Serial DXA Bone and Soft Tissue Estimations in Growing Sheep

Christopher G. Schultz¹, Jodie Dier², Timothy R. Kuchel³, Robert J. Moore⁴ and Barry E. Chatterton¹*

¹Department of Nuclear Medicine, P.E.T and Bone Densitometry, Royal Adelaide Hospital, South Australia, Australia.
²Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia.
³Preclinical Imaging and Research Laboratories, S.A. Health and Medical Research Institute, Adelaide, South Australia, Australia.
⁴The Adelaide Centre for Spinal Research, S.A. Pathology, Adelaide, South Australia, Australia.

Authors’ contributions
This work was carried out in collaboration between all authors. Authors CGS, RJM and BEC designed the study and wrote the protocol, authors CGS performed the statistical analysis and wrote the first draft of the manuscript. Authors TRK and JD managed the animals and assisted CGS in performance of the scans. All authors read and approved the final manuscript.

ABSTRACT

Objectives: We aimed to establish the typical rate of skeletal mineralisation in the growing sheep. The sheep (size, ease of handling, cost and bone physiology) is establishing itself as an appropriate model for bone research.

Materials and Methods: Body composition including bone density at 4 time points in the first two years was performed using DXA on a cohort of 14 developing lambs, as well as measurements in comparable adult sheep.

Results: Adult levels of bone density are reached by 18 months and lean body mass by 2 years in sheep.

Conclusions: Depending on the indications for studying bone and other metabolism in sheep, the rate of development should be considered in selecting the age group of experimental animals.

*Corresponding author: Email: barry.chatterton@gmail.com;
1. INTRODUCTION

Osteoporosis models in animals are required to investigate the efficacy of pharmaceutical and physical treatments, and the United States Food and Drug Administration (FDA) 1994 “Guidelines for pre-clinical and clinical evaluation of agents used in the treatment or prevention of osteoporosis” [1] requires any treatment be tested in two distinct animal species demonstrating modeling and remodeling of bone. The well established ovariectomised rodent is to be used as the modeling system of bone, whilst the second species should be a large animal model with remodeling of bone similar to humans (having a Haversian system [1]), with aged canines, glucocorticoid treated pigs, ewes and primates listed in the guidelines. In both models, oophorectomy is recommended for mimicking post-menopausal osteoporosis.

Davidson et al. [2] have also suggested that an appropriate model should allow for: “1) appropriateness as an analog, 2) transferability of information, 3) genetic uniformity of organisms where applicable, 4) background knowledge of biological properties, 5) cost and availability, 6) generalizability of the results, 7) ease and adaptability to experimental manipulation, 8) ecological considerations, and 9) ethical and societal implications”. Sheep remodel bone tissue similarly to humans [3], they have bones of a similar size, and the existing large animal models of osteoporosis have limitations. Non-human primates are the ideal model with respect to bone metabolism, but are expensive, must be of a suitable age for osteoporosis investigation and difficult to maintain. Of the FDA suggested models, the canine model does not lose bone after oophrectomy and are ethically sensitive to use. The mini pig is metabolically similar to humans, but has much higher bone density and is more difficult to handle. Sheep remodel bone tissue similarly to humans [3], they have bones of a similar size, and the ewe has a similar oestrogen profile to humans, but the results may be affected by seasonal variation. Rabbit models have also been proposed [4], and demonstrate remodeling of trabecular bone, relatively rapid maturation of the skeleton and a Haversian system.

Sheep are extensively used in orthopaedic studies due to the similarity of bone size, bone metabolism, bone strength and ease of handling [3,5,6]. The sheep has also been considered to be a more suitable large animal model for human osteoporosis, although oophorectomy must be supplemented with glucocorticoid treatment [7-9], and seasonal variation may be a confounding factor [3,5]. Knowledge of the age and trajectory of skeletal maturity is important in designing animal models of bone strength, remodeling and metabolism, particularly if younger animals are used.

This study aimed to follow the changes in bone mass and tissue composition in sheep from 4 months to 4 years of age and to determine the rate of change and the time to reach maturity of body composition.

2. MATERIALS AND METHODS

2.1 Animals

A cohort of 14 female Merino lambs had dual energy x-ray absorptiometric (DXA) measurements of the lumbar spine and total body performed at approximately 6 month intervals from 4 months of age to 24 months of age. These sheep were randomly selected from the research flock at the IMVS field station. Cross sectional data from 14 three and 20 four year old ewes randomly selected from the field station flock (not the serial cohort, but related) were included to complete the data acquisition in an acceptable time.

The animals were cared for in the open and had free access to pasture and hay as required to maintain a normal growth rate. All were born between March and May (the Australian autumn). The sheep were supplied from a purpose bred merino sheep flock managed by the Institute of Medical and Veterinary Science (IMVS, now SA Pathology). As far as practicable, the sheep experienced a standardized husbandry and nutrition regime as found in extensive husbandry practice. All authors hereby declare that “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws. All experiments have been examined and approved by the IMVS Animal Ethics committee.

For the densitometry study, animals were anaesthetised with intravenous thiopentone, intubated and anesthesia was maintained via a semi-closed circle with 100% oxygen and
isoflurane for approximately 30 minutes. Scan time per animal was about 10 minutes.

All DXA measurements were performed on the same Hologic QDR2000 bone densitometer (Hologic, Waltham MA, USA. s/n 2263 software version 7.10b) using standard acquisition modes by the same accredited densitometrist (CGS).

The machine was serviced at the recommended intervals and quality assurance performed at least weekly using the approved phantoms.

The sheep were weighed at each densitometry episode.

Commercial DXA software is designed for human anatomy, and techniques need to be adapted for use in large animal systems.

Nevertheless it is still a requirement to image and analyse the lumbar spine or total body in larger animals with different body morphology to maintain general principles of DXA imaging, including:

- Reproducible positioning
- True anterior/posterior images of the skeleton
- Correct and consistent bone edge definition
- Minimal artefact in the scanned region

To maintain a consistent orientation of the spine, foam wedges were placed laterally at the mid-dorsal level for spine scans, allowing imaging of the lumbar spine in a true posterior/anterior orientation. The lower limbs were extended and taped down with paper masking tape to minimise lordosis. The lumbar spine was imaged in Array Spine Medium mode with a scan length of 24 cm.

For the analysis in this study, significant differences in sheep body morphology compared to humans include a very long neck and configuration of the limbs. The lumbar vertebrae are longer and narrower and have longer transverse processes (Fig. 1). The sheep were imaged supine in Array mode, with the hind limbs extended and taped together. The fore limbs were folded into a resting position and taped to the chest and scanning bed as illustrated (Fig. 1).

### 2.2 Analysis

The use of the analysis software must also be adapted for use in an animal model. The sheep lumbar spine typically has 6 vertebrae (but may have 7), with L6 similar to L5 in the human. In this study, L6 was not analysed, and L2-L5 were treated as human L1-L4 (Fig. 1).

Bone mineral density (BMD) is very low in developing sheep. It is important to recognise that at very low BMD levels, software edge detectors may fail. Placing of bone edges in this study required careful attention to anatomical landmarks of bone to ensure the vertebral bodies were analysed with the exclusion of transverse processes.

BMD and bone mineral content (BMC) were calculated using standard Array Spine analysis software (v4.76a).

Enhanced Array Whole Body software (5.73a) was used for total body analysis, but sub-regions of the body could not be analysed reliably using the human software due to the overlap of the upper limbs and the anatomical features of the pelvis. However, total body BMD, BMC and tissue composition may be reliably measured without accurate placement of the regions of interest (Fig. 2).

Scan results were extracted to dBase files and collated using custom written extraction software. The mean and standard deviation and standard error were determined for BMC, BMD, area, and lean and fat results for each age using Microsoft Excel 2003. Student’s t-test was used to establish differences between these variables at the different time points. A probability, p<.05, was regarded as a significant difference between the groups.

Least-squares linear correlation between the estimated weight of the sheep (by DXA) and measured weight was performed using Microsoft Excel as above.

### 3. RESULTS

The serial results are shown in Table 1.

Fig. 3 shows the change in BMD with age in the lumbar spine for L2-L5. This can be seen to increase to a plateau by 18 months, and remain relatively static. BMC initially increased rapidly, but continued to rise (Fig. 4) until year 3. A significant difference exists between time points before 10 month and after 18 month for both BMC and BMD (p<0.01) only. There is no significant difference between other time points.
The composition analysis could not be performed on the 4-month data.

The total body BMD (Fig. 5) also showed similar changes, with non-significant increases after 18 months (p>0.05). BMC increased until 2 years before reaching a plateau (Fig. 6), with no significant change between ages after 2 years (p>0.05).

Examination of the body composition changes with age (Fig. 7) shows lean tissue mass plateauing by 24 months. No significant changes in lean tissue were noted after this (p>0.05). However, fat was observed to remain relatively unchanged after 18 months after the initial increase, with no significant changes after that age (p>0.1).

Total body mass continued to increase after 24 months, although not significantly, until 3 years of age. A comparison of measured body weight to DXA estimated mass showed close agreement. Linear regression analysis showed: Measured mass = 0.97 DXA mass + 598g, with a correlation coefficient of 0.99 (Fig. 8).

Fig. 1. Positioning of animal for scanning. Masking tape was used to secure the animal to maintain correct orientation. The Hologic body composition phantom is adjacent to the animal. (right) lumbar spine analysis. Human software has been adapted for the analysis and labels are not correct with regard to vertebral level, representative spinal images during sheep growth are shown

Fig. 2. Total body analysis. Human software has been adapted for the analysis. Subregions were not used due to forelimb overlap in the upper abdomen. Representative whole body images in the growing sheep are shown
Table 1. Regional BMD

| Age (months) | Bone Composition | |
|--------------|------------------|---|
| Spine BMD (g/cm²) | Total body BMD (g/cm²) | BMC (g) | Fat (kg) | Lean (kg) | Weight estimated by DXA (kg) |
| 4 | 0.519±0.045 | 0.679±0.024 | 298.5±49.4 | n/a | n/a | n/a |
| 10 | 0.525±0.039 | 0.823±0.046 | 457.6±68.2 | 1.4±0.6 | 24.7±1.9 | 26.6±2.4 |
| 16 | 0.869±0.057 | 1.052±0.037 | 1058.4±99.9 | 8.5±2.2 | 33.7±1.6 | 43.2±2.9 |
| 24 | 0.868±0.083 | 1.067±0.051 | 1181.3±92.2 | 7.2±2.4 | 39.3±2.4 | 47.6±3.5 |
| 36 | 0.819±0.073 | 1.079±0.058 | 1278.1±153.4 | 10.2±3.6 | 41.5±4.5 | 53.0±6.2 |
| 48 | 0.907±0.100 | 1.117±0.053 | 1270.1±184.7 | 7.3±3.9 | 51.4±6.7 | 51.4±6.6 |

BMD = bone mineral density, BMC = bone mineral content, DXA = Dual energy X-ray absorptiometry, n/a = not available, variables are reported as mean with standard deviation.

Fig. 3. Change in lumbar spine BMD with age. Near maximal BMD is attained by 18 months of age. Error bars are standard deviation.

Fig. 4. Change in lumbar spine BMC and Area with age. Near maximal BMC and BMD is attained by 18 months of age. Error bars are standard deviation.
Fig. 5. Change in Total Body BMD with age. Near maximal BMD is attained by 18 months of age. Error bars are standard deviation.

Fig. 6. Change in total body BMC and Area with age. Near maximal BMC is attained by 18 months of age but continues to rise until 3 years. Error bars are standard deviation.

3.1 T Scores

The peak BMD in the lumbar spine at age 4 (corresponding to a T score of 0) is 0.87 g/cm$^2$ with a standard deviation (T score) of 0.08 g/cm$^2$. The peak BMD in the total body is 1.08 g/cm$^2$ with a standard deviation of 0.06 g/cm$^2$. These results are constrained by the small size of the group, nevertheless, the relatively small Standard estimate of the mean (SEM) suggests these values are of a sufficiently small range to be useful.

4. DISCUSSION

The composition of growing sheep is usually of major concern in the meat industry to maximise growth with the minimum resource input. Most assessment methods have included slaughter of the sheep, with regard to the skeleton, bone markers [8] or sizing of bones using x-ray [10]. Imaging techniques, such as peripheral quantitative computed tomography (pQCT) have been used in excised specimens [11,12].
Fig. 7. Change in total body composition with age. Near maximal fat is attained by 18 months of age, lean mass continues to increase to 3 years. Error bars are standard deviation.

Fig. 8. Correlation of measured mass and DXA estimated body mass. The correlation coefficient was 0.99 with a slope of 0.97.

For research purposes using sheep as a model for human bone disease, greater emphasis on the skeleton is necessary with higher precision and preferably a non-invasive or non-destructive assessment to allow the monitoring of intervention. Turner [13] showed an acceptable precision for repeated DXA studies in sheep, with good correlation between different bony sites and densitometers. [14] using DXA found a coefficient of variation (CV) of smaller than 1.6% in DXA measurements of sheep spine BMD and accuracy lower than 5% when compared with excised vertebrae. Mercier et al. [15] found only moderate correlation with bone mass with DXA and dissected sheep.

We have described serial DXA-estimated body composition in a cohort of 14 ewes of a single breed of sheep and also valued from older sheep of the same breed. We believe this is the first description of such a data set.

Near maximal bone mineral values were observed in the lumbar spine and total body at 18 months of age. This occurs at approximately the body mass at which Merino ewes reach sexual maturity and is similar to other mammals (e.g. Orwoll et al. [16]). Despite this, at sexual maturity, the ewe is not yet mature from the skeletal point of view, and bone growth ends a long time after puberty. Closure of the vertebral
body epiphysis may not occur until age 5 [5]. Nevertheless, the sheep at a younger age is acceptable for human-related research [17-21].

In this study, we observed that the maximum spinal measured BMD is 13% lower than the human lumbar spine and 3% lower than the human total body using the pre-menopausal range from the Hologic software (v46a). Although direct measurements suggest that the density in sheep is higher [22], some of this variation may be due to the different configuration and geometry of the ovine vertebral body and posterior elements affecting the DXA assessment. In studies of osteoporotic bone and prosthetic devices this variation may not be significant and so the sheep model may be applied to humans.

If sheep are to be used as a model for bone metabolism, variations in physiology and mechanical loading need to be considered. Sheep have similar bone physiology to humans [22,23]. Mechanical loading is different in the spine because the animal is a quadruped.

The sheep is an appropriate model for inducing osteoporosis and trialing orthopedic procedures [24,25], and it is possible to induce osteoporosis in sheep by hormonal/pharmacological means [26].

Limitations of this study include the fact that the later time points were from sheep related to, but not the sheep from the younger cohort, the adaptation of human-based software to an anatomically distinct creature, and the selected animals were Australian Merino sheep (other varieties may have different growth and maturation rates). The relatively narrow range of the data suggests that the relatively small sample size is not an important issue.

5. CONCLUSION

We conclude that studies requiring maximum average bone density should use sheep at least 18 months old, whilst maximum lean mass requires sheep at least 24 months old.

CONSENT

It is not applicable.

ACKNOWLEDGEMENTS

The study was funded by the special purposes fund of the Royal Adelaide Hospital.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Guidelines for Preclinical and Clinical Evaluation of Agents Used in the Treatment of Postmenopausal Osteoporosis 1st ed. Maryland: Food and Drug Administration. Maryland: April, 1994, United States Food and Drug Administration.

2. Davidson MK, Lindsey JR, Davis JK. Requirements and selection of an animal model. Isr J Med Sci. 1987;23:551-555.

3. Turner S. Animal Models of Osteoporosis — necessity and limitations. Eur Cell Mater. 2001;22:1:66-81.

4. Casañeda S, Calvo E, Largo R, González-González R, de la Piedra C, Díaz-Curiel M, Herrero-Beaumont G. Characterization of a new experimental model of osteoporosis in rabbits. J Bone Miner Metab. 2008;26:53-9.

5. Martini L, Fini M, Giavaresi G, Giardino R. Sheep model in orthopedic research: a literature review. Comp Med. 2001;51:292-9.

6. Lill CA, Fluegel AK, Schneider E. Sheep model for fracture treatment in osteoporotic bone: A pilot study about different induction regimens. J Orthop Trauma. 2000;14:559-65.

7. Lill CA, Gerlach UV, Eckhardt C, Goldhahn J, Schneider E. Bone changes due to glucocorticoid application in an ovariectomized animal model for fracture treatment in osteoporosis. Osteoporos Int. 2002;13:407-14.

8. Nicodemo Mlf, Scott D, Buchan W, Duncan A, Robins SP. Effects of variations in live weight gain on bone growth and composition and on markers of bone turnover in lambs Experimental Physiology. 1999;84:579-587.

9. Zarrinkalam M, Schultz CG, Parkinson IH, Moore RJ. Osteoporotic Characteristics Persist in the Spine of Ovariectomized Sheep after Withdrawal of Corticosteroid Administration Journal of Osteoporosis. 2012;ArticleID 182509. Available:http://dx.doi.org/10.1155/2012/182509

10. Krausgrill DI, Tulloh NM, Hopkins DL, Growth of sheep to the age of three years
after a severe nutritional check in early post-natal life. The Journal of Agricultural Science. 1997;128:479-494.

11. Kaymakci B, Wark JD. Precise accurate mineral measurements of excised sheep bones using X-ray densitometry. Bone Miner. 1994;25:231-46.

12. Schorlemmer S, Gohl C, Iwabu S, Ignatius A, Claes L, Augat P. Glucocorticoid treatment of ovariectomized sheep affects mineral density, structure, and mechanical properties of cancellous bone. J Bone Miner Res. 2003;18:2010-5.

13. Turner AS, Mallinckrodt CH, Alvis MR, Bryant HU. Dual-energy X-ray absorptiometry in sheep: Experiences with In vivo and Ex vivo studies. Bone. 1995;17(4 Suppl):381S-387S.

14. Pouilles JM, Collard P, Tremollieres F, Frayssinet P, Railhac JJ, Cahuzac JP, Autefage A, Ribot C. Accuracy and precision of In vivo bone mineral measurements in sheep using dual-energy X-ray absorptiometry. Calcif Tissue Int. 2000;66:70-3.

15. Mercier J, Pomar C, Marcoux M, Goulet F, Theriault M, Castonguay F. The use of dual-energy X-ray absorptiometry to estimate the dissected composition of lamb carcasses: Meat Science. 2006;73:249–257.

16. Orwoll ES, Belknap JK, Klein RF. Gender specificity in the genetic determinants of peak bone mass. J Bone Miner Res. 2001;16:1962-71.

17. Magarey AM, Boulton TJ, Chatterton BE, Schultz C, Nordin BE, Cockington RA. Bone growth from 11 to 17 years: relationship to growth, gender and changes with pubertal status including timing of menarche. Acta Paediatr. 1999;88:139-46.

18. Goodfellow LR, Earl S, Cooper C, Harvey NC. Maternal diet, behaviour and offspring skeletal health. Int J Environ Res Public Health. 2010;7:1760-72.

19. Jackowski SA, Erlandson MC, Mirwald RL, Faulkner RA, Bailey DA, Kontulainen SA, Cooper DM, Baxter-Jones AD. Effect of maturational timing on bone mineral content accrual from childhood to adulthood: evidence from 15 years of longitudinal data. Bone. 2011 1;48:1178-85.

20. Wren TA, Kim PS, Janicka A, Sanchez M, Gilsanz V. Timing of peak bone mass: discrepancies between CT and DXA. J Clin Endocrinol Metab. 2007;92:938-41.

21. Aerssens J, Boonen S, Lowet G, Dequeker J. Interspecies differences in bone composition, density, and quality: Potential implications for In vivo bone research Endocrinology. 1998;139:663-70.

22. Kennedy OD, Brennan O, Mahony NJ, Rackard SM, O’Brien FJ, Taylor D, Lee CT. Effects of high bone turnover on the biomechanical properties of the L3 vertebra in an ovine model of early stage osteoporosis. Spine (Phila Pa 1976). 2008;33:2518-23.

23. Wu ZX, Lei W, Hu YY, Wang HQ, Wan SY, Ma ZS, Sang HX, Fu SC, Han YS. Effect of ovariectomy on BMD, micro-architecture and biomechanics of cortical and cancellous bones in a sheep model. Med Eng Phys. 2008;30:1112-8.

24. Zarrinkalam MR, Beard H, Schultz CG, Moore RJ Validation of the sheep as a large animal model for the study of vertebral osteoporosis. Eur Spine J. 2009;18:244-53.

25. Reinwald S and Burr D, Review of Nonprimate, Large Animal Models for Osteoporosis Research J Bone Miner Res. 2008;23:1353–1368.

26. Phillips FM, Turner AS, Seim HB 3rd, MacLeay J, Toth CA, Pierce AR, Wheeler DL. In vivo BMP-7 (OP-1) enhancement of osteoporotic vertebral bodies in an ovine model. Spine J. 2006;6:500-6.