 0.235	*137*	–0.22	0.83
	multi	0.072	–0.167 – 0.311	*86*		
Femur length (cm)	null	0.075	–0.095 – 0.246	*145*	0.33	0.74
	multi	0.028	–0.210 – 0.266	*92*		
Proximal CSA (cm²)	null	–0.061	–0.263 – 0.140	*136*	–0.28	0.78
	multi	–0.014	–0.299 – 0.271	*82*		
Mid-shaft CSA (cm²)	null	–0.028	–0.250 – 0.195	*143*	–0.94	0.35
	multi	0.139	–0.129 – 0.407	*91*		
Distal CSA (cm²)	null	–0.007	–0.218 – 0.204	*132*	–0.98	0.33
	multi	0.160	–0.104 – 0.423	*82*		

Fetal measurement growth velocity and maternal parity status for boys and girls.

Appendix IX

BOYS	**Maternal smoking status**					
Fetal growth velocity (z scores)		mean	95% CI	n	paired t- test	
					t	p
Thigh volume (cm^3)	non-smoker	0.045	–0.125 – 0.215	*130*	0.99	0.33
	smoker	–0.119	–0.440 – 0.202	*61*		
Fat / skin volume (cm^3)	non-smoker	–0.043	–0.236 – 0.150	*128*	–0.21	0.84
	smoker	–0.002	–0.409 – 0.404	*61*		
Muscle volume (cm^3)	non-smoker	0.110	–0.073 – 0.293	*128*	1.55	0.12
	smoker	–0.153	–0.459 – 0.153	*62*		
Femur volume (cm^3)	non-smoker	0.033	–0.156 – 0.223	*159*	0.68	0.50
	smoker	–0.089	–0.411 – 0.233	*72*		
Femur length (cm)	non-smoker	–0.011	–0.172 – 0.151	*169*	0.67	0.51
	smoker	–0.111	–0.373 – 0.151	*76*		
Proximal CSA (cm^2)	non-smoker	0.138	–0.067 – 0.344	*156*	**1.95**	**0.05**
	smoker	–0.217	–0.510 – 0.076	*72*		
Mid-shaft CSA (cm^2)	non-smoker	–0.069	–0.280 – 0.143	*162*	–0.17	0.87
	smoker	–0.038	–0.317 – 0.241	*76*		
Distal CSA (cm^2)	non-smoker	0.130	–0.089 – 0.349	*150*	1.76	0.08
	smoker	–0.200	–0.478 – 0.077	*70*		
GIRLS	**Maternal smoking status**					
Thigh volume (cm^3)	non-smoker	0.134	–0.028 – 0.296	*140*	1.66	0.10
	smoker	–0.167	–0.509 – 0.175	*37*		
Fat / skin volume (cm^3)	non-smoker	0.171	–0.031 – 0.373	*140*	**2.06**	**0.04**
	smoker	–0.308	–0.758 – 0.142	*35*		
Muscle volume (cm^3)	non-smoker	0.014	–0.137 – 0.166	*141*	0.42	0.68
	smoker	–0.059	–0.392 – 0.275	*35*		
Femur volume (cm^3)	non-smoker	0.064	–0.110 – 0.239	*174*	0.33	0.74
	smoker	0.003	–0.306 – 0.312	*49*		
Femur length (cm)	non-smoker	0.128	–0.022 – 0.278	*184*	1.90	0.06
	smoker	–0.189	–0.523 – 0.144	*53*		
Proximal CSA (cm^2)	non-smoker	–0.080	–0.270 – 0.109	*169*	–0.824	0.41
	smoker	0.084	–0.247 – 0.415	*49*		
Mid-shaft CSA (cm^2)	non-smoker	0.029	–0.170 – 0.227	*182*	–0.18	0.86
	smoker	0.067	–0.270 – 0.404	*52*		
Distal CSA (cm^2)	non-smoker	0.026	–0.163 – 0.215	*168*	–0.71	0.48
	smoker	0.170	–0.166 – 0.505	*46*		

Fetal measurement growth velocity and maternal smoking status for boys and girls.

APPENDIX X

Method for 25OHD concentration

Serum 25OHD is measured by radioimmunoassay using Diasorin RIA kit (Diasorin, Stillwater, Minnesota, USA). In this method 25OHD in the serum is extracted with acetonitrile. An aliquot of the extracted sample is incubated with an antibody (goat) specific for 25OHD and an iodinated $25OHD_3$ for 90 minutes at room temperature. The bound and free tracer are separated by using a mixture of donkey anti-goat serum and PEG (polyethylene glycol). After centrifugation the supernatant is discarded and the radioactivity in the precipitate is counted using a gamma counter. This assay has a detection limit of 4nmol/L and the analytical CV of the method is 9.1% at 22nmol/L.

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Appendix XI

Below is tabulation of the significant results obtained for fetal bone size at 19 and 34 weeks and the growth velocity between those two points correlated with maternal variables. Positive significant associations are shown with + after the fetal acronym and significant negative associations are shown with (-) after the fetal acronym. All significant results are those of p = 0.05 and below.

	Bone determinants and significant results								
	19 weeks			**34 weeks**			**Growth velocity**		
Maternal variable	**both**	**boys**	**girls**	**both**	**boys**	**girls**	**both**	**boys**	**girls**
BW						**FV + PCSA +**	**FL +**	**FL +**	**FV + FL +**
Age		**PCSA (-)**							
Height				**FL +**	**FL +**	**FL + FV +**	**FL +**		
BMI	**PCSA (-)**		**PCSA (-)**	**FV (-)**		**PCSA (-) MSCS A (-)**			**MSCS A (-)**
SSF			**PCSA (-)**	**FL+**	**FL +**	**FL+ MSCS A (-)**			**MSCS A (-)**
AMA									
Parity					**FV +**			**PCSA +**	
Smoking					**DCSA (-)**			**DCSA (-)**	
Vitamin D	**PCSA (-) DCSA (-)**	**DCSA (-)**	**PCSA (-)**	**DCSA (-)**		**DCSA (-)**			

Appendix XII

Below is tabulation of the significant results obtained for fetal muscle size at 19 and 34 weeks and the growth velocity between those two points correlated with maternal variables. Positive significant associations are shown with + after the fetal acronym and significant negative associations are shown with (-) after the fetal acronym. All significant results are those of p = 0.05 and below.

	Muscle determinants and significant results								
	19 weeks			**34 weeks**			**Growth velocity**		
Maternal variable	**both**	**boys**	**girls**	**both**	**boys**	**girls**	**both**	**boys**	**girls**
BW									
Age									
Height				**MV +**	**MV +**	**MV + FV +**	**MV +**	**MV +**	
BMI				**MV +**	**MV +**	**FV (-)**	**MV +**	**MV +**	
SSF				**MV +**	**MV +**		**MV +**	**MV +**	
AMA	**TV +**			**TV + MV +**	**TV + MV +**	**MV +**	**TV + MV +**	**TV + MV +**	**MV +**
Parity				**TV + SV +**	**FV +**				
Smoking									
Vitamin D									

Appendix XIII

Below is tabulation of the significant results obtained for fetal fat / skin size at 19 and 34 weeks and the growth velocity between those two points correlated with maternal variables. Positive significant associations are shown with + after the fetal acronym and significant negative associations are shown with (-) after the fetal acronym. All significant results are those of p = 0.05 and below.

	Fat / skin determinants and significant results								
	19 weeks			**34 weeks**			**Growth velocity**		
Maternal variable	**both**	**boys**	**girls**	**both**	**boys**	**girls**	**both**	**boys**	**girls**
BW				**BW +**	**TV + SV +**			**TV +**	
Age									
Height					**SV +**			**TV +**	
BMI		**TV +**		**TV +**	**TV +**	**TV +**	**TV +**	**TV +**	
SSF				**TV + SV +**	**TV + SV +**	**TV + SV +**	**TV + SV +**	**TV + SV +**	**TV + SV +**
AMA	**TV +**			**TV + MV + SV +**	**TV + MV + SV +**	**TV + MV + SV +**	**TV + MV +**	**TV + MV +**	**MV +**
Parity				**TV + SV +**	**SV +**				
Smoking									
Vitamin D									**SV +**

Glossary of Technical Terms

absorption - the loss of (electromagnetic) energy to a medium. For instance, an electromagnetic wave which propagates through a plasma will set the electrons into motion. If the electrons make collisions with other particles, they will net energy from the wave.

acoustic impedance - the resistance that a material offers to the passage of a sound wave (colloquial); a property of a medium computed as the product of density and sound propagation speed (characteristic acoustic impedance). Discontinuities in acoustic impedance are responsible for the echoes on which ultrasound imaging is based.

amplitude - the extent of a movement measured from the starting point or position of equilibrium; applied especially to vibratory movements

anlage – in embryology an anlage is a precursor or forerunner of a more mature structure

antero-posterior (AP) - in medical imaging, describing the direction of the ultrasound or x-ray beam, through the patient, from anterior to posterior.

asphyxia - a lack of oxygen or excess of carbon dioxide in the body that is usually caused by interruption of breathing and that causes unconsciousness.

axial - in physics and especially imaging, it refers to the direction along which the central axis of the beam travels, which is perpendicular to the wave front of an ultrasound beam.

bandwidth - the bandwidth is often defined as the width of the frequency response (transducer efficiency vs. frequency relationship) at half maximum transducer output.

biometric - measurement of life; calculation of the probable duration of human life.

bone - the hard, calcified tissue of the skeleton of vertebrate animals, consisting very largely of calcium carbonate, calcium phosphate, and gelatine. Even in the hardest parts of bone there are many minute cavities containing living matter and connected by minute canals, some of which connect with larger canals through which blood vessels ramify.

cancellous – denoting bone that it lattice-like or spongy.

diaphysis - the shaft of a long bone.

distal – pertaining to the furthest part of a limb or bone, away from the midline of the body.

electro-magnetic field – the region in which electro-magnetic radiation from a source exerts an influence on another object with or without there being contact between them.

electro-mechanical coupling coefficient - which is a measure of the efficiency of conversion of sound to electrical energy.

epiphysis - the part of a long bone where growth bone growth occurs from and can be seen as an independent bony structure at the ends of long bones before puberty.

erythema – reddening of the skin caused by dilation of blood vessels.

excitation – a process by which radiation imparts energy to an atom or molecule without causing damage and is dissipated as heat within tissue.

femur - the large bone in the thigh that articulates with the pelvis above and the knee below.

fetus - the developing unborn offspring of an animal that gives birth to its young (as opposed to laying eggs). From approximately three months after conception the offspring take on a recognisable form (all parts in place, etc.). In human development, the period after the seventh or eighth week of pregnancy is the fetal period.

frequency – the number of complete cycles of an electromagnetic wave in a second, measured in Hertz (1 Hz = 1 cycle per second).

gestation sac - the fluid-filled sac in which the fetus develops.

hydramnios – increased amniotic fluid within the gestation sac. (See polyhydramnios and olighydramnios).

insonation – to introduce ultrasound into a material by artificial means, as in the use of a medical ultrasound transducer.

IU – International Unit; a pharmacological unit of measurement for the amount of a substance, based on biological activity or effect. Used for vitamins and for vitamin D the biological equivalent of 0.025 μg cholecalciferol/ergocalciferol (1/40 μg exactly).

lateral – in anatomy it denotes a position farther from the median plane or midline of the body or of a structure. It can also mean pertaining to a side.

longitudinal- the word come from the Latin *longitudo* meaning length. Hence, longitudinal means along the length, running lengthwise, or (by extension) over the course of time.

metaphysis - a conical section of bone between the epiphysis and diaphysis of long bones.

neo-natal - of, relating to, or affecting the newborn and especially the human infant during the first month after birth.

placenta - an organ characteristic of true mammals during pregnancy, joining mother and offspring, providing endocrine secretion and selective exchange of soluble, but not particulate, blood–borne substances through an apposition of uterine and vascularised parts. The human placenta is discoid and contains many vessels. After birth, it weighs about 600 g, is about 16 cm in diameter and 2 cm thick.

post-natal – after delivery.

pre-natal – before delivery.

quantitative ultrasound – the use of ultrasound to calculate density and strength in bone, most often using the adult calcaneum.

oligohydramnios – reduced amniotic fluid within the gestation sac.

osteogenesis - production of bone.

pixel – picture element (pix for picture, el for element). A single, finite-sized element of a digitised video picture. A pixel is defined by its X and Y coordinates and its grey level (luminance), commonly expressed by binary numbers.

placental abruption – where the placental attachment to the uterine wall has been lost and is usually accompanied by pain or bleeding.

polyhydramnios – excessive amniotic fluid within the gestation sac.

pre-eclampsia - a serious condition developing in late pregnancy that is characterized by a sudden rise in blood pressure, excessive weight gain, generalized oedema, proteinuria, severe headache, and visual disturbances and that may result in eclampsia if untreated.

pre-term labour – onset of labour before gestational maturity, usually considered to be under 37 weeks.

proximal – pertaining to the nearest end of a limb or bone, to the midline of the body.

radiation – the process of emitting energy as waves or particles. The energy thus radiated.

raster pattern – mechanical movement pattern of a piece of equipment, such as the scanning head of a DXA machine, which traverses a straight line before advancing superiorly or inferiorly, before completing the next line in the opposite direction to the previous.

reflection - the act of reflecting, or turning or sending back, or the state of being reflected. Specifically, the return of rays, beams, sound, or the like, from a surface.

refraction - bending of waves as they pass from a medium having one refractive index to a medium (or region within a medium) having a different refractive index.

resolution - complete return to normal structure and function: used, for example: of an inflammatory lesion or of a disease

reverberation - multiple echoes or reflections seen in ultrasonography, as an artifactual image caused by delay of an echo which has been reflected back and forward again before returning to the transducer.

rickets - a condition caused by deficiency of vitamin D, especially in infancy and childhood, with disturbance of normal ossification. The disease is marked by bending and distortion of the bones under muscular action, by the formation of nodular enlargements on the ends and sides of the bones, by delayed closure of the fontanelles, pain in the muscles and sweating of the

head. Vitamin D and sunlight together with an adequate diet are curative, provided that the parathyroid glands are functioning properly.

scatter - spread in irradiation away from its target.

skeletal muscle – it is the striated muscles that have fibres connected at either or both extremities with the bony framework of the body.

skeleton - a solid or fluid system which allows muscles to relax after contracting (in general, because there is an opposing muscle which pulls the skeletal part in the opposite direction when it contracts). In mammals, it is the framework of bone onto which muscles and tissues are attached.

stem cells – specialised mammalian tissue cells that can both renew themselves and give rise to more specialised daughter cells.

thigh - the proximal segment of the hind limb between the knee and the trunk.

trabecular – divided into partitions, as in the inner part of a bone.

transducer - a device that transforms one type of energy to another.

transverse or transverse section (TS) - lying or being across, or in a crosswise direction, often opposed to longitudinal.

ultrasound (US) - a type of medical imaging technique which uses high-frequency sound waves.

uterus - the hollow muscular organ in female mammals in which the fertilised ovum normally becomes embedded and in which the developing embryo and fetus is nourished. In the non-gravid human, it is a pear shaped structure, about 6 cm in length, consisting of a body, fundus, isthmus and cervix. Its cavity opens into the vagina below and into the uterine tube on either side at the cornu. It is supported by direct attachment to the vagina and by indirect attachment to various other nearby pelvic structures.

volumetric - of or pertaining to the measurement of volume.

wavelength – the distance between successive crests of an electromagnetic wave passing through a given material, measured in meters.

X-ray - a type of radiation used for imaging purposes. It uses energy beams of very short wavelengths (0.1 to 1000 Angstroms) that can penetrate most substances except heavy metals. This is the commonest form of imaging technique used in clinical practice to capture internal images on photographic film (radiograph).

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